CYCLE XXXVI

February 2024

Study of the Fruit Inhibitory Mechanism on Citrus Flowering. Nutritional, Hormonal and Genetic Factors

Programma di Dottorato in Biodiversity in Agriculture and Forestry (UNIPA)

Programa de Doctorado en Recursos y Tecnologías Agrícolas (UPV)

PhD. Candidate
Andrés Marzal Blay

Supervisor
Dr. Carlos Mesejo Conejos

Co-Supervisor
Dr. Manuel Agustí Fonfría
Dr. Riccardo Lo Bianco

Università degli Studi di Palermo

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Als meus pares

Als meus germans

A Raquel

Agradecimientos/ Ringraziamenti

A mi director, profesor y sobre todo amigo Carlos, con el que descubrí el increíble mundo de la investigación. Empezamos a trabajar juntos hace ya 7 años, con el trabajo final de carrera, pasando por la tesina de máster y terminando hoy, con la Tesis Doctoral. He aprendido mucho de ti, lo que significa verdaderamente el esfuerzo, la constancia, el rigor, la exigencia… pero sobre todo otras cosas como la comprensión, la amistad y la ayuda desinteresada. Gracias por no dejar que me desanimara nunca y ayudarme a seguir adelante. Gracias por todo.

A Manuel Agustí, por abrirme las puertas del IAM y tratarme siempre como uno más del equipo, gracias por tus consejos y ayuda incondicional. A Amparo, por ser mi amiga, por su paciencia infinita en el laboratorio, su actitud positiva y su alegría constante. A Carmina por transmitirme su buena actitud y consejos. A Vicent por su buena disponibilidad siempre.

A todos aquellos que han pasado por el IAM todos estos años: María, Camilo, Vicente, Javi, Guillem, Mar, Carlos, Elena, Antonio y en especial a mi compañero de experimentos Paco, por su alegría y gran amistad.

Del IBMCP quiero agradecer a Miguel Blázquez su ayuda e infinita paciencia durante toda la parte de interpretación de resultados del RNA-seq. A Vicente Balanzá y Cristina Ferrándiz por abrirme las puertas de su laboratorio y hacerme todo tan fácil. A Guille y Mar, mis compañeros de hibridación in situ, que entre los “tres” conseguimos hacer “uno”.

A mis compañeros de doctorado de la UPV e IVIA: Ramón, María y Yaiza por cada congreso y buen momento vivido. A Natalia, la mejor compañera de estancia de investigación posible. Sin ti, estoy seguro de que Palermo no habría sido lo mismo, gracias por tu felicidad infinita y saber animarme en los momentos difíciles. Gracias por todo.

Grazie a Riccardo per il suo aiuto incondizionato e per avermi fornito tutto ciò di cui avevo bisogno all'università.

A tutte le persone di UNIPA, in particolare a Roberto, Eugenia, Dario, Valeria, Daniele, Marco, Eliseo, Sofia e Lucia. Grazie per avermi fatto sentire a casa.

Alla mia piccola famiglia italiana Roberta e Simone. Che fortuna avervi conosciuto, so che sarò sempre un piccolo palermitano grazie a voi.

A mi maravillosa familia, en especial a mis padres y hermanos, por estar siempre a mi lado mostrándome apoyo y cariño. Porque con todo su esfuerzo me han podido dar esta gran oportunidad, por creer en mí y ayudarme a ser la persona que soy hoy en día.

A mis abuelos, en especial a Ricardo y Miguel, que despertaron en mi la pasión por la agricultura. Por ser unos de mis principales referentes.

A Raquel, por ser la mejor compañera de vida posible. Gracias por apoyarme y nunca dejar que me rindiera. Si todo esto ha sido posible, también es gracias a ti. Por todo lo que nos queda.

Index

Abbreviations

AB-G. Apical basal girdling

A-G. Apical girdling

ATX1. TRITHORAX 1

a/b. annual/biennial

ABA. Abscisic acid

ACT. Actine

AG. AGAMOUS

AGL7. AGAMOUS LIKE 7

AGL24. AGAMOUS LIKE 24

An. net photosynthesis

AP1. APETALLA1

AP2. APETALLA2

AS1. ASYMMETRIC LEAVES 1

α-AMY. alpha-amylase

α-SnRK1. Serine-threonine kinase (subunit α)

B-G. Basal girdling

BP. Biological process

bZIP. Basic Leucine Zipper

β-SnRK1. Serine-threonine kinase (subunit β)

CC. Cellular component

CcFLC. Citrus clementina FLOWERING LOCUS C

CcMADS19. FLC ortholog

CDKB2. Cyclin kinase dependent

CEN. CENTRORADIALIS

CiFT3. Citrus FLOWERING LOCUS T ortholog

CIN. Cytosolic Invertase

CKs. Cytokinins

CLF. PRC2 protein member

CO. CONSTANS

CsLFY. Citrus sinensis LEAFY

CT. Control

cvs. cultivars

CWIN. Cell-wall Invertase

CYCB2. Cyclin

D Jul. Defruiting July

D Sep. Defruiting September

D Nov. Defruiting November

D Jan. Defruiting January

DAT. Days after treatment

DEF. Defruiting

DEGs. Differential gene expressed

DELLA-like. DELLA protein

DNA. Deoxyribonucleic acid

ELF6. EARLY FLOWER 6

ELF8. EARLY FLOWER 8

Exp. Expected

FC. log2 Fold Change

FD. FLOWERING LOCUS D

FLC. FLOWERING LOCUS C

FM. Floral meristem

FT. FLOWERING LOCUS T

GA. Gibberellin

GAs. Gibberellins

GA2ox2. Gibberellin 2-oxidase 2

GA3ox1. Gibberellin 3-oxidase 1

GA3ox2. Gibberellin 3-oxidase 2

GA20ox. Gibberellin 20-oxidase 2

GA_3 Gibberellic acid

GA3ox. Gibberellin 3-oxidase

ChIP. Chromatin immunoprecipitation

GI. GIGANTEA

GIDB1. Gibberellin receptor

GO. Gene Ontology

γ-SnRK1. Serine-threonine kinase (subunit γ)

H3. Histona 3

H3K4me3. Histone H3 lysine 4

H3K27me3. Histone H3 lysine 27

HPLC. High-performance liquid chromatography

I. Reproductive shoots without leaves, inflorescence

IAA. Indol acetic acid

IM. Inflorescence meristem

JA. Jasmonic acid

L-I. Mixed shoots with inflorescence

L-SF. Mixed shoot with a solitary flower

LAX3. Auxin influx carrier

LC/MS/MS. Liquid chromatography–mass spectrometry

LD. Long-Day

LFY. LEAFY

lp. Leave primordia

LSD. Least significant difference

LYC. β -lycopene cyclase

m. meristem

MADS. MCM1/AGAMOUS/DEFICIENS/SRF

MALDI, MS/MS. Mass spectrometry

MF. Metabolic function

ML. leafless inflorescences with more than one flower

MLY. leafy inflorescences with more than one flower

MP. MONOPTEROS protein

N. Nitrogen

NF-YA1. nuclear factor YA

OFF. Without fruits

ON. With fruits

P/I. promoter/inhibitor

PBZ. Paclobutrazol

PCR. Polymerase chain reaction

PI. PISTILATA

PRC2. Polycomb Repressive Complex 2

RNA. Ribonucleic acid

RNA-Seq. Massive transcriptome sequencing

RT-qPCR. Real time – polymerase chain reaction

SA. Salicylic acid

SAM. Shoot apical meristem

sc. scales

SD. Short-Day

SE. Standard error

SEP. SEPALLATA gene

SF. small fruit generated in an extemporaneous flowering in summer

SL. leafless inflorescences with one flower

SLY. Single leafy shoot

SOC. Super Optimal broth with Catabolite repression

SOC1. SUPPRESSSOR OF OVEREXPRESSION OF CONSTANS 1

SPL3. SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3

SUS1. Sucrose Synthase

SVP. SHORT VEGETATIVE PHASE

T6P. trehalose-6-phosphate

TEM1. TEMPRANILLO 1

TF. Transcription Factor

TFL. TERMINAL FLOWER 1 ortholog

TFL1. TERMINAL FLOWER 1

Theo. Theoric

TIBA. 2,3,5-triiodobenzoic acid

TRN2. Tetraspanin protein

Tz. Trans-zeatina

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

V. Vegetative shoot

VIN. Vacuolar invertase

VIN3. PHD protein member

VRN. VERNALISATION

VS. Vegetative shoot

YUC4. Indole-3-pyruvate monooxygenase

2,4-D. 2,4-D dimethylamine salt

Abstract

Abstract

In citrus, low temperature promotes flower induction in autumn-winter by increasing the expression of the *CiFT3* promoter gene (citrus homologue of the *FLOWERING LOCUS T* gene). The presence of large numbers of fruits on the tree at this time inhibits *CiFT3* expression and flowering, but the inhibitory signal produced by the fruits is unknown. The most widely accepted hypotheses are that the signal is hormonal or nutritional. In the first case, the inhibitory effect is attributed to hormones produced and exported by the fruit during development. In the second case, the inhibitory effect is attributed to the high demand and consumption of carbohydrates by the developing fruit. The two hypotheses are complementary and not mutually exclusive. In addition, it has been shown that the fruit promotes the epigenetic activation of the flowering repressor *CcMADS19* (citrus homolog of the *FLOWERING LOCUS C* gene), which inhibits the expression of the *CiFT3* gene. To determine which signal is produced by the fruit to inhibit flowering, the following hypothesis is proposed in this thesis: The fruit inhibits flowering through the synthesis and export of auxins, which activates the synthesis of gibberellins and, in turn, the expression of *CcMADS19*.

Experiments with exogenous treatments of auxins, gibberellins and their antagonists, fruit thinning, and disruption of phloem transport between fruit and buds indicate that neither gibberellins nor auxins are consistently associated with the activation of *CcMADS19* expression in leaves. In buds, gibberellins are associated with the activation of the flowering inhibitor *CENTRORADIALIS* (CEN), in the presence of fruit by increasing GA_4 synthesis, and in the absence of fruit by its exogenous application. The presence of fruit increases the concentration of auxin in the stem and bud at the time of induction and suppresses its synthesis and transport. However, this does not prevent the epigenetic silencing of the *CcMADS19* gene in the bud, which is transmitted to the leaves of the new vegetative shoots. These shoots flower in the following cycle, where floral differentiation is associated with an increase in auxin synthesis and transport and a decrease in gibberellin synthesis in the bud.

Resumen

En los cítricos, la baja temperatura promueve la inducción floral en otoño-invierno aumentando la expresión del gen promotor *CiFT3* (homólogo en los cítricos del gen *FLOWERING LOCUS T*). La presencia de un gran número de frutos en el árbol durante ese momento inhibe la expresión de *CiFT3* y la floración, pero se desconoce la señal inhibitoria que genera el fruto. Las hipótesis mayormente aceptadas proponen que la señal puede ser hormonal o nutricional. En el primer caso, el efecto inhibidor se atribuye a las hormonas que el fruto produce y exporta durante su desarrollo. En el segundo caso, el efecto inhibidor se atribuye a la alta demanda y consumo de carbohidratos por los frutos en desarrollo. Ambas hipótesis son complementarias y no excluyentes entre sí. Además, se ha demostrado que el fruto promueve la activación epigenética del represor de la floración *CcMADS19* (homólogo en los cítricos del gen *FLOWERING LOCUS C*), que inhibe la expresión del gen *CiFT3*. Con el objetivo de determinar qué señal produce el fruto para inhibir la floración, en esta Tesis se propone la siguiente hipótesis: El fruto inhibe la floración a través de la síntesis y exportación de auxinas que activa la síntesis de giberelinas y, a su vez, la expresión de *CcMADS19*.

Mediante experimentos con tratamientos exógenos de auxinas, giberelinas, y sus antagonistas, aclareo de frutos, y la interrupción del transporte por el floema entre el fruto y las yemas, los resultados indican que ni las giberelinas ni las auxinas se relacionan de forma consistente con la activación de la expresión de *CcMADS19* en las hojas. En las yemas, las giberelinas se relacionan con la activación del gen inhibidor *CENTRORRADIALIS* (*CEN*), cuando hay fruto por aumento de la síntesis de GA_4, y cuando no hay fruto por su aplicación exógena. La presencia del fruto aumenta la concentración de auxinas en el tallo y la yema en el momento de la inducción, y reprime su síntesis y trasporte. Pero esto no impide que, en la yema, el gen *CcMADS19* esté epigenéticamente silenciado y que el silenciamiento se transmita a los nuevos brotes vegetativos. Estos brotes florecen en el siguiente ciclo, y, en sus yemas, la diferenciación floral se relaciona con un aumento de la síntesis y trasporte de auxinas y una reducción de la síntesis de giberelinas.

Resum

Als cítrics, les baixes temperatures promouen la inducció floral a la tardor i l'hivern augmentant l'expressió del gen promotor *CiFT3* (homòleg en els cítrics del gen *FLOWERING LOCUS T*). La presència d'un gran nombre de fruita a l'arbre en aquest moment inhibeix l'expressió de *CiFT3* i la floració, però es desconeix la senyal inhibidora que genera la fruita. Les hipòtesis majoritàriament acceptades proposen que la senyal pot ser hormonal o nutricional. En el primer cas, l'efecte inhibidor s'atribueix a les hormones que la fruita produeix i exporta durant el seu desenvolupament. En el segon cas, l'efecte inhibidor s'atribueix a la alta demanda i consum de carbohidrats per part de la fruita en desenvolupament. Ambdues hipòtesis són complementàries i no es descarten mútuament. A més, s'ha demostrat que la fruita promou l'activació epigenètica del repressor de la floració *CcMADS19* (homòleg en els cítrics del gen *FLOWERING LOCUS C*), que inhibeix l'expressió del gen *CiFT3*. Amb l'objectiu de determinar quina senyal produeix la fruita per inhibir la floració, en aquesta Tesi es proposa la següent hipòtesi: La fruita inhibeix la floració mitjançant la síntesi i exportació d'auxines que activa la síntesi de giberelines i, al seu torn, l'expressió de *CcMADS19*.

Mitjançant experiments amb tractaments exògens d'auxines, giberelines i els seus antagonistes, aclarida de fruita i la interrupció del transport pel floema entre la fruita i les brots, els resultats indiquen que ni les giberelines ni les auxines es relacionen de manera consistent amb l'activació de l'expressió de *CcMADS19* a les fulles. A les gemmes, les giberelines es relacionen amb l'activació del gen inhibidor *CENTRORRADIALIS* (*CEN*) quan hi ha fruita per l'augment de la síntesi de GA_4 i quan no hi ha fruita per la seua aplicació exògena. La presència de la fruita augmenta la concentració d'auxines a la tija i la gemma en el moment de la inducció i reprimeix la seua síntesi i transport. Però això no impedeix que, a la gemma, el gen *CcMADS19* estigui epigenèticament silenciat i que el silenciament es transmeti als nous brots vegetatius. Aquests brots floreixen al següent cicle i, a les seues gemmes, la diferenciació floral es relaciona amb un augment de la síntesi i transport d'auxines i una reducció de la síntesi de giberelines.

Introduction

1. *The flowering process*

Citrus are polycarpic and evergreen species that flower once or several times a year, depending on the genotype and climatic conditions. In Mediterranean climates, citrus flowers in spring after the winter dormancy. Later, in summer and autumn, the buds release only developing vegetative shoots. However, these vegetative shoots contribute significantly to the return of flowering, as the flowers originate from the axillary buds located on the previous year's shoots (Fig. 1; Agustí et al., 2022). Flower induction is mainly triggered by low temperatures and occurs 2-3 months before bud break. Flower differentiation occurs at the same time as bud break after winter rest. Bud break occurs 3-4 weeks earlier in flowering shoots than in vegetative shoots (Fig. 1; Lord and Eckard, 1985).

Flowering buds develop determinate shoots, which may be mixed or generative. In the former, the apical meristem of a leafy shoot develops into a terminal flower, and the axillary buds may in turn sprout, and when this occurs, these buds carry only flowers (Schneider, 1968; Lord and Eckard, 1985). In the generative shoots, the leaf primordia are inhibited from sprouting, resulting in flowering shoots without leaves. Thus, the presence or absence of leaves and flowers in combination gives rise to the five shoot types: 1) leafless inflorescences with one (SL) or 2) more flowers (ML), 3) leafy inflorescences with one flower (SLY) in a terminal position and 4) many axillary flowers (MLY), and 5) vegetative shoots (VS) (Agustí et al., 2022; Fig. 2).

Some species and cultivars flower once or more a year (*regular bearing*), while others alternate years of heavy flowering and production with years of little or no flowering. *Alternate bearing* is an important horticultural problem in many *Citrus* varieties but also in other unrelated evergreen and deciduous fruit tree species such as olive, avocado, mango, apple, etc. (Davis, 1957; Monselise and Goldschmidt, 1982; Dag et al., 2010; Muñoz-Fambuena et al., 2011; Ziv et al., 2014; Guitton et al., 2016; Durand et al., 2017; Elsysy and Hirst, 2017; Muñoz-Fambuena et al., 2018; Das et al., 2019; Dastkar et al., 2020; Goldschmidt and Sadka, 2021; Gottschalk et al., 2021; Haim et al., 2021; Agustí et al., 2022).

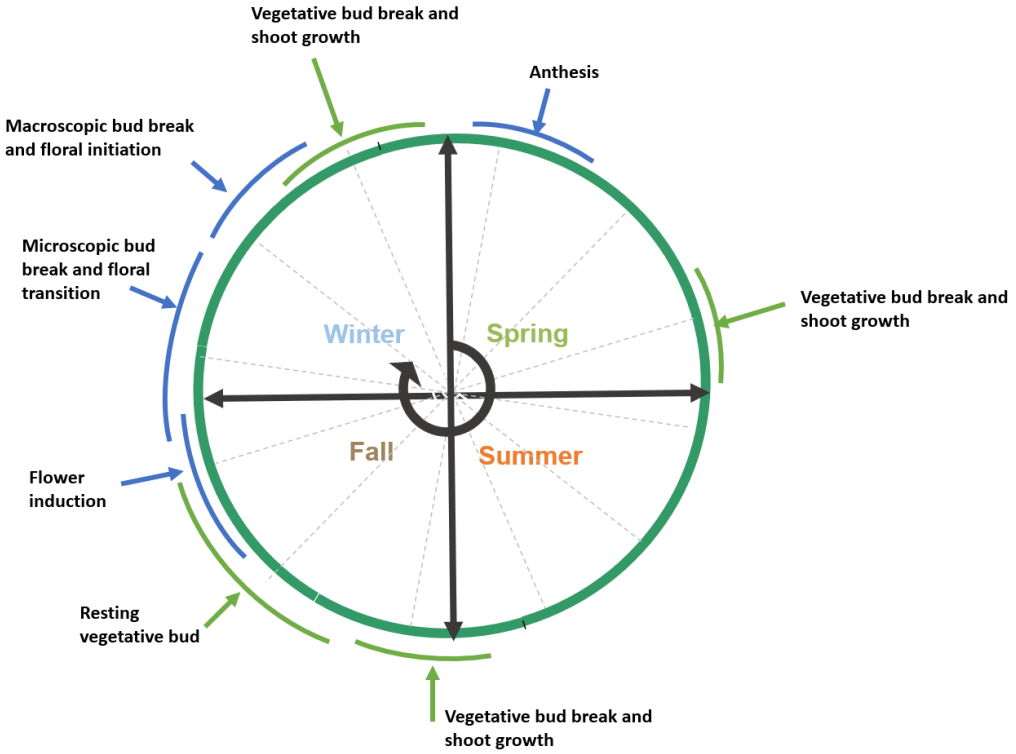


***Figure 1**. Chronology of flowering (blue) and vegetative growth (green) in Citrus grown in the Mediterranean-climate (Adapted from Lord and Eckard, 1985 and Agustí et al., 2022). The time of phenological stages depends on the environment and species or varieties.*

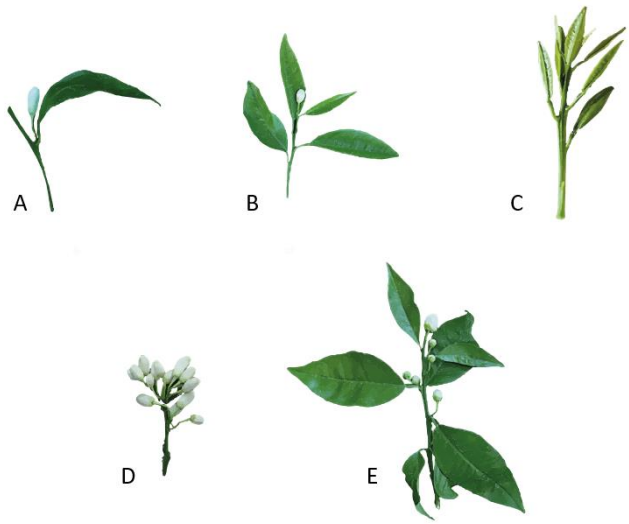


***Figure 2**. Type of shoots in Citrus. (A) Single-flowered leafless shoot; (B) Single flowered leafy shoot; (C) Vegetative shoot; (D) Multiflowered leafless shoot; (E) Multiflowered leafy shoot. Source: Agustí et al., 2022.*

The effect of the citrus fruit on flowering can, under certain circumstances, inhibit it completely (Moss, 1971). The intensity of this phenomenon is highly species and cultivar dependent, and in some mandarins and their hybrids it is a general rule that can cause severe economic damage. The inhibition of flowering by the fruit is particularly important in those mandarins and oranges whose fruits contain seeds and in those that are late harvested; in the first case because of their higher percentage of initial fruit set, which hinders the self-control mechanism of fruit abscission in the plant and produces a large quantity of fruit (Monselise, 1979); in the second case because the prolonged permanence of the fruit on the tree aggravates the problem (Martínez-Fuentes et al., 2010). Among the cvs of sweet orange (*Citrus sinensis* L. Osbeck), 'Salustiana' and 'Valencia Late', within the Balncas group, and 'Navelina', within the Navel group, show a certain degree of alternation (Gravina et al., 2000; Martínez-Fuentes, 2010). In mandarins, the clementine 'Hernandina' (*Citrus clementina* Hort. Ex Tan.) and most of the hybrids and triploid cvs. show a remarkable tendency to alternate bearing (Martínez-Fuentes et al., 2012).

According to current knowledge, all the buds of a tree have the competence to flower, and if they do not, it is due to the presence of inhibitory factors that prevent their floral development (Agustí et al., 1982; Goldschmidt et al., 1997; Martínez-Fuentes et al., 2004). Since some branches of the same tree flower while others do not, the inhibitory factors must be endogenous. Among these, fruit load and harvest time are strong inhibitors of flowering and sprouting in many citrus species, although the response varies between cultivars (Monselise and Goldschmidt, 1982; Valiente and Albrigo, 2004; Martínez-Fuentes, 2010). The time at which flowering is inhibited coincides with the time at which the fruit reaches its final size and starts colour change (Martínez-Fuentes, 2010).

The process of flower induction has been extensively studied in annual/biennial (a/b) plants and considerable progress has been made in elucidating the endogenous mechanism that leads to the transition from the vegetative to the reproductive stage. Thus, many studies in fruit trees are based on this knowledge, despite the differences

between a/b and perennial plants (reviewed by Wilkie et al., 2008; Bangerth, 2009; Andrés and Coupland, 2012; Bratzel and Turk, 2015; Soope et al., 2021). In mature fruit trees, flower induction is a quantitative process, with a significant proportion of meristems remaining vegetative or dormant. Reproductive competence varies between branches, so that when exposed to inductive environmental conditions, only competent meristems respond to floral induction signals and differentiate into inflorescences and flowers. In a/b plants, on the other hand, flower induction is a qualitative process because it is the transition from the juvenile (vegetative) stage to the adult (reproductive) stage and all meristems are induced to flower at once, ending the life of the plant (Fig. 3).

Despite these differences, the basis of flower induction and floral organogenesis appears to be conserved between a/b and perennial plants. For example, the expression of several Arabidopsis 'floral integrators', 'flowering time' and 'flowering identity' genes also occurs in citrus, apple, olive, etc. (Haberman et al., 2016, 2017; Pilliteri et al., 2004;

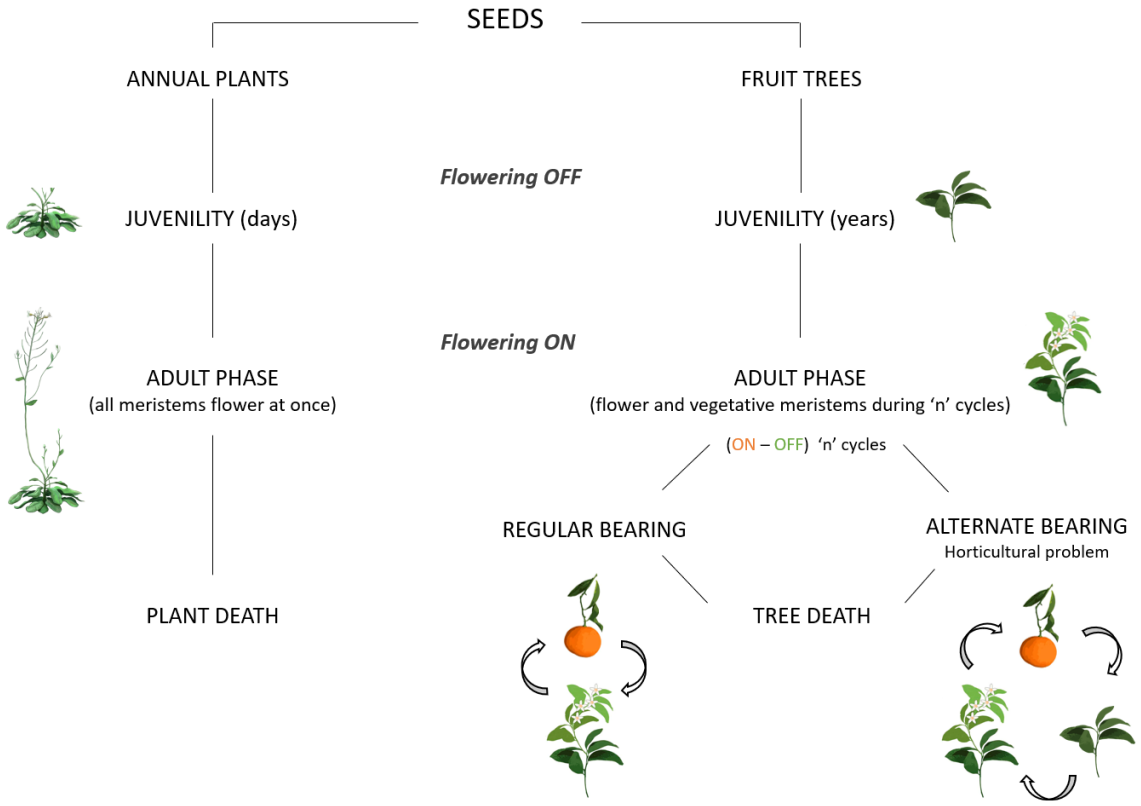


Figure 3. *Induction of flowering in annual plants and fruit trees. In annual plants, all meristems are induced to flower. In adult fruit trees, some meristems are induced to flower while others are not. This can cause the problem of alternate bearing.*

Böhlenius et al., 2006; Nishikawa et al., 2007; Wang et al., 2009; Muñoz-Fambuena et al., 2011, 2012, 2019).

2. Genetic control of flowering

During the juvenile stage in Arabidopsis, flowering is repressed by *TERMINAL FLOWER 1* (*TFL1*) in the shoot apical meristem (SAM) (Bradley et al., 1997). *TFL1* forms a floral repression complex that includes the basic leucine zipper (bZIP) transcription factor *FLOWERING LOCUS D (FD)* (Abe et al., 2005). This repression complex maintains the vegetative stage by inhibiting the flowering promoter *LEAFY* (*LFY*). In *Citrus*, *CsTFL1* expression is upregulated in young seedlings (less than 1 year old), while *CsLFY* is barely detected (Pilliteri et al., 2004; Muñoz-Fambuena et al., 2019). However, in adult trees, *CsTFL1* is barely detected during the vegetative bud stage (see Fig. 1) and *CsLFY* is upregulated after the 8-week induction period (Pilliteri et al., 2004; Muñoz-Fambuena et al., 2019). *CENTRODIALIS* (*CsCEN*), a *CsTFL1* homolog gene, is expressed in axillary meristems of juvenile *Citrus* seedlings, where it interacts with *CsFD*, keeping them indeterminate. This pattern of *CsCEN* and *CsFD* co-expression and their interactions suggests that these genes act together to regulate axillary bud development (Zhang et al., 2021). However, it is unknown how the vegetative bud stage is maintained in the bud of adult *Citrus* trees.

When a vegetative meristem receives the flowering signal, its genetic programme is altered to become a reproductive meristem that produces flowers. This signal activates floral induction and establishes the start of the transition from vegetative to reproductive development. In this sense, the leaves are essential for flowering as they receive the stimulus, produce the inductive signal and transmit it to the bud. In citrus, flower induction occurs during the autumn-winter and the role of the leaves was demonstrated by defoliating the trees in autumn and evaluating flowering in spring (Sánchez-Capuchino and Casanova, 1973).

The onset of flowering relies on specific genes, known as floral pathway integrators, that combine external and internal signals to trigger the process of flowering, including flower induction, determination, and organogenesis. The florigen

signal that triggers flower induction is produced in the leaf and was discovered in *Arabidopsis thaliana* (the model plant) as *FLOWERING LOCUS T (FT)* (Kobayashi et al., 1999). In the development of floral meristem identity, *APETALA1 (AP1)* and *LFY* genes are essential, while the *SEPALLATA (SEP)* genes of the E-class, among others, operate in floral patterning regulation (Pelaz et al., 2000). The FT protein is synthesized in the phloem companion cells of the induced leaf and transported through the sieve tubes to the meristem. Here, it binds with the transcription factor FD to form the FD/FT heterodimer, which activates the expression of *LFY* and *AP1*, thereby inducing floral initiation (Abe et al., 2005; Wigge et al., 2005). *LFY* is crucial for flower development, playing a pivotal role in both its temporal function and floral identity (Blázquez et al., 1997). In *Citrus*, the transgenic *CcFT1* overexpressing lines did not flower, whereas those overexpressing *CcFT3* flowered (Soares et al., 2020). Thus, it is the *CcFT3* paralog the one that induces flowering. The *FD-like (CcFD and CsFD*) genes (Zhang et al., 2021), *CsLFY*, *CsAP1* (Pillitteri et al., 2004a), and the *SEPALATA* genes (*CiSEP1* and *CiSEP3*) (Endo et al., 2006) have been also isolated and characterized.

In *Citrus*, water shortage and low temperature have been shown to trigger *CiFT3* expression and flower induction. Chica and Albrigo (2013) show that subjecting Citrus to periods of water stress (- 2 MPa) between 45 and 60 days induces flowering. Water shortage leads to an increase in the transcription of the *CiFT3* gene, while the transcription of the *CsAP1* and *CsLFY* genes decreases. However, when the water status is restored by irrigation, the expression levels of *CsCiFT3* return to the initial values before treatment, whereas *CsAP1* and *CsLFY* experience an increase in expression, triggering flowering in water-stressed trees.

Exposure of citrus plants to periods of low temperature for 6 to 8 weeks can induce flowering (García-Luís et al., 1992; Pilliteri et al., 2004; Nishikawa et al., 2007; Cho et al., 2017). Adult Satsuma mandarin (*Citrus unshiu* Marc.) trees artificially induced by exposure to 15°C for 45 days showed increased expression of *CiFT3* in their leaves and flowered, whereas those exposed to 25°C for two and a half months did not increase gene expression or flower (Nishikawa et al., 2007).

In addition to *TFL1*, other flowering repressors are involved in maintaining the vegetative stage. For example, an important regulatory mechanism in winter annual Arabidopsis accessions is that the repressor *FLOWERING LOCUS C (FLC)* MADS box transcription factor determines the appropriate time for flowering in coordination with cold. Thus, the transition from the vegetative to the reproductive stage is not completed until vernalisation (6-12 weeks at low temperatures ~ 5ºC) down-regulates the *FLC* gene (Sheldon et al., 2000). During vernalisation, *FLC* is progressively down-regulated by a mechanism involving RNA-mediated chromatin silencing, after which flowering is allowed (Whittaker and Dean, 2017). Thus, it can be said that repression of the flowering inhibitor allows flowering in these plants.

Recent studies in *Citrus* show that fruits promote the epigenetic activation of the transcription factor *CcMADS19* (the *Citrus clementina FLC* ortholog), which inhibits flowering (Agustí et al., 2020). This finding was subsequently highlighted and explained by (Hoijemberg and Cerdán, 2020, Fig. 4). At the time of the flowering transition, the expression of *CiFT3* increases in OFF (without fruits), but not in ON (with fruits) trees, in the leaves formed in the previous spring (Muñoz-Fambuena et al., 2011). Interestingly, the expression of *CcMADS19* follows an opposite pattern, being higher in ON than in OFF trees at the end of winter, just when the flowering transition is established in OFF trees.

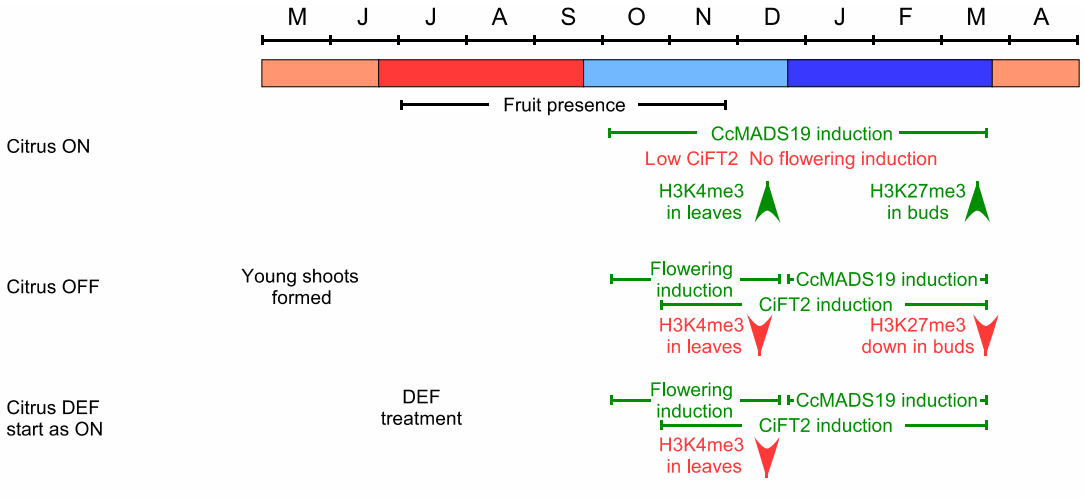


Figure 4. *A citrus flowering timeline. A whole year, from May to April, is shown, with the four seasons in colour, corresponding to the northern hemisphere. Increased processes are shown in green and decreased processes in red. ON: with high fruit load; OFF: without fruit; DEF, defruited; (Source: Hoijemberg and Cerdán, 2020).*

An enrichment of histone 3 trimethylated at lysine 4 (H3K4me3) found in the *CcMADS19* promoter of leaves of ON trees, compared to OFF and defruited trees, explains the higher expression of *CcMADS19* in leaves of ON trees. However, these results do not explain the ability of ON trees to flower in the next (OFF) season (Agustí et al., 2020; Hoijemberg and Cerdán, 2020, Fig. 4).

According to Bangerth (2009), these epigenetic modifications may require long-distance signals, and Goldschmidt and Sadka (2021) propose two main hypotheses to explain how the fruit inhibits flower induction, both plausible and not necessarily contradictory:

1. The carbohydrate/energy hypothesis suggests that depletion of resources during the ON year limits reproductive activity in the following year.

2. The hormonal hypothesis proposes that currently developing fruits release hormones that interfere with the flowering induction of next year's crop.

3. Hormonal control of flowering

In fruit trees, gibberellins (GAs) have always been considered the main inhibitors of flowering. Gibberellic acid (GA_3) treatments reduce flowering in either evergreen (i.e. citrus, loquat, avocado, mango, olive, etc.) or deciduous fruit trees (apple, peach, cherry, plum, etc.) and are now a common cultural practice to regulate fruit tree production (Southwick et al., 1995; El-Otmani et al., 2000; Atay et al., 2016). In citrus, sprays of gibberellic acid (GA_3) in late autumn or winter reduce flowering the following spring (Halevy et al., 1964). Selective inhibition of leafless inflorescence shoots appears to be the basic mechanism, since GA_3 does not alter the number of flowers per inflorescence (Guardiola et al., 1982). A second mechanism involves an increase in the number of vegetative shoots that develop, which has been interpreted as a reversion of the meristems to a vegetative state. This all-or-nothing effect of GA_3 sprays on the bud contrasts with the quantitative control of flowering for the whole tree. In fact, when flowering is extremely heavy, a GA_3 application to reduce flowering intensity is

ineffective unless the GA_3 concentration is greatly increased (Martínez-Fuentes et al., 2004).

At the genetic level, GA_3 treatments reduce the expression of *CiFT3* in the leaf (Muñoz-Fambuena et al., 2012; Goldberg-Moeller et al., 2013), *AP1, AP2, SEPALLATAs (SEP1 and SEP3), PISTILATA (PI) and AGAMOUS (AG)* in the axillary buds (Goldberg-Moeller et al., 2013; Tang and Lovatt, 2019).

However, in these species, the endogenous role of GA and the interaction between GA and the environment are not yet understood. Since in most species the fruit also inhibits flowering at the time GA_3 treatments are effective, the main hypothesis since the 1960s has been that GA moves from the fruit down the stem to inhibit flowering (Bangerth, 2009). To test this hypothesis, most experiments have used the GA synthesis inhibitor paclobutrazol (PBZ). PBZ promotes flowering in *Citrus* (Delgado et al., 1985), but this effect is fruit load dependent (Martínez-Fuentes et al., 2013). In particular, flowering intensity increased significantly in the following spring in trees with medium to low fruit load treated with either 1-10 g PBZ $tree^{-1}$ applied to the soil or 15 g $tree^{-1}$ sprayed on the canopy. In contrast, high fruit load nullified the effect of PBZ irrespective of the time of treatment (induction period or bud burst), the dose applied (1, 10 or 15 g $tree^{-1}$) or the treatment method (soil or canopy spray). These results suggest that the fruit inhibits flowering without altering endogenous GA metabolism. However, in October and December (in the NH), the content of GA-like substances is higher in the buds ('Valencia' sweet orange) and leaves (Satsuma mandarin) of fruit-bearing shoots that do not flower than in the vegetative non-bearing shoots that flower abundantly; later, up to February, the differences become very small (Jones et al., 1977; Koshita et al., 1999). Furthermore, when the fruit changes colour (coinciding with the flower induction period), GA_1 and GA_4 concentrations decrease significantly in the peel and increase in the stem bark, especially for GA_4 (Gambetta et al., 2012). However, there is no evidence that the target of these GAs is the lateral bud or the leaf. Furthermore, the gibberellin precursor GA_{12}, which acts as a long-distance growth signal (Regnault et al., 2015), has not been found in citrus buds during the induction period. Recent studies still show inconclusive results for the endogenous influence of GA on apple flower bud

formation (Milyaev et al., 2022). Thus, it remains unclear whether GA has a true physiological role in the regulation of flowering in woody perennials.

Because of these doubts, and the ability of auxin to polar transport, an alternative signalling pathway has been proposed: GA would be the first messenger present at the shoot apex, where it would stimulate indole-3-acetic acid (IAA) synthesis, and polar auxin transport would act as the second messenger, which would be the true transported signal capable of inhibiting flowering (see Bangerth, 2009). In Citrus, ON crop buds have higher levels of IAA than OFF crop buds (Shalom et al., 2014), i.e. the fruit induces strong polar auxin transport that is reduced by fruit removal, allowing auxin release from the bud; this suggests that the fruit could generate an auxin signal in the bud and apical meristem that would interfere with flower induction (Haim et al., 2021).

In Arabidopsis, GA play an inhibitory role at the time of floral formation (Yamaguchi et al., 2014). To overcome the inhibitory effect of GAs, the *LFY* transcription factor activates the expression of *ELA1*, an enzyme involved in the catabolism of these hormones. Thus, an increase in *LFY* expression leads to a reduction in GA levels. The low level of GAs in the meristem allows up-regulation of the *AP1* gene and flowering. Furthermore, a transient auxin peak is essential for the emergence of the floral meristem. Auxin releases the MONOPTEROS (MP) protein, which acts at several levels, including directly inducing the expression of *LFY* (Denay et al., 2017).

4. Nutritional control of flowering

The carbohydrate/energy hypothesis suggests that depletion of resources in the ON year limits reproductive activity in the following year (Goldschmidt and Sadka, 2021). In citrus, multi-flowering leafy shoots (MLY), and especially single flowering leafy shoots (SLY), with a terminal flower, contribute the most fruit to the crop. As these fruits grow, they become the main sink of nutrients in the tree, resulting in a reduction in starch content in nearby leaves compared to non-fruiting shoots. Thus, after a year of high crop load, carbohydrates are depleted in all tree organs in alternate-bearing citrus cultivars and flowers are not formed in the following season (Goldschmidt et al., 1985; Li et al.,

2003; Martínez-Alcántara et al., 2015). In fact, defruiting at an early stage of fruit development partially restores carbohydrates and allows flowers to form (Goldschmidt and Golomb, 1982). Nevertheless, the regulatory role of carbohydrates in citrus flowering is still controversial, and some studies have shown no correlation between carbohydrates and flowering. For example, shading of Satsuma mandarin trees significantly reduces the carbohydrate content, corresponding to its effect on photosynthesis, but its effect on emergence and flowering is not significant (García-Luis et al., 1995). The relationship between carbohydrates and flowering is not fully understood in citrus, as it is not clear whether their function is to stimulate or inhibit the process, or simply to provide an essential source of energy to sustain cell division and growth.

In other plant systems, A transient increase in sucrose in the phloem has been observed prior to the onset of mitotic activity in the meristem (Corbesier et al., 1998), it has been demonstrated the role of sucrose as a primary signal in bud release and apical dominance (Mason et al., 2014). With respect to flowering, plants with abnormal trehalose-6-phosphate (T6P) levels have been shown to have changes in flowering time, such that FT expression is reduced in plants with low T6P levels (Wahl et al., 2013). In addition, T6P inhibits miRNA156 expression, which in turn allows SPL (flowering-related transcription factor) to stimulate flowering induction (Matsoukas et al., 2012). It is therefore possible that T6P, or other carbohydrates, plays a signalling role in flower induction, but all this is currently unknown in citrus.

5. *Convergence of hormonal, genetic and epigenetic inhibitory signals in citrus flowering*

With the aim of explaining the relationship between exogenous hormone treatments and endogenous factors regulating Citrus flowering, Martínez-Fuentes et al (2004) proposed a hypothesis suggesting three levels of flowering response depending on the promoter/inhibitor (P/I) ratio, which can be updated according to current knowledge (*CcMADS19*; Agustí et al., 2020) and the qualitative/quantitative theory of

Bangerth (2009) (Fig. 5). Considering the whole tree, only when the endogenous P/I ratio is close to equilibrium can flowering be exogenously *reduced* by applying inhibitors (GA_3) or *increased* by applying promoters (PBZ) (*quantitative response*). This is produced in trees of medium yield and only some buds are responsive to the treatment.

However, extreme high-null flowering is regulated by the endogenous high-low P/I ratio, which cannot be counteracted by exogenous treatments (*qualitative response*). This is produced in high-yield (ON) and low-yield (OFF) trees. Flowering is *switched off* (hindered) in all the buds near a fruit in ON trees by epigenetic activation of *CcMADS19* in the leaves (Agustí et al., 2020). This could be a short-distance effect transmitted within the shoot or branch (*qualitative effect*). The more fruits in the tree, the less flowering (*quantitative effect*). Therefore, in this PhD thesis, the mechanism of flowering inhibition will be mainly investigated using the single leafy shoot (SLY) with one terminal flower, as a non-flowering model, compared to vegetative shoots. If the fruit inhibits flowering through a signal involving auxins and/or GAs, treatments with 2,4-D, GA_3 and PBZ could alter the expression of *CcMADS19, CiFT3, LFY* and *CEN* genes, and the presence of fruit could be related to the regulation of GA and auxin metabolism in leaves and buds.

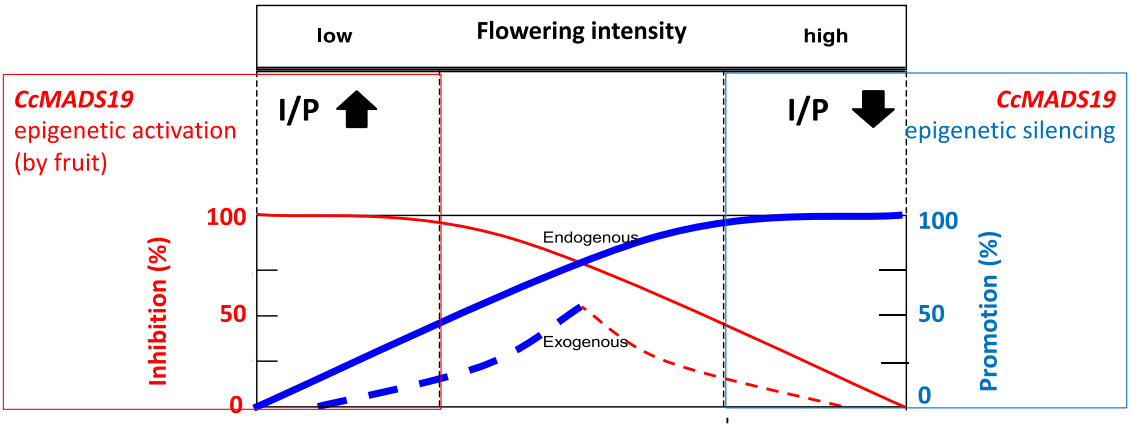


Figure 5. *A hypothesis for the relationship between endogenous and exogenous flowering regulators in citrus (adapted from Martínez-Fuentes et al., 2004). The ration between endogenous inhibitors (I) and promoters (P) outweighs the exogenous inhibitors and promoters of flowering.*

Hypothesis and objectives

In this PhD thesis the following hypothesis was tested:

"The fruit inhibits flowering through auxin synthesis and export, which activates gibberellin synthesis and thus *CcMADS19* expression."

General objective:

To determine the fruit signal for citrus flowering inhibition.

Specific objectives:

1. To determine the effect of GA_3 and 2,4-D treatments on the expression of *CcMADS19* and other flowering genes.

2. To determine the hormonal composition of the fruit exudate during the flower induction period.

3. To determine the effect of the fruit on the synthesis and concentration of gibberellin and auxin.

4. To determine the effect on flowering of intercepting phloem transport to avoid the fruit-derived signal to the buds.

5. To determine the epigenetic silencing of *CcMADS19* in vegetative shoots during the OFF season.

6. To characterize the main metabolic pathways altered by the fruit using RNA-seq analysis.

Material and Method

1. Plant Material

All the experiments in this thesis were carried out on mature trees (10-20 years old) from commercial fields located in the province of Valencia, in the experimental plot of the Universitat Politècnica de València (Spain) and in the experimental plot of the Università degli studi di Palermo (Italy). The species and varieties used were: sweet orange cvs '*Navelate*', *'Lane-late'* and *'Valencia late'* [*Citrus sinensis* (L.) Osb.], lemon '*Eureka*' (*Citrus limon* L.), ruby grapefruit (*Citrus paradisi* Macf.), clementine mandarin '*Clemenules*' and '*Oronules*' (*Citrus clementina* Hort. Ex Tan), satsuma mandarin '*Owari'* (*Citrus unshiu* Marc.) and mandarin '*Nadorcott*' or '*Afourer*' (*Citrus sinensis* (L.) Osb.), Raf. X *Citrus reticulata* Blanco] (a hybrid, a nucellar selection or a spontaneous mutation of '*Murcott'*). All trees were grafted on Carrizo lemon rootstock [*Citrus sinensis* (L.) Osb. X *Poncirus trifoliata* (L.)], except for the lemon trees, which were grafted on bitter orange (*Citrus aurantium* L.). The '*Nadorcott*' mandarin was used as a reference variety in all the chapters because of its late ripening, its alternate bearing behaviour and because it was the genotype used to characterise the epigenetic activation of *CcMADS19* in previous studies (Agustí et al., 2020). The other species, but mainly sweet orange, were used to generalise the agronomic and physiological response of the experiments. All the fields were irrigated and fertilised by localised irrigation and were in perfect nutritional and sanitary condition.

Table M1 summarises the species used in each experiment, their location, and the chapter of the thesis to which they refer. Depending on the experiment, the experimental unit was the whole tree, a branch, or a shoot. Trees were either high-yielding (ON) or had reduced or no yield (OFF). Branches had about 200 nodes with fruit (ON) or without fruit (OFF). Shoots were either vegetative (OFF), summer flush shoots with at least 5 nodes in length, or canopy shoots (ON) with a fruit at the end of a spring shoot with at least 5 nodes in length. ON trees, branches and shoots do not flower the following spring, whereas OFF trees, branches, and shoots flower profusely.

***Table M1**. Species and varieties used in the experiments. Locations in the province of Valencia (VLC) have practically the same average temperature and rainfall. Ordered from coldest to warmest area: Pedralba, Godelleta, Torrent, UPV, Almussafes.*

Species	Variety	Chapter	Experiment	Localization	Year
C. sinensis	Lane-late	Gibberelins	1	Torrent (VLC)	2020
			3	Torrent (VLC)	2021
			7	Almussafes (VLC)	2021
			8	Torrent (VLC)	2021
	Navelina	Gibberelins	2	UPV (VLC)	2020
		Auxins	9	UPV (VLC)	2022
	Valencia-late	Auxins	10	USP (Palermo)	2022
C. sinensis x *C. reticulata*	Nadorcott	Gibberelins	4	Pedralba (VLC)	2020
			5	Pedralba (VLC)	2020
			6	Pedralba (VLC)	2021
			8	Torrent (VLC)	2021
		Auxins	12	Torrent (VLC)	2022
		Girdling	13	Torrent (VLC)	2018
			14	Godelleta (VLC)	2019
			15	Torrent (VLC)	2021
			16	Godelleta (VLC)	2022
C. clementina	Clemenules	Gibberelins	2	UPV (VLC)	2020
		Auxins	9	UPV (VLC)	2022
	Oronules	Auxins	11	Pedralba (VLC)	2022
C. unshiu	Owari	Gibberelins	2	UPV (VLC)	2020
C. limon	Eureka	Gibberelins	2	UPV (VLC)	2020
		Auxins	9	UPV (VLC)	2022
C. paradisi	Star Ruby	Gibberelins	2	UPV (VLC)	2020

2. *Treatments*

Growth regulator treatments were carried out as follows, depending on the experiment:

- Treatments localized to the leaf, bud, or shoot/branch. Leaf application was made with a thick brush, moistening both the upper and lower sides until run-off; bud

application was made with a micropipette, placing a 20 µl drop of solution in each axillary bud. Application to the shoot or branch was made with a sprayer, using approximately 100 ml per branch.

- Treatments to the entire tree were made with a spray gun connected to a 100-litre tank (at 40 atm pressure), wetting the tree until the point of run-off, using 6 litres per tree.

A 0.05% nonionic surfactant (*nonylphenol polyethylene glycol ether*) was added to all treatments.

3. Sampling

Axillary buds, leaves and stem bark, including phloem and cortex tissues were sampled for hormonal, metabolite, and gene expression analyses. To obtain sufficient plant material for these analyses, the limiting factor for these organs is the axillary bud. Therefore, each biological replicate required a minimum of 4 shoots with 5 nodes each. The established method was to sample 12 shoots per treatment and date for the analysis of 3 biological replicates. Each biological replicate consisted of 20 axillary buds, 3 leaves and 2 g of phloem tissue. The different shoots were randomly distributed in the field. No more than 4 shoots per tree were selected. All samples were transported to the laboratory at 4°C where they were processed, frozen in liquid nitrogen and stored at -80°C until analysis.

4. Phenotypic evaluation

4.1. *Sprouting and flowering evaluation*

The number of new shoots sprouted per bud, the number of flowers and the number of leaves on each shoot were counted and analysed according to the protocol established by Guardiola et al. (1982). The non-sprouting nodes were also recorded. Then, to calculate the total number of flowers on each branch, the number of flowers per shoot was multiplied by the number of shoots developed on that branch. The results

are expressed as flowers per 100 nodes to account for differences in the size of the branches selected for counting.

4.2. *Photosynthesis*

The net photosynthetic rate (An) was assessed in fruit-bearing shoots and in shoots with double stripping (AB-G) at the end of January using the portable photosynthesis system (*Li-COR*).

5. *Laboratory methods*

5.1. *RNA extraction*

Total RNA was extracted from frozen tissues according to the conditions of the *TAKARA* amplification kit (*TAKARA bio USA*). DNase digestion was not required. The amount of RNA was quantified by spectrophotometric analysis using a *NanoDrop NDB1000 spectrophotometer* (*Thermo Fisher, USA*). The purity of the RNA samples was confirmed by a non-retrotranscription assay using PCR with primers for citrus *ACTIN (ACT)* or *β-LYCOPENE CYCLASE (LYC)* (Table M2), and no amplified products were detected. The extracted RNA was stored at -80°C until further use.

5.2. *Gene expression analysis by RT-qPCR*

Transcripts present in 1 µg of total RNA were subjected to reverse transcription using the *PrimeScriptTM RT Reagent Kit* (*Perfect Real Time; TAKARA bio Europe*) in a total volume of 10 µl. For each amplification reaction, a 2 µl aliquot of four-fold diluted first-strand cDNA was used. Real-time quantitative PCR was performed on a *Rotor Gene Q 5-Plex (Qiagen, USA)* using the *TB Green® Premix Ex TaqTM PCR Kit (TAKARA bio Europe)*. The mix configuration and reaction conditions were adjusted according to the manufacturer's recommendations with some modifications. The PCR mix included 2 µl of diluted cDNA, 12.5 µl of *TB Green Premix Ex Taq II* (2X) (*TAKARA bio Europe*), 1 µl of 10 µM forward primer (F), 1 µl of 10 µM reverse primer (R), and 8.5 µl of sterile purified water to reach a final volume of 25 µl. The cycling protocol for amplification consisted of a 15-minute pre-incubation stage at 95°C, followed by 40 cycles of denaturation for 15

seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C. RT-qPCR reactions were performed three times for each gene and monitored in real-time using the Rotor Gene Detector. After amplification, a melting curve analysis was conducted to exclude potential unwanted amplifications. The relative expression of RNA transcripts was quantified using the threshold cycle (Ct) values obtained from each sample, following the 2^-DDCt method (Livak and Schmittgen, 2001). Expression levels were calculated relative to the reference gene *ACT* in adult leaves or *LYC* in buds (see Table M2). The choice of the reference gene depends on the tissue type and the sampling time. Actin is a gene associated with cell division processes, so its expression level in adult leaves is basal. However, in buds, the meristems are in development. Therefore, choosing actin could result in anomalous results. Hence, the reference gene chosen is lycopene, related to color change processes, which are not occurring at this time or in this tissue (Alquezar et al., 2009). The relative gene expression level was determined using the 2^-DDCt value and normalized to the lowest value among the samples for each experiment. Three independent biological samples were evaluated under each experimental condition, and three technical replicates were performed.

***Table M2**. Primer sequence used in RT-qPCR amplification reactions.*

Gene	ID	Description	Sequence (5'-3')	Reference
CiFT3	Ciclev10012905m	FLOWERING LOCUS T ortholog	F: AGACTGTTTATGCACCGGGG R: AGTTGAAGTAGACAGCGGCC	Mesejo et al., 2022
CiNF-YA1	orange1.1g017825m	nuclear factor YA	F: ACCAAGGAAATGCTAATGAGTG R: AATACGAATCGGAATACGGA	Zhou et al., 2022
CcMADS19	Ciclev10033420m	FLC ortholog	F: GGCAACTTGAAGGTCCAAAC R: GCCCAATGAGCATAGGAATG	Agustí et al., 2020
ATX1-like	Ciclev10031846m	Histone methyltransferase	F: GCAAATGTCTTGTGCTGGAA R: TGTGCTTCCTAGCATATCG	Mesejo et al., 2022
CLF-like	Ciclev10024826m	PRC2 protein member	F: GAGCGAATTAGTGCCGGAGA R: CACGACCACTTGAAGGACCA	Mesejo et al., 2022
VIN3-like	Ciclev10007575m	PHD protein member	F: CAATGGCCAGTGGAGGAAGT R: ACATCAAGCGGCATCACTGA	Mesejo et al., 2022
ELF6-like	Ciclev10030491m	Lysine-specific demethylase	F: CGTTGCCAAAACCCTCGAAG R: CCACGTTCACAACAACCCAC	Mesejo et al., 2022
CsLFY	Ciclev10033942m	LEAFY	F: TCTTGATCCAGGTCCAGAACATC R: TAGTCACCTTGGTTGGGCATT	Muñoz-Fam et al., 2011
CsAP1	Ciclev10032490m	AGAMOUS-LIKE MADS-BOX ROTEIN	F: CAAAACCAGGTTCCCAACAC R: ACGAACATACGGGTTCAAGG	Muñoz-Fam et al., 2011
CsTFL	Ciclev10013475m	TERMINAL FLOWER 1 ortholog	F: TCCGTCCACAGTTGTTTCAA R: TCACTAGGGCCAGGAACATC	Muñoz-Fam et al., 2011
CsCEN	Ciclev10029782m	Phosphatidylethanolamine protein	F: CCAGCTTCAAGGGATCGGTT R: GAGCAGCAGTCTCCCTTTGT	Zhang et al., 2021

Gene	ID	Description	Sequence (5'-3')	Reference
GA20ox2	Ciclev10020694m	Oxidoreductase family (Gas)	F: GGTGACACCTCCGAACAACT R: AATGCGTTGAGGGTTTTCAC	Mesejo et al., 2016
GA3ox1	Ciclev10027153m	Gibberellin 3beta-hydroxylase 1	F: CAACGCAAGATGTCAAATGG R: CAGGCCGGGTAGTAATTCAA	Mesejo et al., 2016
GA3ox2	Ciclev10010629m	Gibberellin 3beta-dioxygenase 2	F: CCTGTTGATGGTGCCCTAGT R: CCGCTTTTGATTCACAGACA	Mesejo et al., 2016
GA2ox2	Ciclev10024433m	Gibberellin 2beta-hydroxylase	F: GAAACATCGGCTTCAATGGT R: GCGCGACTGAATTTTAAAGG	Mesejo et al., 2016
GIDB1	orange1.1g019235m	Gibberellin receptor	F: CCGCCGCCTTGTTAACATTT R: AACCCACTTAAGAGCAGCCC	Unpublished data
DELLA-like	orange1.1g009607m	DELLA protein GRAS43	F: CCGCTGGCTAAATGGAGGAA R: AGTAACCCTCGGCCGAAAAG	Unpublished data
CYCB2	Ciclev10025564m	Cyclin	F: GAAAGCAGCAACAGGGAAGC R: TCCAAAAGAAATTGCGCCGG	Mesejo et al., 2022
CDKB2	Ciclev10002132m	Cyclin dependent kinase	F: GAGGTCTCCATCTTGCGCAT R: CGTCCTGCCTTCCTTGTTCT	Mesejo et al., 2022
TRN2	Ciclev10032401m	Tetraspanin protein	F: ACTCTACCTCGTTGCCATGC R: AGCTCTACTGGGTGGAAGGT	Unpublished data
YUC4	Ciclev10008466m	Indole-3-pyruvate monooxygenase	F: GGAAAGACGCCGGTACTTGA R: TCTTTCACACCACCCACCAC	Unpublished data
LAX3	Ciclev10001072m	Auxin influx carrier	F: GGATTTGCCTGCACTCCTCT R: GATGGGGCCAAAGAAAGGGA	Unpublished data
PIN3	orange1.1g007420m	Auxin efflux carrier	F: CTCTCTTTGCAGTCCCCTTG R: CCCTTTTGCTCAACTTGCTC	Mesejo et al., 2021
α-SnRK1	Ciclev10011600m	Serine-threonine kinase (subunit α)	F: TGCATCCCATTGAACCTGCT R: TGGATGGGCTCGAGACTGTA	Mesejo et al., 2019
β-SnRK1	Ciclev10016186m	Serine-threonine kinase (subunit β)	F: TGGGAAATGCAAACGGGAGA R: TGAATGATCACCGCCGTTGA	Unpublished data
γ-SnRK1	Ciclev10020027m	Serine-threonine kinase (subunit γ)	F: TGAAATACTGGCCCAACACA R: CAGCAATTCCTGCAAACTCA	Unpublished data
CWIN	Ciclev10025259m	Cell-wall Invertase	F: ACCATTTCCAGCCTCCCAAG R: GTTGCCCCATACTGCTCCTT	Unpublished data
CIN	Ciclev10003220m	Cytosolic Invertase	F: TGAAGCACGATGGGAGGAAC R: GACTGGCCAAGAACCTCCAT	Unpublished data
VIN	Ciclev10019134m	Vacuolar Invertase	F: CAATGGCCAGTGGAGGAAGT R: ACATCAAGCGGCATCACTGA	Unpublished data
SUS1	orange1.1g003661m	Sucrose Synthase	F: CAAATCCTCATTATTACCCGTCTC R: ACTTGCGGACAACTCCCTTC	Hussain et al., 2020
α-AMY	Ciclev10001266m	alpha – amylase	F: ACCGAACTGCAGAGAGGAAA R: ATGTTCCACCCTCGAAAATG	Mesejo et al., 2019
ACT	Ciclev10025866m	Reference gene	F: TTAACCCCAAGGCCAACAGA R: TCCCTCATAGATTGGTACAGTATGAGA	Mesejo et al., 2022
LYC	Ciclev10025866m	Reference gene	F: GAACCAGGAGCTTAGGTCTG R: GCTAGGTCTACAACAAGGCC	Alquezar et al., 2009

	Flowering
	Gibberellin metabolism
	Cell division
	Auxin metabolism
	Carbohydrate metabolism

5.3. *Sequence analysis and phylogenetic trees*

The sequences of *CiFT3, CcMADS19, ATX1, CLF, VIN3* and *ELF6* (Mesejo et al., 2022) were used to study floral induction processes in leaves. In addition, the sequence *NF-YA1*, recently implicated in FT protein transcription (Zhou et al., 2022), was included. Furthermore, genes involved in floral organogenesis, such as *CsLFY* and *CsAP1*, along with the repressor Cs*CEN and CsTFL* (Muñoz-Fambuena et al., 2019; Zhang et al., 2021), were selected to study the differentiation stage. Sequences *GA20ox2, GA3ox1, GA3ox2* and *GA2ox2* (Mesejo et al., 2016) and *GIDB1 and DELLA* were selected to study the metabolism of gibberellins (GAs). On the other hand, genes of cyclins and cyclins dependent kinases from the cell cycle (*CYCB2, CDKB2*), genes involved in auxin metabolism and transport (*TRN2, YUC4, LAX3*), and genes involved in carbohydrate metabolism (*SnRK1, CWINV, CIN, VIN, SUS1, α-AMY*) were obtained from the NCBI database (*www.ncbi.nlm.nih.gov*). Sequences were aligned to the *Citrus clementina* genome using the TBLASTN tool from the *Phytozome v13* database (*www.phytozome-next.jgi.doe.gov*). Based on this sequence similarity, some putative homologues of these genes were identified in the genomes of *Citrus clementina* and *Citrus sinensis*.

The sequence of the auxin transporter *PIN (orange1.1g007420m)* has been used in recent *Citrus* studies (Mesejo et al., 2021; 2022). The PIN transporter family is quite large. In this work, this *PIN (Cs2g16620.1; orange1.1g007420m)* was characterised on the basis of similarity to identified amino acid sequences from *Arabidopsis thaliana, Citrus clementina, Citrus sinensis, Malus domestica* and *Oryza sativa*. The selected sequence, *orange1.1g007420m*, was found to be an ortholog of *PIN3 (AT1G70940; Arabidopsis thaliana)*. The primers used for the qRT-PCR analysis are listed in Table M2.

5.4. *Metabolomic analysis (Carbohydrates & Aminoacids)*

Three replicates per treatment were taken at the time of flowering induction (03/12/18). The samples were ground using liquid nitrogen. Approximately 100 ± 5 mg of each replicate were used to measure carbohydrates, amino acids, and polyamines to study the role of these compounds in the floral initiation mechanism.

A total of 11 primary metabolites directly involved in growth, development and reproduction of the organism were measured. These included sugars (fructose, glucose, glucose-6P, sucrose, maltose and trehalose), amino acids (tryptophan, glutamine and methionine) and polyamines (spermidine and putrescine). Metabolite quantification was performed at the Metabolomics Service of the Instituto de Biología Molecular y Celular de Plantas (IBMCP). The protocol used involves the analysis of volatile compounds (GC-MS) in plant tissues by gas chromatography coupled to mass spectrometry (Roessner et al., 2000). This analysis includes sample extraction and derivatisation, as well as a targeted analysis involving the identification and relative quantification of the 11 metabolites mentioned, available in the database of the IBMCP.

5.5. *In situ Hibridization*

Probes were designed using a fragment of 372 bp from the cDNA region of the *CEN* gene *(CENTRORADIALIS; Ciclev10029782m)*. The fragment was amplified by PCR and sense and antisense probes were synthesised using SP6 and T7 RNA polymerase (Table M3). Control experiments were performed in which expression was detected in the sense probes, indicating CEN expression. The antisense probes showed no expression.

Citrus sinensis *'Lane late'* buds with fruit (ON) were harvested on 1 September, when the relative expression quantified by RT-qPCR was highest. They were immediately fixed in 3.7% FAA and vacuum pumped to ensure thorough penetration of the reagent into the buds (every 5 minutes, 3 times). They were then dehydrated through a series of tertiary butyl alcohol up to 70% v/v and stored at -20°C until the next dehydration step. Dehydration was continued (70%, 85%, 95% and 100% v/v) and the samples were embedded in paraffin. The paraffin-embedded material was sectioned at 6-8 mm using a Microm HM330 rotary microtome, and the obtained sections were rehydrated prior to staining (3 washes in Histoclear [CellPath, Hemel, UK], one in Histoclear:ethanol [1:1, v/v], and a series of ethanol [100%, 70% and 40%, v/v]). The 6-8 mm thick sections were mounted on polysine slides (*Menzel-Glaser*).

The *in situ* hybridization procedure is based on the method developed by Jackson (1992) and optimised for Arabidopsis reproductive tissues (Ferrándiz et al., 2020;

2021). Antisense RNA probes labelled with digoxigenin (negative control) and sense RNA probes were synthesised using T7 and SP6 RNA polymerases. After hybridization, the slides were washed twice for 1 hour in 2× SSC, once for 1 hour in 1× SSC and for 30 minutes in 0.5× SSC. The digoxigenin-labelled probe was detected using alkaline phosphatase anti-digoxigenin antibodies diluted 10:1000 and NBT/BCIP as substrate. The reaction was stopped with sterile *Milli-Q* water and the slides were visualised and photographed using a bright field microscope. No antibody activity was detected in the control *in situ* hybridization using the sense probe.

***Table M3**. Probe sense and anti-sense CsCEN gene for in situ hybridization analysis.*

Oligo Name	***Sequence 5'-3'***
CsCEN Left	ATTTAGGTGACACTATAGACTCCATCTGTGAAAATGA
CsCEN Right	TAATACGACTCACTATAGGGTTAAACCGATCCCTTGAA

Red: SP6 sequence

Green: T7 sequence

5.6. *RNA-seq. Whole Transcriptome Sequencing*

The RNA-seq study was carried out on '*Nadorcott*' mandarin trees, taking leaf and bud samples from four different treatments with different flowering ability. RNA quality was first assessed using the *Agilent Eukaryote Total RNA Nano Kit*. Libraries were generated using the *TruSeq RNA Sample Prep Kit (Illumina)*. Mass sequencing of the library was performed on the *Illumina HiSeq 2500 system* at the Centre for Genomic Regulation (CRG; Barcelona). The quality of the raw reads was then analysed using the *FastQC v.0.10.1 tool (http://bioinformatics.babraham.ac.uk/projects/fastqc/)*. The reads were aligned against the latest version of the *Citrus clementina* reference genome *(www.citrusgenomedb.org/organism/Citrus/clementina)*. Finally, the *Bioconductor program edgeR v.3.12.1* on the *R platform v.3.2.2* (Robinson et al., 2010) was used to visualise the distribution of the raw data, normalise and quantify the differential

expression. Data normalisation was performed using the Reads Per Kb of transcript per Million mapped reads (RPKM) method. Statistical analysis was performed using *Student's t-test* ($p < 0.05$).

5.6.1. *Enrichment Analysis and Gene Ontology Term Assignment*

The genes with differential expression were examined to assign *Gene Ontology (GO)* terms and determine their distribution in the categories of *Molecular Function (MF), Biological Process (BP)* and *Cellular Component (CC)*. These results were graphically presented in a horizontal bar chart based on the enrichment of the categories and the number of differentially detected genes in each category. The genes detected in the *Citrus clementina* genome were mapped to the ID of the model plant *Arabidopsis thaliana* to enable categorisation using *ShinyGO 0.77 software (bioinformatics.sdstate.edu/go/)*. In addition, the main *KEGG pathways* were determined for the differentially expressed genes. For a more in-depth analysis of the different comparisons generated from the libraries, different tools and visualisation methods were used, including *Venn diagrams (bioinfogp.cnb.csic.es/tools/venny/), Circos diagram,* heat maps *(www.heatmapper.ca/)* and transcription factor (TF) prediction *(bioinformatics.psb.ugent.be/webtools/TF2Network/).*

5.7. *Hormone isolation, purification, and quantification*

Material (leaves, buds and phloem) frozen in liquid nitrogen was ground to a fine powder. Aliquots (approximately 50 mg fresh and dry weight) of the 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 the quantification of the different phytohormones were the deuterium labelled hormones. The extraction protocol used was that described in Seo et al. (2011) with some modifications. Briefly, for desalting, the extracts were passed through HLB reverse phase columns (Waters). The phytohormones were eluted with 80% methanol containing 1% acetic acid and subsequently applied to MCX cation exchange columns (Waters). The fraction containing acidic hormones was applied on ion exchange WAX columns (Waters). The final residue was dissolved in 5% acetonitrile-1% acetic acid and the

hormones were separated using an autosampler and reverse phase UPHL chromatography (2.6 µm Accucore RP-MS column, 50 mm 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. Hormones were analysed by selected ion monitoring (SIM) using a *Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific*). Hormone concentrations in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs. Quantification was carried out at the IBMCP hormone quantification service.

5.8. *Chromatin immunoprecipitation*

ChIP was performed as described previously (Agustí et al., 2020). The crude nuclear pellet was resuspended in nuclear lysis buffer and sonicated in a Covaris M220 focused ultrasonicator for 8 min at 6ºC with a 5% duty cycle. The soluble chromatin solution was incubated with 1 µg anti-H3K27me3 (Millipore 07-449) for 4 h, and chromatin-antibody complexes were captured with protein A/G magnetic beads (Thermo Scientific). The destaining reaction was performed with Chelex slurry (Biorad) as described. To identify the H3K27me3 regulated regions, we first divided the *CcMADS19* promoter (5000 bp) and genomic region (13800 bp) into bins of 1000 bp and designed primers to amplify approximately 180 bp within each bin. A total of 19 pairs of primers were tested by qPCR against the input. We then performed a comparative analysis between induced and non-induced samples. This analysis was carried out by Dr Miguel de Lucas from the University of Durham (UK).

6. Experimental Design

6.1. *Experiments from Chapter I & II. Gibberellins*

In order to demonstrate whether the fruit inhibits flowering by gibberellins (GAs), it was studied using various experimental approaches: 1) the effect of GA application on flowering gene expression; 2) the relationship between the presence of fruit and gibberellin synthesis, signalling and concentration.

- ***Experiment I:*** *Effect of repeated applications of GA and PBZ during the floral induction and differentiation periods on the expression of the CiFT3 and CcMADS19 genes and on flowering control.*

Six OFF and six ON *'Lane late'* orange trees were selected, from which 200 OFF and 200 ON shoots, respectively, were selected. Gibberellic acid (GA_3, 25 mg l^{-1}) and the GA synthesis inhibitor paclobutrazol (PBZ, 50 mg l^{-1}) were applied locally to the shoots to inhibit and promote flowering, respectively. The first treatment was applied at the end of October (29/10/2020) and repeated weekly for a total of 9 times.

Shoot and spring flowering were quantified in 30 shoots per treatment, and 12 shoots per treatment were sampled on three different dates: the start of treatment (29/10/2020), the flower induction phase (17/12/2020) and the flower differentiation phase (19/01/2021). For each treatment, the expression of genes responsible for promoting/inhibiting flowering (*CiFT3, CcMADS19, NF-YA1, CsLFY, CsAP1*) was determined in leaves and buds by RT-qPCR. Table E1 summarises the experimental design.

Table E1*. Design of experiment I. GA_3 (25 mg l^{-1}) and PBZ (50 mg l^{-1}) were applied weekly up to 9 times to ON and OFF branches of 'Lane late' sweet orange. Treatments started on 29 October. Leaf and bud samples for RT-PCR analysis were collected on 29th October, 17th December, and 19th January.*

Treatments	**N**	
	Phenotype evaluation	**RT-PCR**
ON	30	66
ON+GA_3	30	66
ON+PBZ	30	66
OFF	30	66
OFF+GA_3	30	66
OFF+PBZ	30	66
Total	180	396

- ***Experiment II:*** *Generalization of the response to GA_3 application on the expression of the CiFT3 and CcMADS19 genes during the floral induction period in the main citrus species.*

Sixty OFF shoots were selected for each citrus species (Lemon, Grapefruit, Clementine Mandarin, Sweet Orange, and Mandarin), uniformly distributed across the trees (one per species). Half of the shoots were treated with GA3 at a concentration of 50 mg l^{-1} on 28th November 2020, while the other half of the shoots were left untreated as a control.

Periodic sampling of three leaves per date and species was performed to determine the expression of the *CiFT3* and *CcMADS19* genes. In Sweet Orange, samples were also taken at 12, 24 and 48 hours after treatment to assess the immediate effect of the treatment. Shoot and flowering were assessed the following spring on 30 shoots per treatment. Table 2E summarises the experimental design.

Table E2*. Design of experiment II. GA_3 (50 mg l^{-1}) was applied to OFF branches of C. limon, C. paradisi, C. clementina, C. unshiu and C. sinensis. Leaf samples for RT-PCR analysis were collected on the day of treatments 28th November, and weekly during December.*

Treatments for each species	**N**	
	Phenotype evaluation	**RT-PCR**
OFF	30	30
OFF+GA_3	30	30
Total	60	60

- ***Experiment III:*** *Effect of localized application of GA_3 to leaves and buds, before, during, and after flowering induction, on the expression of the CiFT3 and CcMADS19 genes and flowering control.*

The interaction between the temporal effect of GA_3 treatment (before, during and after the floral induction period) and the spatial effect (leaf or bud) on flowering and the expression of floral induction regulatory genes (*CiFT3, CcMADS19* and the transcription factor *NF-YA1*) and meristem identity genes (*CsLFY* and *CsCEN*) was studied.

Six ON and six OFF '*Lane late*' orange trees were selected, from which 45 ON and 520 OFF shoots were selected. Treatments were applied before flower induction (6/9/2021), during flower induction (24/11/2021) and during flower differentiation, before sprouting (13/01/2022). At each date, GA3 (25 mg l^{-1}) was applied locally to the leaf, bud or sprayed on the branch, for a total of 40 shoots per treatment.

Samples were taken from 12 shoots per treatment and date 48 hours after each application. The remaining shoots, 20 per treatment, were used to assess flowering the following spring. Table E3 summarises the experimental design.

***Table E3**. Design of experiment III. GA_3 (25 mg l^{-1}) was applied before, during or after the flower induction period to OFF branches of 'Lane late' sweet orange. Treatments were applied locally to the leaf, bud, or the whole branch. Dates of treatments 6/9, 24/11 and 13/1. Leaf and bud were sampled for RT-PCR analysis 48h after treatment.*

Treatments for each date	**N**	
	Phenotype evaluation	**RT-PCR**
ON	30	12
OFF	30	12
OFF+GA_3 (to the leaf)	30	12
OFF+GA_3 (to the bud)	30	12
OFF+GA_3 (to the branch)	30	12
Total	150	60

- ***Experiment IV:** Study of the evolution of gibberellin synthesis and content in leaves of OFF trees in relation to the expression of the CiFT3 and CcMADS19 genes.*

The evolution of endogenous GA content was studied in OFF branches of '*Nadorcott'* mandarin throughout the period of floral induction and differentiation. This study was carried out in relation to the expression of the *CiFT3* and *CcMADS19* genes, as well as the regulatory enzymes of the GA biosynthesis pathway, *GA3ox2* and *GA20ox2*, in leaves. Sixty OFF shoots were selected and six leaves were randomly sampled once a month from the first week of September 2020 to February 2021. All shoots flowered the following spring.

- ***Experiment V:*** *Effect of time of fruit on tree on gibberellin metabolism in buds and its relation to flowering.*

In experiment V, 40 *'Nadorcott'* mandarin trees were used, with 130 OFF shoots and 450 ON shoots selected and marked in July 2020. In July (21/7), September (7/9), November (10/11) and January (11/1), fruit was removed from 78, 66, 54 and 42 ON shoots, respectively, using pruning shears. Samples were collected 48 h after each thinning, consisting of 12 shoots per treatment and an additional 12 shoots from the previous thinning dates. The study included the expression of genes responsible for meristem identity (*LFY* and *AP1*), GA synthesis and signalling (*GA20ox2, GA3ox2, GIDB1* and *DELLA*), and the concentration of GAs and indole-3-acetic acid (IAA). In addition, shoot and flowering were evaluated in spring (15/03/2021). The table E4 gives a summary of the experimental design.

Table E4*. Design of experiment V. Timing of the inhibitory effect of fruit on GA synthesis and signaling. Fruit thinning was applied to Nadorcott mandarin ON shoots and buds were sampled 48h after thinning and along the following thinning dates. Flowering was evaluated in Spring.*

Treatments for each date	**N**	
	Phenotype evaluation	**RT-PCR and hormone analysis**
ON	30	96
OFF	30	96
ON+ fruit thinning (July)	30	96
ON+ fruit thinning (September)	30	72
ON+ fruit thinning (November)	30	48
ON+ fruit thinning (January)	30	24
Total	180	432

- ***Experiment VI:*** *Evaluation of the hormonal content of the fruit exudate.*

Four *'Nadorcott'* mandarin trees were selected, and in November (induction) and December (floral differentiation), two complete branches with a high fruit load were cut and transported refrigerated (4°C) to the laboratory. Nine fruits were cut with a scalpel, leaving about 5 cm of the peduncular zone. With the fruit placed upside down,

the peduncular section was immersed in an extraction buffer (5mM EDTA). The fruits remained in a sterile environment at room temperature (20°C) for 24 hours. Using a 200 µl pipette, the buffer, together with the substances released by the fruit, was collected, frozen and lyophilized. The concentration of GAs, jasmonic acid (JA), IAA, and cytokinins (CKs) was determined.

- ***Experiment VII:*** *GA signaling in ON and OFF buds.*

Five ON and five OFF late orange trees were selected. To determine the effect of fruit presence on GA signalling, shoots from both ON and OFF trees were sampled between the beginning of the inductive period (November) and the floral differentiation period (January). At each time point, 12 complete shoots per treatment were sampled. The expression of the genes *GIDB2, DELLAs, TFL1* and *LFY* was analysed.

- ***Experimento VIII****. Time-course and spatial localization of the expression of flowering genes in the buds of ON and OFF trees.*

Ten ON and ten OFF trees of *'Lane late'* orange and *'Nadorcott'* mandarin were selected for a long-term study to investigate the effect of fruit on the expression of the *LFY, CYCB2, TFL1* and *CEN* genes. Monthly samples of 12 shoots per treatment and date were taken from June to January. In addition, to determine the expression site of these genes in the meristem using *in situ* hybridization, 20 axillary buds were taken per treatment and date. These buds were chemically fixed with FAA at 8°C for 24 hours and stored in an 80% ethanol solution at -30°C.

6.2. *Experiments from Chapter III. Auxins*

In order to demonstrate whether the fruit inhibits flowering through auxins, it was studied using different experimental approaches: 1) the effect of the application of 2,4-D or TIBA on the expression of flowering genes; 2) the relationship between the presence of fruits and the synthesis, transport and concentration of IAA.

- ***Experiment IX:*** *Effect of 2,4-D application during the induction period on the expression of the CcMADS19 and CiFT3 genes in leaves and their relationship with flowering.*

In lemon tree *'Eureka'*, sweet orange tree *'Navelina'* and clementine mandarin tree '*Oronules*', 40 OFF branches were marked. Half of them were treated with 12 mg l^{-1} 2,4-D dimethylamine salt (2,4-dichlorophenoxyacetic acid) (10% w/v) in November and the other half was used as control. To evaluate the effect of application on the *CiFT3* and *CcMADS19* genes, 3 leaves (1 leaf per biological replicate) from each treatment, date and species were sampled every 15 days until the onset of flower differentiation in January. Sprouting and flowering were assessed the following spring. Table E5 summarises the experimental design.

Table E5*. Design of experiment IX. 2,4-D (12 mg l^{-1}) was applied to OFF branches of C. limon, C. clementina, and C. sinensis. Leaf samples for RT-PCR analysis were collected on the day of treatments, 25th November, and every other week during December and January.*

Treatments for each species	Number of branches for phenotype evaluation and sampling
OFF	20
OFF+2,4-D	20
Total	40

- ***Experiment X:*** *Effect of repeated applications of 2,4-D and TIBA during the periods of flower induction and differentiation on the expression of the CiFT3 and CcMADS19 genes and flowering control.*

The experiment was conducted on 20 Valencia Late orange trees. 70 ON shoots and 210 OFF shoots were selected for the study. Out of the 210 OFF shoots, 70 were used as a control, another 70 were treated with 2,4-D (25 mg l^{-1}), and the remaining 70 were treated with *2,3,5-triiodobenzoic acid* (TIBA; 1g l^{-1}). The treatments were started before the floral induction period on 31st October 2022, and were repeated every 20 days in November, December, and January (4 treatments in total). 12 shoots were sampled per treatment 48 hours after each application. The effects of the treatments on

the expression of the genes *CiFT3* and *CcMADS19* in leaves, *LFY* and *TFL1* in buds, and genes responsible for auxin homeostasis and transport, also in buds, were assessed. Bud emergence and flowering were assessed the following spring. Table E6 summarises the experimental design.

***Table E6**. Design of experiment X. 2,4-D (12 mg l^{-1}) and TIBA (1 g l^{-1}) were applied every 20 days up to 4 times to OFF branches of Valencia late sweet orange. Treatments started on 31st October. Leaf and bud samples for RT-qPCR analysis were collected 48h after each treatment.*

Treatments	**N**	
	Phenotype evaluation	**RT-PCR**
ON	22	48
OFF	22	48
OFF+2,4-D	22	48
OFF+TIBA	22	48
Total	88	192

- ***Experiment XI:** Study of the auxin-gibberellin relationship in the mechanism of flowering inhibition in buds.*

Ten ON and OFF '*Oronules*' clementine trees were initially selected as controls, and on 15th November another 10 trees were treated with GA_3 at a dose of 25 mg l^{-1}, while another 10 trees were treated with *2,4-D dimethylamine salt* at 12 mg l^{-1}. For each treatment, 12 shoots were sampled on four different dates, from the time of treatment to one and a half months later. The study focused on assessing the effect of these treatments on the expression of flowering regulatory genes such as *LFY, AP1* and *CEN*, as well as *GA20ox* and *GA3ox* genes involved in gibberellin synthesis, *TRN2* and *YUC4* genes involved in auxin synthesis, *LAX3* and *PIN3* genes involved in auxin transport and *CYCB2* involved in cell division. Bud sprouting and flowering were assessed the following spring on a branch with at least 200 nodes, representative of the flowering intensity of each tree. Table E7 summarises the experimental design.

***Table E7**. Design of experiment XI. 2,4-D (12 mg l^{-1}) and GA3 (25 mg l^{-1}) were applied once to OFF branches of 'Oronules' Clementine mandarin trees. The treatment was made on 15th November. Bud samples for RT-qPCR analysis were collected 4 times from the date of treatment until 45 days later.*

Treatments	**Number of branches**	
	Phenotype evaluation	**RT-PCR**
ON	10	48
OFF	10	48
OFF+2,4-D	10	48
OFF+GA_3	10	48
Total	40	192

- ***Experiment XII:** Effect of fruit presence on auxin synthesis, transport and concentration.*

Ten hybrid '*Nadorcott*' mandarin trees, 5 with fruit (ON) and 5 without (OFF), were selected for the study. To determine the effect of fruit presence on the endogenous mechanism of auxin regulation and response, 12 shoots from both ON and OFF trees were sampled at the beginning of the floral differentiation period in November. Subsequent samples were taken every 15 days until January. One sample from December (on 20/12) was duplicated to measure the concentration of IAA in axillary buds. The study assessed the concentration of IAA and the expression of genes related to floral differentiation (*LFY*), auxin synthesis (*TRN2* and *YUC4*), and auxin transport (*PIN3*).

6.3. *Experiments from Chapter IV. Girdling*

- ***Experimento XIII**. Effect of apical girdling (A-G) on bud break and flowering. Epigenetic silencing of CcMADS19 in vegetative buds.*

The aim of this experiment was to disrupt phloem transport between fruit and axillary buds and determine the effect on 1) sprouting and flowering and 2) flowering promoter and inhibitor signals in leaves and buds. In 10 '*Nadorcott*' hybrid mandarin trees, 375 ON shoots and 125 OFF shoots were marked. The 125 ON shoots were ringed with a scalpel by removing a 3-5 mm wide ring of bark between the calyx and the first axillary bud. This was referred to as apical girdling (A-G). Care was taken not to damage the xylem. The treatment was carried out in July ('A-G Jul') and September ('A-G Sep'). In the days following girdling and in the following spring, sprouting and flowering were assessed on 50 shoots per treatment. From October to January, leaves from ON, OFF and A-G shoots, as well as from new vegetative shoots produced in autumn as a result of girdling, were sampled for mRNA extraction and quantification of the expression of *CiFT3* and *CcMADS19* and their homologous genes in Citrus, which are responsible for the epigenetic regulation of FLC in Arabidopsis*: CcVIN3, CcATX1, CcCLF, CcELF6*. Finally, the level of H3K27me3 was determined in the same leaves by chromatin immunoprecipitation (ChIP) in two regions of the promoter of the *CcMADS19* locus. Table E8 summarises the experimental design.

Table E8*. Design of experiment XIII. Apical girdling (A-G) was applied to ON shoots of 'Nadorcot' mandarin trees to promote bud sprouting and flowering. Ungirdled ON shoots and OFF shoots were used as control. The treatment was made in July and September. Bud samples for RT-qPCR analysis were collected monthly from the date of treatment until January.*

Treatments	**Number of shoots**	
	Phenotype evaluation	**RT-PCR**
ON	50	72
OFF	50	72
ON+ apical girdling (July)	50	72
ON+ apical girdling (September)	50	60
Total	200	276

- ***Experiment XIV.*** *Isolation of axillary buds from the influence of the fruit and the rest of the plant. Apical girdling (A-G), basal girdling (B-G), and double girdling (AB-G).*

The aim of this experiment was to disrupt phloem transport between the fruit and the axillary buds, and between the axillary buds and the rest of the tree, and to determine its effect on 1) sprouting and flowering, and 2) the promoters and inhibitors of flowering in leaves and buds. The experiment was conducted on 20 *'Nadorcott'* hybrid mandarin trees. In September 2018, 320 ON shoots and 80 OFF shoots were marked. Girdling of 80 ON shoots was done as described above (apical girdling, A-G). In addition, 80 ON shoots were ringed between the last bud and the main branch (basal girdling, B-G) and 80 ON shoots were ringed in both previous positions (double girdling, AB-G).

Leaf samples were collected every two weeks (7 samples) to study the effect of treatments on amino acid and carbohydrate content by metabolomics, as well as the expression of the genes *CiFT3* and *CcMADS19*. Complete shoot samples (24 shoots per treatment) were also collected at the time of flowering induction (12/03/18), where buds, phloem and leaves were separated. Six biological replicates were obtained, 3 of which were freeze-dried for amino acid, carbohydrate and hormone analysis (IAA, GAs, ABA and CKs), and the other 3 biological replicates were used to analyse the expression of the genes *LFY, AP1, CYCB2, CDKB2, TFL1, CEN, PIN3, GA20ox, GA3ox, GA2ox, SNRK1, α-AMY, CWIN, CIN, SUS1, VIN* by RT-qPCR. Finally, flowering and sprouting were evaluated in spring (13/03/19). Table E9 summarises the experimental design.

Table E9*. Design of experiment XIV. Apical girdling (A-G), basal girdling (B-G) and double girdling (AB-G) was applied to ON shoots of 'Nadorcot' mandarin trees to promote bud sprouting and flowering. Ungirdled ON shoots and OFF shoots were used as control. The treatment was made in September. Bud samples for hormones and RT-PCR analysis were collected periodically from the date of treatment until January.*

Treatments	**Number of shoots**	
	Phenotype evaluation	**Hormones and RT-PCR**
ON	30	50
OFF	30	50
ON+ apical girdling (A-G)	30	50
ON+ basal girdling (B-G)	30	50
ON+ double girdling (AB-G)	30	50
Total	150	250

- ***Experiment XV.*** *Effect of double girdling (AB-G) performed before, during, and after the floral induction period.*

The aim of this experiment was to determine the influence of the timing of double girdling (AB-G) on flowering. Twenty *'Nadorcott'* hybrid mandarin trees were selected and 240 ON shoots and 60 OFF shoots were marked in September 2021. Double girdling (AB-G) of the ON shoots was carried out in September, December and January as described above, with 60 shoots for each date.

At all three dates, the short-term effects of AB-G were determined by sampling the shoots 8 days after treatment and assessing hormone concentrations and flowering gene expression. In addition, two further samples were taken in December (3/12, 20/12) and in January net leaf photosynthesis was measured 2 and 24 hours after AB-G treatment using the portable photosynthesis system (*Li-COR*). The first measure was taken at midday. Flowering and sprouting of all treatments were assessed in the following spring. Table E10 summarises the experimental design.

Table E10*. Design of experiment XV. Double girdling (AB-G) was applied to ON shoots of 'Nadorcot' mandarin trees to promote bud sprouting and flowering. Ungirdled ON shoots and OFF shoots were used as control. The treatment was made in September, December, and January. Bud samples for hormones and RT-qPCR analysis were collected periodically from the date of treatment until January.*

Treatments	**Number of shoots**	
	Phenotype evaluation	**Hormones and RT-PCR**
ON	20	40
OFF	20	40
ON+ double girdling (Sept)	20	40
ON+ double girdling (Dec)	20	40
ON+ double girdling (Jan)	20	40
Total	100	250

- ***Experiment XVI.*** *Massive transcriptome sequencing (RNA-Seq) of flowering phenotypes (OFF shoots, AB-G, and SF) vs. ON shoots.*

Although the double girdling technique promotes flowering in all axillary buds of the shoot, similar to an OFF shoot, its mechanism of action may differ significantly from the natural process of flower induction for two reasons: 1) the injury caused by the interruption of phloem transport, and 2) the presence of the fruit during the induction period. Therefore, in order to gain a comprehensive understanding of the pathways that AB-G modifies to promote flowering, while excluding other effects of the technique, RNA-seq analysis was performed during the floral induction period with samples from four phenotypes (1 non-flowering, 3 flowering) compared to each other: 1) ON shoot with a spring fruit, incapable of flowering the following spring; 2) OFF shoot without fruit, which flowers naturally; 3) ON shoot with girdling (AB-G 'Sep'), which flowers like the OFF shoot; and 4) shoot with a small autumn fruit from a late summer flowering (SF), which flowers in spring but with less intensity.

The study was carried out on leaves and buds of the tangor '*Nadorcott'.* Sampling was carried out during the induction period, when the temperature began to fall (5th December 2019). The large-scale sequencing was carried out at the National *Centre for Genomic Analysis* (*CNAG-CRG*) in Barcelona, Spain. Table E11 summarises the experimental design.

Table E11*. Design of experiment XV. Double girdling (AB-G) was applied to ON shoots of 'Nadorcot' mandarin trees to promote bud sprouting and flowering. Ungirdled ON shoots and OFF shoots were used as control. The treatment was made in September, December, and January. Bud samples for hormones and RT-qPCR analysis were collected periodically from the date of treatment until January.*

Treatments	**Number of shoots**	
	Phenotype evaluation	**RNA for RNA-seq**
ON	20	12
OFF	20	12
ON+ double girdling (Sept)	20	12
ON+ small summer fruit (SF)	20	12
Total	100	68

7. Statistical analysis

In all cases, randomised block experiments were designed with a minimum of three blocks and three replicates per treatment. In addition, all analyses (gene expression, metabolomics, and hormone quantification) included three additional technical replicates.

Results were statistically evaluated using analysis of variance (ANOVA) or regression analysis, with a confidence interval between $P = 0.01$ and $P = 0.05$, and the least significant difference (LSD) was used to separate means. Percentages were transformed (√p) to normalise the sample. All experiments were analysed using *Statgraphics Centurion XVI software (Statistical Graphics, Englewood Cliffs, NJ, USA)*.

Results

Chapter I. *Gibberellic acid and fruit do not affect the same mechanism in Citrus flowering inhibition. Effect in the leaf*

In *Citrus*, the fruit is the main inhibitor of flowering induction. As the fruit reaches its final size and the colour change begins, the fruit activates the flowering inhibitor *CcMADS19*, repressing the expression of the flowering time genes. However, it is not known which signal throughout the fruit activates this repressive process. It has been suggested that this action of the fruit may be mediated by GAs. During fruit ripening, GAs, particularly GA_4, are transported to the plant and this generally coincides with the period of flowering induction, during which application of GA_3 reduces the expression of *CiFT3* and, consequently, subsequent flowering. The late ripening varieties have major problems with alternate bearing because the fruit is present throughout the induction period. For this reason, we used *'Lane late'* sweet orange and '*Nadorcott*' mandarin trees as an experimental model. The colour change of the fruit stars in November and the harvest lasts until February-March.

Therefore, the aim of this chapter is to determine the specific role of GAs during the floral induction process.

1.1. Effect of Gibberellic Acid (GA_3) and Paclobutrazol (PBZ) treatments on Citrus flowering. The fruit interference

Gibberellic acid (GA_3, 25 mg l^{-1}) was applied repeatedly (9 treatments) to OFF branches of '*Lane late*' sweet orange during flowering inductive period. Applications started on October 29th and finished on January 13th. Moreover, ON branches were treated with PBZ (1000 mg l^{-1}). If the fruit acts via GAs, this treatment may induce flowering. GA_3 and PBZ were also applied to ON and OFF branches, respectively, for comparison. Untreated ON and OFF trees were used as controls.

In the OFF branches, GA_3 treatments reduced flowering by 70% compared to the control (170 and 657 flowers per 100 nodes in GA and control branches, respectively) (Fig. 1.1A). Paclobutrazol, however, significantly increased flowering by 12%. The fruit

also reduced flowering, but to a greater extent, almost eliminating it (Fig. 1.1A). Furthermore, the fruit almost cancelled out the effect of paclobutrazol as well. This effect, both in GA_3 and in fruit, was paralleled to that on bud sprouting, although the fruit does not get to nullify it (Fig. 1.1B). GA_3 significantly reduced bud sprouting even in the presence of the fruit (Fig. 1.1B).

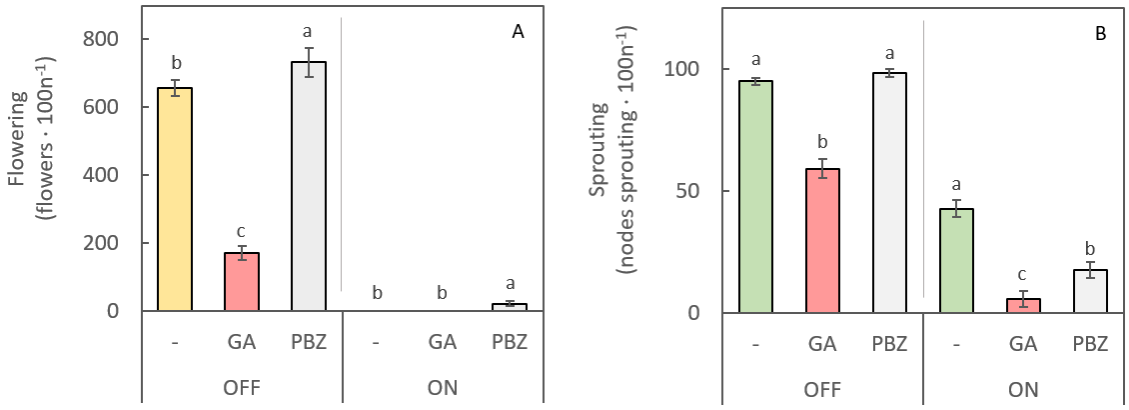


***Figure 1.1**. Effect of GA_3, PBZ and the presence of the fruit on flowering and sprouting intensity of 'Lane late' sweet orange. ON (with yield), OFF (without yield), GA (25 mg l^{-1} of gibberellic acid) and PBZ (50 mg l^{-1} of paclobutrazol). Treatments were started at the end of October (29/10/2020) on 70 OFF and ON shoots, (with a minimum of 5 axillary buds), distributed on 6 trees per treatment. Treatments were repeated weekly (for a total of 9 applications). A: flowering intensity. B: sprouted nodes. Each value is the mean of 30 shoots per treatment. Flowering and sprouting were evaluated in spring. The experiment was carried out in València, Spain. Different letters indicate significative differences.*

The effect is not species dependent. All *Citrus* species used in our experiment responded when GA_3 was applied once in the flowering inductive period as in the experiments of Guardiola et al. 1977, García-Luis 1986 and Martínez-Fuentes et al. 2003 (Fig. 1.2A). The GA_3 treatment also modified the type of shoots, as found by Agustí et al., 1980 in orange. Leafless flowering shoots were more sensitive to reduction by GA_3 than leafy shoots, whereas vegetative shoots increased with the treatment, regardless of the ssp. (Fig. 1.2B).

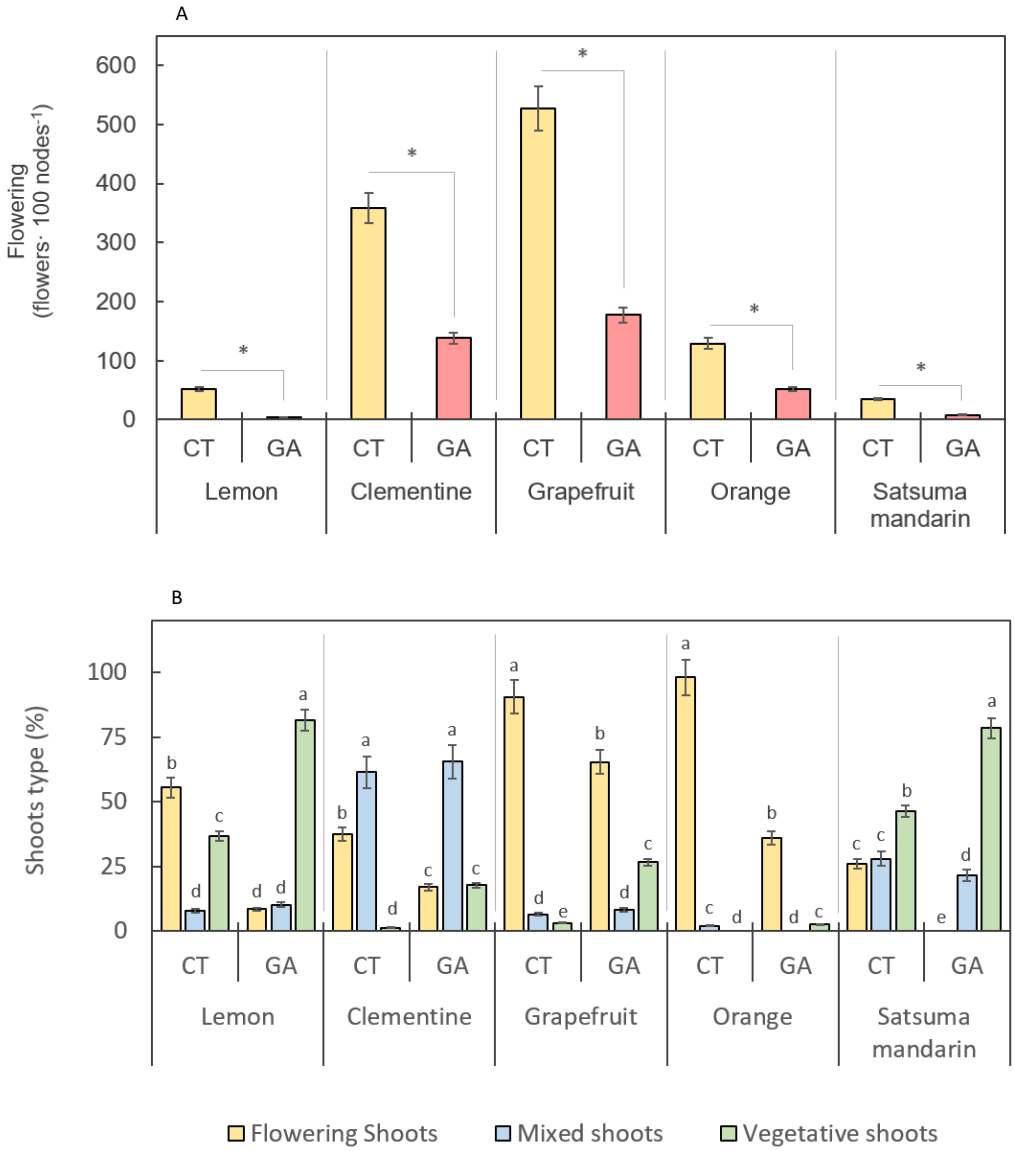


Figure 1.2. *Effect of GA on flowering intensity and the type of shoots formed of 5 citrus species. CT (OFF shoots), GA (15 OFF shoots treated with 50 mg l^{-1} gibberellic acid). The treatment was carried out on 28th November, when temperature dropped below 15°C. A: flowering intensity. B: type of sprouted shoots in percentage: Flowering shoots; Mixed shoots (flowers and leaves); Vegetative shoots. Each value is the mean of 15 shoots. Flowering and type of shoots were evaluated in Spring. Different letters or asterisk indicate significative differences.*

1.2. Spatial control of flowering inhibition by GA_3

Trying to identify where the inhibitory effect of GA_3 is located, localised applications were done in leaves and buds at three times: before, during and at the end of the flowering induction period.

Both localised treatments significantly reduced flowering intensity compared to the control, regardless of the time of treatment. The effect of GA_3 applied in September and November was higher in leaves than in buds (25% and 10% more, respectively). However, in January (bud differentiation stage), the effect of localised GA_3 treatments in the buds was higher, inhibiting flowering in 92% of treated shoots (Fig. 1.3C). Thus, the results suggest that GA_3 interferes with both the floral induction mechanism in the leaves (Chapter I) and the bud differentiation (Chapter II). However, it is not known how endogenous GAs regulate these processes.

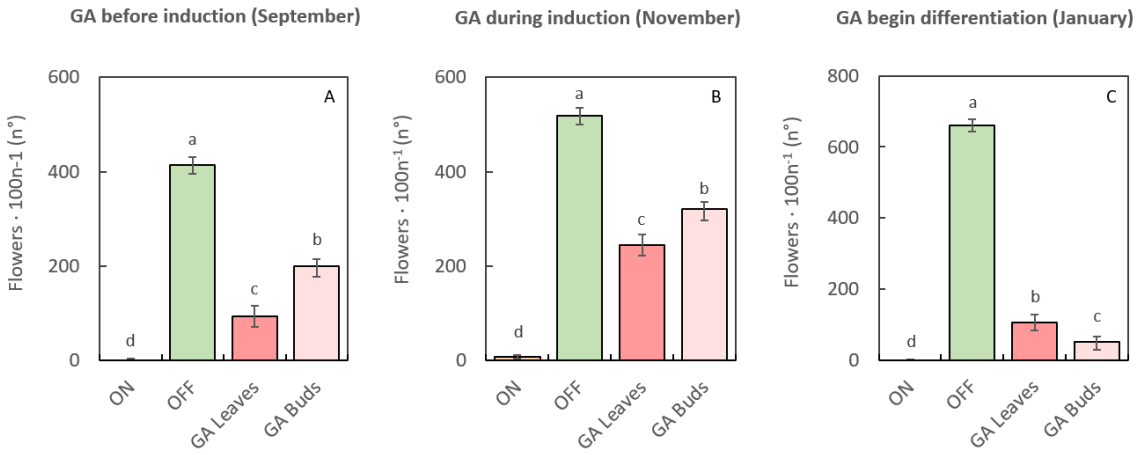


***Figure 1.3**. Effect of gibberellic acid (GA_3) locally applied to leaves and buds on the flowering intensity of 'Lane late' sweet orange. GA_3 treatments (25 mg l^{-1}) were carried out to branches without fruits (OFF; 60 shoots were selected per treatment) A: 6th of September, before flowering induction; B: 24th of November during flowering induction; C: 13th of January, beginning differentiation period. Branches with fruits (ON) were used for comparison. Flowering was evaluated in Spring; each value is the mean of 20 shoots. Different letters indicate significative differences.*

1.3. Correlation between GA synthesis and concentration in the leaves and CiFT3 gene expression

In OFF shoots, GAs biosynthesis in the leaf was reduced due to cold. Both *GA20ox2* and *GA3ox2* dramatically reduced their expression at the beginning of November (Fig. 1.4E, D), just when the temperature dropped below 12-15°C (Fig. 1.4A). Correspondingly, a significant reduction in the levels of $GA_{12,}$ GA_{19}, GA_{20} and GA_1 was also observed. The concentration of GA_{12} and GA_{19} decreased rapidly from 17,93 ng g^{-1} and 2,07 ng g^{-1}, respectively, in September to became null in October. During October and November GA_{20} was accumulated up to 2,08 ng g^{-1} and 2,96 ng g^{-1} respectively, and decreased to practically zero in the following months, which correlated with the expression of the *GA20ox2* enzyme. At the end of the 13-hydroxilation pathway, GA_1 concentration was also reduced from 2,6 ng g^{-1} to 0, coinciding with the downregulation of *GA3ox2* in the decrease of minimum temperatures. On the other hand, the concentration of GA_4, and other GAs from the non-hydroxylation pathway, was zero regardless of the time of analysis (Fig. 1.5).

Interestingly, *CiFT3* expression increased coinciding with the decrease in the concentration of GA_{20} and GA_1, peaking in early December (Fig. 1.4B). *CcMADS19* expression slightly decreased during cold period (Fig. 1.4C).

Finally, to better establish a cause and effect relationship between fruit, GAs and *CiFT3*, the fruit was removed before, during and after flowering induction to promote flowering and to determine the role of GA synthesis in this process. Only early thinning (July) fully restored flowering ability (Fig. 1.6A). Thinning resulted in a significant increase in *CiFT3* expression on leaves of ON branches 48 h after fruit removal in both July and November, reaching the expression level of OFF branches. The relative increase was slightly higher for the November thinning (Fig. 1.6B).

However, the consistent response of thinning to *CiFT3* gene expression was not observed for GA metabolism. First, while the July thinning significantly increased the relative expression of *GA3ox2* and, to a lesser extent, *GA3ox1*, no differences were found in the November thinning (Fig. 1.6C & D).

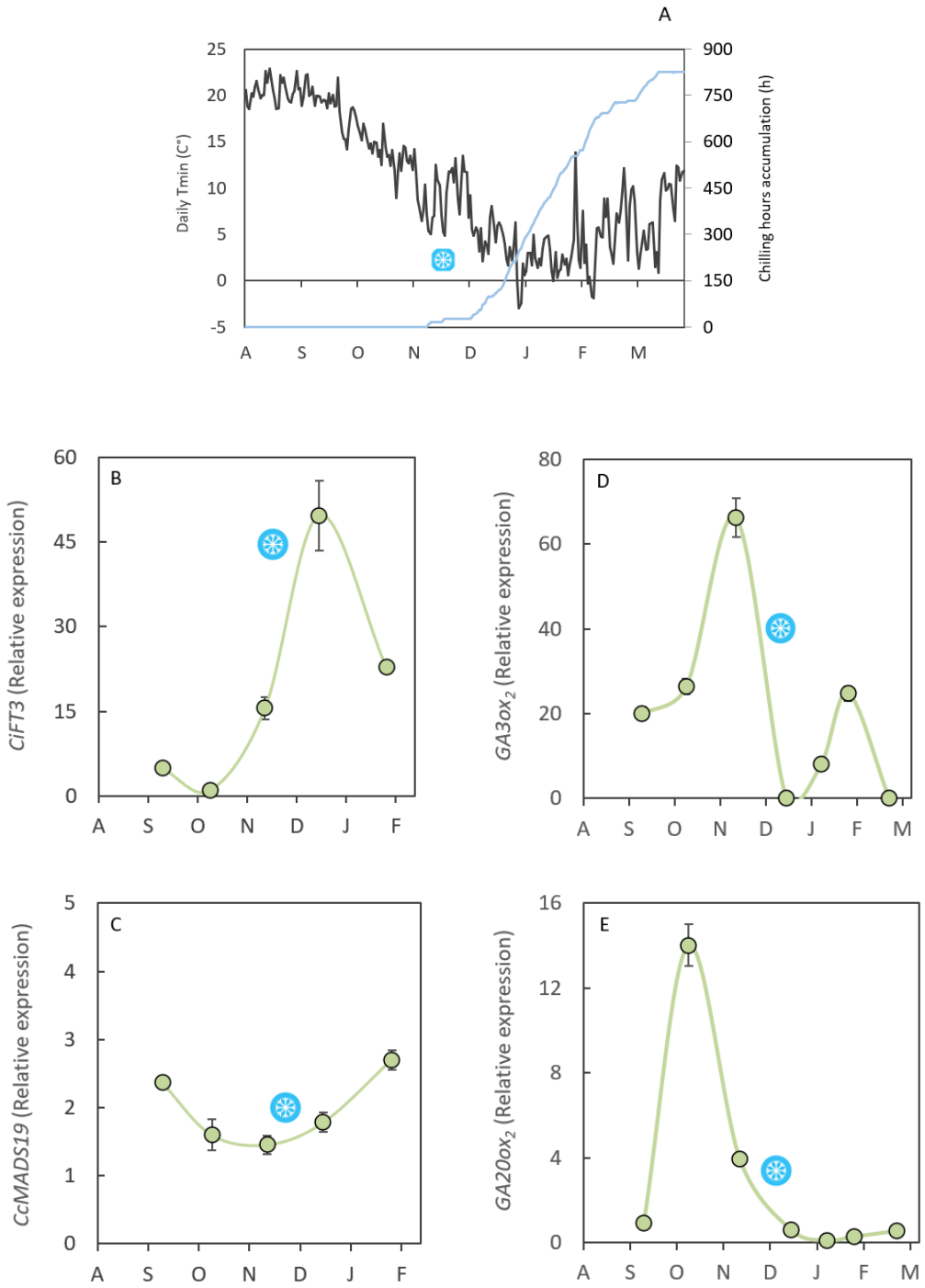


***Figure 1.4**. Relationship between the daily minimum temperature in Pedralba (València, Spain) during the induction period (A) and the time-course of the relative gene expression of CiFT3 (B), CcMADS19 (C) and GA3ox2 (D), GA20ox2 (E). Expression was related to chilling hours accumulation (hours below 7,2°C) in OFF trees of 'Nadorcott' mandarin (snowflake symbol). Temperature data obtain from the IVIA (Institut Valencià d'Investigacions Agràries) climate station in Pedralba. The data of gene expression are means of 3 mature leaves from 3 different shoots. The PCR reference gene was Actin. Standard errors (SE) are given as vertical bars; in some cases, SE is smaller than symbol size.*

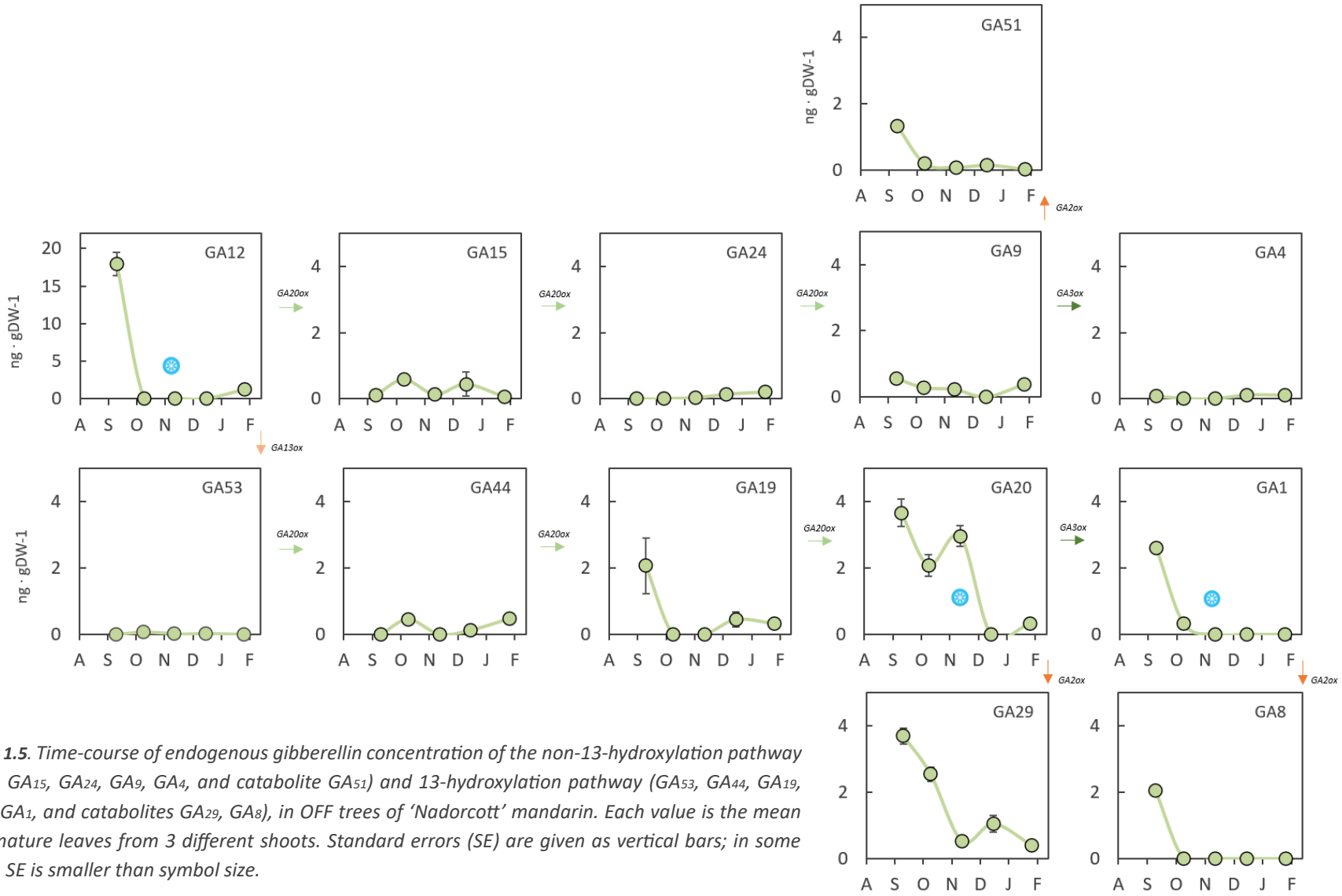


***Figure 1.5**. Time-course of endogenous gibberellin concentration of the non-13-hydroxylation pathway (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, and catabolite GA_{51}) and 13-hydroxylation pathway (GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, and catabolites GA_{29}, GA_8), in OFF trees of 'Nadorcott' mandarin. Each value is the mean of 3 mature leaves from 3 different shoots. Standard errors (SE) are given as vertical bars; in some cases, SE is smaller than symbol size.*

The evolution of GA_1 and GA_4 levels did not correlate with *CiFT3* expression. Indeed, GA_1, whose concentration was much higher than GA_4, showed a basal content in the leaves of ON branches from September until the end of the flowering induction period (around 2,5 ng g-1 on average). In contrast, fruit thinning in July and November led to an increase in leaf GA_1 content coinciding with autumn and spring budbreak, respectively (Fig.1.7).

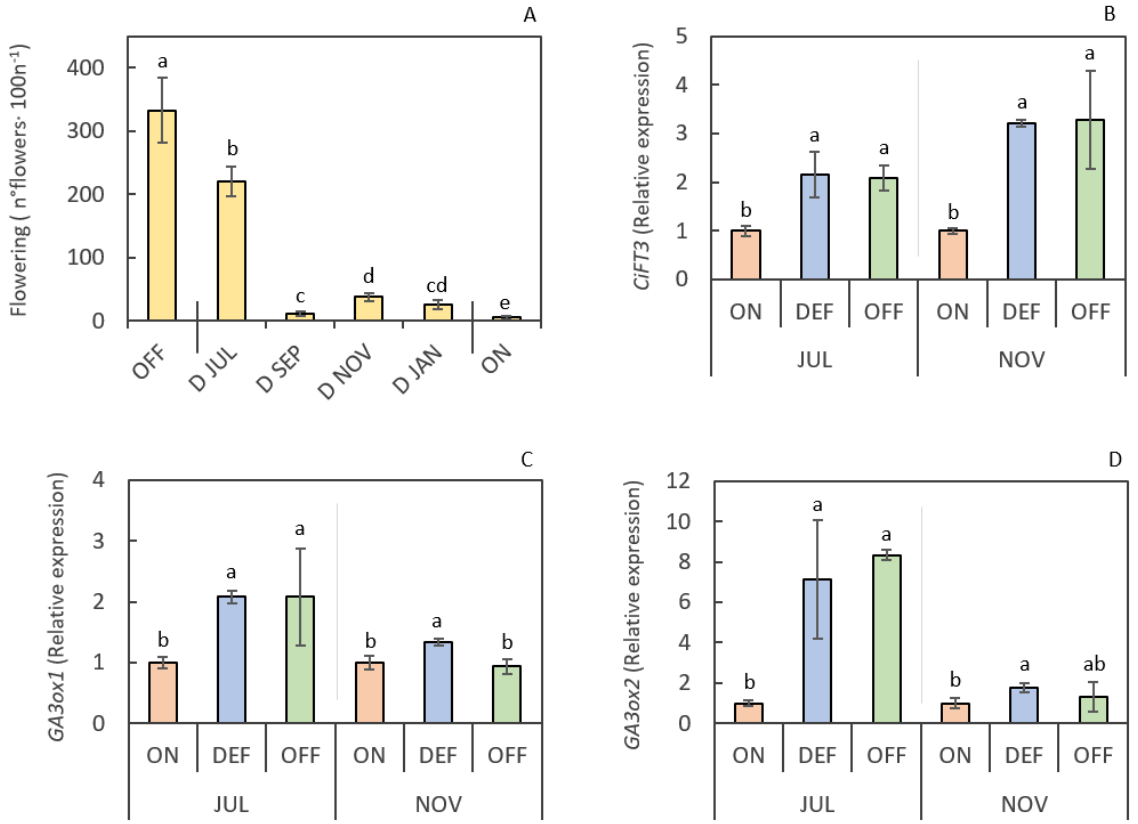


***Figure 1.6**. Influence of the time the fruit remained on the tree of flowering (A) in 'Nadorcott' mandarin. Defruiting was carried out in 78 ON shoots on 21st July, 66 on 7th September, 54 on 10th November and 42 on 11th January. Flowering was evaluated in the following spring; each value is the mean of 30 shoots. Relative expression of CiFT3 (B) and gibberellin biosynthesis enzymes: GA3ox1 (C) and GA3ox2 (D) was also assessed in ON, OFF and 48h after defruited shoots (DEF) in July and November. Gene expression data are mean values of 3 mature leaves from 3 different shoots. The PCR reference gene was Actin. Different letters denote statistical differences.*

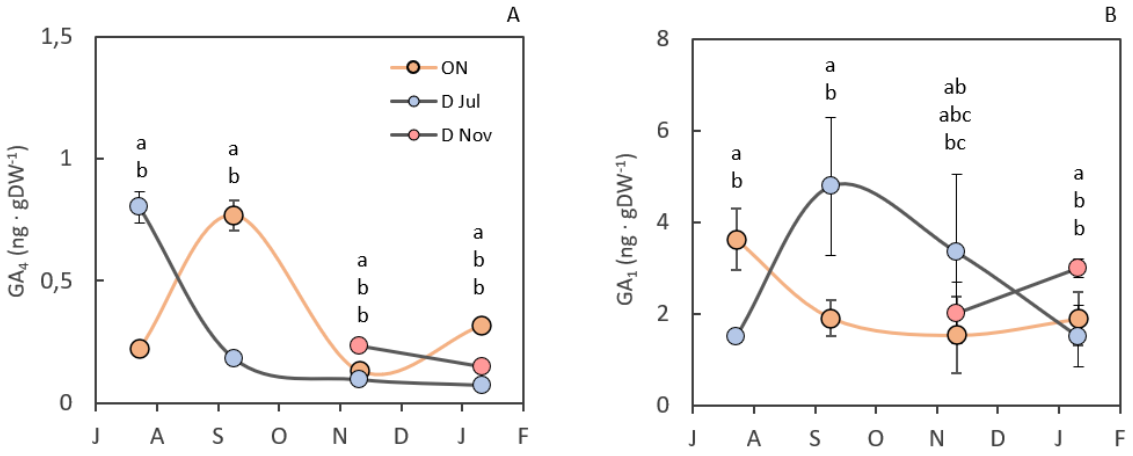


Figure 1.7. *Influence of the time the fruit remained on the tree of bioactive gibberellin metabolites GA_4 (A) and GA_1 (B) in 'Nadorcott' mandarin. Defruiting was carried out in 78 ON shoots on 21st July (D Jul) and 54 on 10th November (D Nov). Each value is the mean of 3 mature leaves from 3 different shoots. The first sample was taken 48 hours after fruit thinning. Subsequent samples were taken every two months approximately. Different letters denote statistical differences.*

Thus, the results suggest an inverse relationship between endogenous GA and *CiFT3* expression in OFF shoots. Furthermore, *CcMADS19* decreased slightly during cold, coinciding with GAs depletion. However, this relationship was not observed in ON shoots. This raises a new question: Are GAs really the activators of *CcMADS19* to inhibit *CiFT3* and flower induction?

1.4. Effect of GA_3 in flowering-time genes expression

The behaviour of the natural floral induction mechanism was altered throughout the GA_3 treatments. Significant reductions in *CiFT3* mRNA transcription were detected for each GA_3 application, regardless of the date of treatment and the number of treatments (Fig. 1.8A, B, F). When treated in September, before the induction period, the relative expression of *CiFT3* in GA-treated trees was similar to that of the control trees in the short term (48h after treatment). However, in the long term, GA_3 significantly reduced *CiFT3* expression by an average of 64% in November and maintained this trend even 113 DAT compared to control trees (Fig. 1.8A).

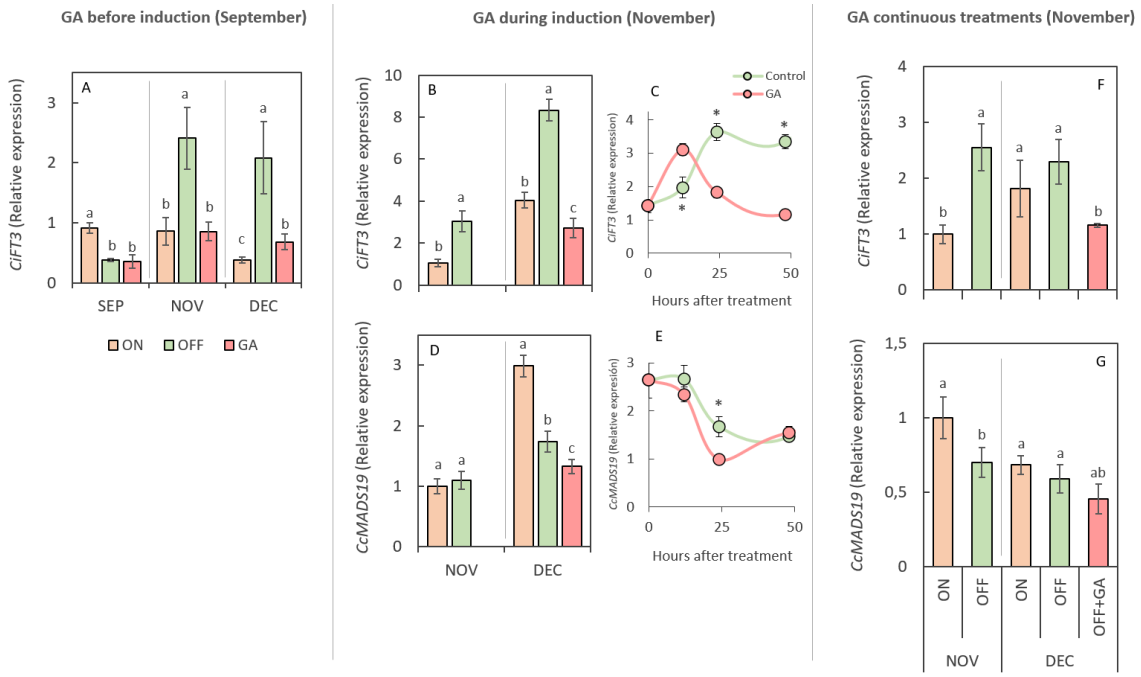


***Figure 1.8**. Effect of gibberellic acid (GA) treatments before, during and at the end of flowering inductive period on CiFT3 and CcMADS19 expression in 'Lane late' sweet orange. A: treatment with GA_3 (GA; 25 mg l^{-1}) was carried out on the 6th of September sampling 48h after application of GA_3, during inductive period (Nov) and after induction (Dec). B, D: Treatment with GA_3 (GA; 25 mg l^{-1}) was carried out on the 26th of November, sampling 10 days after treatment and 12h, 24h and 48h after application (C & E), evaluating the immediately effect of GA. F, G: 6 continuous GA treatments (OFF+GA; 25 mg l^{-1}) between 1st of November and 17th of December (1 application per week, sampling 22nd of December). Each value is the mean of 3 mature leaves of 3 different shoots. The PCR reference gene was Actin. Different letter or asterisk indicates statistical differences.*

When trees were treated with GA_3 in November, during the induction period, an immediate effect on *CiFT3* expression was detected 24 h after treatment (Fig. 1.8C), which was consistently maintained thereafter, reducing *CiFT3* expression 10 DAT (Fig. 1.8B). Finally, 6 continuous GA_3 applications (one per week in an attempt to keep hormone levels high), produced the same downregulation effect (Fig. 1.8F). As expected, the expression pattern of *CiFT3* in ON trees remained at basal levels.

The expression of the flowering repressor *CcMADS19* was upregulated in ON trees compared to OFF trees (Fig. 1.8D, G), as previously reported for mandarin trees (Agustí et al., 2020). GA_3 treatments did not increase *CcMADS19* gene expression in any of the experiments, neither in the short term (Fig. 1.8E) nor in the long term (Fig. 1.8D, G). Even after 6 applications of GA_3, the relative expression was not upregulated, but slightly downregulated. (Fig. 1.8G).

In other citrus species treated with GA_3 in November, the relative expression of *CiFT3* was downregulated by an average of 75% in *'Citrus limon'*, 77% in *'Citrus paradisi'* and 40% in *'Citrus clementina'* at 9 DAT. Furthermore, as in '*Citrus sinensis'*, no GA_3 effect was observed on *CcMADS19* expression in *'Citrus paradisi'* and *'Citrus clementina',* which remained almost constant. Only the treatments in *'Citrus limon'* reduced the expression of *CcMADS19* (Fig. 1.9).

To sum up, endogenous gibberellin concentration drops coinciding with *CiFT3* upregulation during the inductive period, and the application of GA_3 before, during or continuously throughout the induction period of flower buds reduces *CiFT3* gene expression in the leaves, suggesting that GAs depletion is required to increase *CiFT3* expression. But the application of GA_3 was not correlated with an upregulation of the flowering repressor *CcMADS19*. Thus, the results suggest that GAs and the fruit do not use the same mechanism to repress flowering in Citrus. At this point, a question needs posing: How do GAs regulate flowering time in *Citrus*?

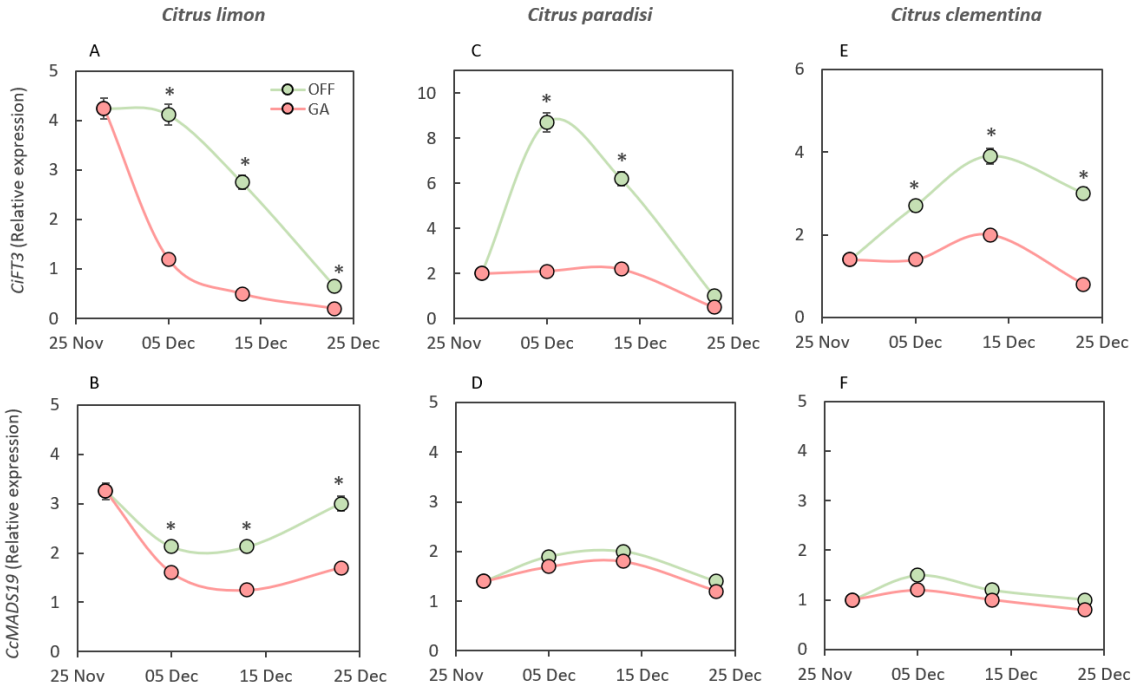


***Figure 1.9**. Time-course of gibberellic acid (GA; 25 mg l^{-1}) effect on the CiFT3 and CcMADS19 gene expression in 3 citrus species: 'Citrus limon' (A, B), 'Citrus paradisi' (C, D) and 'Citrus clementina' (E, F). Treatment done on the 28th of November. Each value is the mean of 3 mature leaves from 3 different shoots. The PCR reference gene was Actin. Asterisk indicates statistical differences. Standard errors (SE) are given as vertical bars; in some cases, SE is smaller than symbol size.*

Recently, Zhou et al., 2022, showed the importance of other flowering transcription factors (TFs), in particular the nuclear factor YA (*NF-YA1*). This TF binds to the *CiFT3* promoter to activate its expression under inductive conditions such as drought or low temperature.

In our experiments GA_3 significantly reduced the relative expression of *NF-YA1*, a transcription factor that activates *CiFT3* expression by binding to the promoter. In both September and November, GA_3 treatments significantly reduced NF-YA1 expression by an average of 70% (Fig. 1.10A and B). Furthermore, six continuous GA_3 treatments during induction period also resulted in a slight decrease in the relative expression of *NF-YA1* in December (Fig. 1.10C).

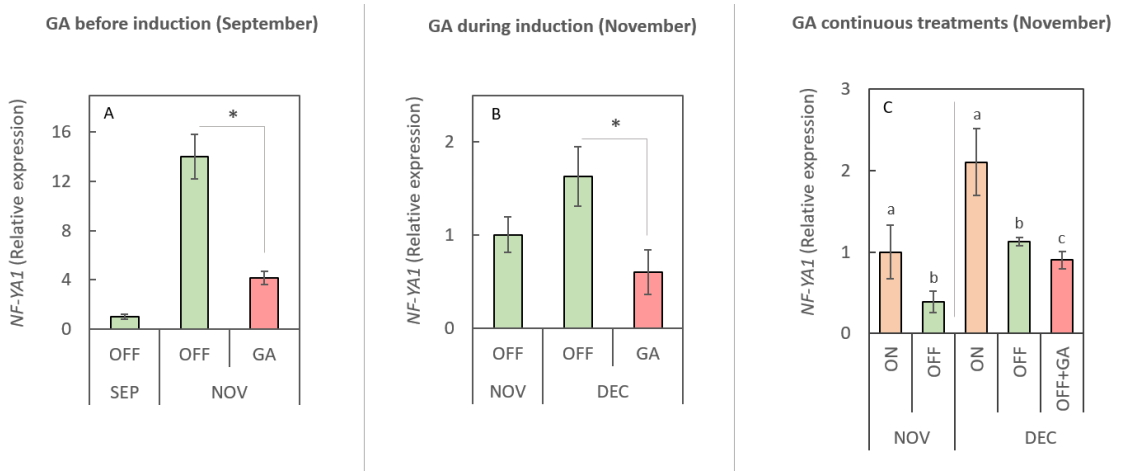


***Figure 1.10**. Effect of gibberellic acid (GA) treatments (25 mg l^{-1}): before, during and at the end of flowering inductive period on the relative expression of transcription factor NF-YA1, applied to 'Lane late' sweet orange shoots. A: treatment applied the 6th of September, sampling during inductive period (Nov). B: treatments carried out 26th of November, sampling after induction (Dec). C: 6 continuous GA treatments between 1st of November and 17th of December (1 application per week, sampling 22nd of December). Each value is the mean of 3 mature leaves from 3 different shoots. The PCR reference gene was Actin. Different letter or asterisks indicates statistical differences.*

But more importantly, the relative expression of *NF-YA1* increased in ON trees during the induction period (Fig. 1.10C), unlike GAs. Thus, the results suggest that the inhibitory effect of GA is mediated through a different pathway than that of the fruit, affecting the transcription factor NF-YA1, which indirectly regulates the flowering process by modulating the florigen *CiFT3* (Fig. 1.11). However, as it is not known how gibberellins regulate *NF-YA1* expression, further research is needed to establish this causal relationship.

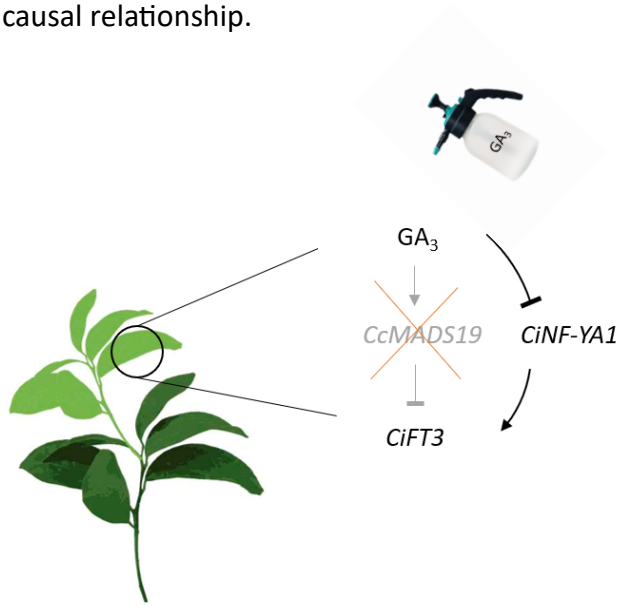


***Figure 1.11**. Diagrammatic representation of an alternative model for the inhibitory effect of gibberellins on the genetic mechanism of flowering induction in 'Citrus sinensis'. Exogenous treatment with GA_3 prevents CiFT3 expression, but not by up-regulating CcMADS19. GA follows another pathway of the fruit to repress flowering and a possible mechanism is blocking the CiFT3 transcription factor CiNF-YA1.*

Chapter II. *The role of GAs in Citrus flowering inhibition. Effect in the bud*

Localised application of GA_3, both to the leaf and to the bud, significantly reduces flowering (Fig. 1.3; Chapter I). In the previous chapter, it was shown that in the leaf, the decrease in the concentration of bioactive gibberellins correlates with the onset of *CiFT3* expression in the flowering phenotypes, the OFF trees. Furthermore, when the same trees are immediately treated with GA_3, flowering is suppressed by a reduction in the expression of the *NF-YA1* and *CiFT3* genes. However, the inhibition of flowering by the effect of the fruit represents a different mechanism, activating the repressor *CcMADS19*, which prevents the expression of *CiFT3* and thus flowering.

This chapter examines the regulatory action of GAs in the bud, particularly during the period of floral differentiation, when the meristem acquires its reproductive character. The following questions will be addressed 1) Is the mechanism of action of exogenous treatments with GA_3 on the buds the same as that produced by the presence of the fruit, or is it different, as in the case of the leaves? 2) Does the fruit have a regulatory effect on endogenous GAs levels? And if so, 3) What is the relationship between these endogenous gibberellins and the regulation of flowering?

2.1. The temporal evolution of gibberellin metabolism in the phloem and buds of ON, OFF, and thinned trees

To establish a cause and effect relationship between fruit, gibberellins (GAs) and flowering, a fruit thinning experiment was carried out. Spring bud sprouting and flowering showed an inverse relationship with the duration of fruit presence on the tree, with a threshold between the months of July and September. Branches that flowered most intensively were those from OFF shoots and those thinned in July (JUL). However, from September onwards, the presence of fruit significantly reduced flowering compared to the July thinning treatment. Out of a total of 230 flowers per 100 nodes, flowering was 11.4 in SEP, 37.4 in NOV, 25.7 in JAN and 4.8 flowers per 100 nodes in ON shoots (Figure 1.6A; Chapter I). The type of buds formed also showed qualitative differences between July and September. Buds on OFF branches and those thinned in

July (JUL) showed a high number of leafless reproductive shoots and mixed shoots, whereas branches where the fruit remained until September or later produced mainly vegetative shoots (Fig. 2.1).

In general, this experimental model is consistent. In fact, these results are similar to those obtained by Martinez-Fuentes et al. (2010), in which the threshold for fruit-induced flowering inhibition in sweet orange *'Valencia Late'* is established in November, later than in *'Nadorcott'* mandarin (Fig. 2.1).

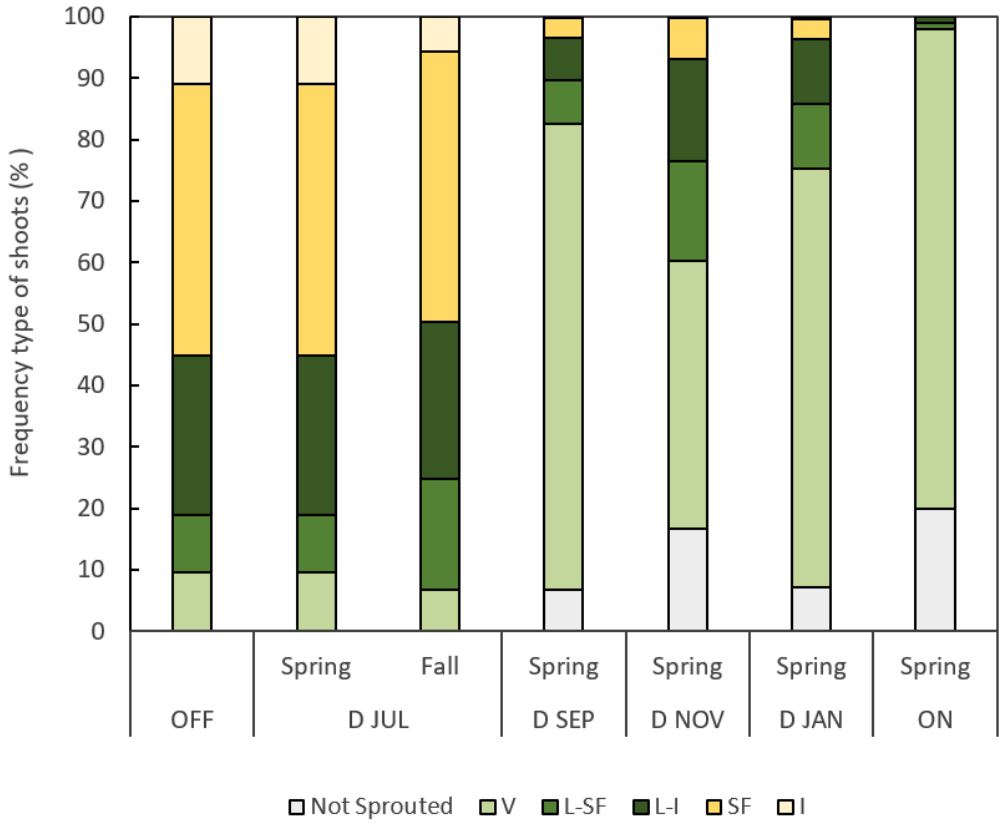


Figure 2.1*. Influence of the time the fruit remained on the tree of the percentage of shoot types developed in 'Nadorcott' mandarin. 78 ON shoots were defruited in July (21/7), 66 in September (7/9), 54 in November (10/11) and 42 in January (11/1). The distribution of shoot types was obtained from the evaluation of 30 shoots per treatment in autumn and spring. Vegetative shoots (V); Reproductive shoots without leaves (I, inflorescence and SF, solitary flower); Mixed shoots (L-I, inflorescence with leaves and L-SF, vegetative shoot with solitary flower in apical position).*

In the ON trees, the concentration of gibberellins in the buds was higher than in the OFF trees, and the trees were thinned in September, November and January, especially in the *non-hydroxylation* pathway (Fig. 2.2). The concentration of GA_{12} decreased rapidly from 3.45 ng g^{-1} in September to 0.32 ng g^{-1} in November (Fig. 2.2C). At the same time, in the *13-hydroxylation* pathway, the concentration of GA_{20} and GA_1 increased significantly from 0.0 ng g^{-1} in September to 1.11 and 0.25 ng g^{-1}, respectively, in January (Fig. 2.2D, F). In the *non-hydroxylation* pathway, the metabolites GA_9 and GA_4 also increased from values close to 0 ng g^{-1} in September to 45.16 and 579.71 ng g^{-1}, respectively, in January (Fig. 2.2G, H). The inactive catabolites of both pathways, GA_8 and GA_{34}, increased in parallel with the biologically active metabolites GA_1 and GA_4 (Fig. 2.2F, I). The expression of the synthesis enzymes *GA20ox2* and *GA3ox2* doubled in ON trees during the month of January (Fig. 2.2A, B), correlating with the increase in endogenous GAs concentration.

However, the level of GAs in the buds of the OFF trees was lower than in the ON trees during the periods of flower induction and differentiation, as well as the relative expression of the genes encoding the synthesis enzymes (Fig. 2.2). The concentration of GAs in the buds of the thinning treatments carried out in September, November and January did not differ from that of the buds of the OFF trees.

The repetition of the experiment with *'Moncada'* mandarin gave similar results (Fig. 2.3).

The concentration of GA_{12}, GA_{20} and GA_1 metabolites from the *13-hydroxylation* pathway detected in the phloem did not correlate with the GAs content in the buds (Fig. 2.4). GAs content from fruit exudate was also analysed in November (colour change/flower induction) and December (flower differentiation); in these samples the concentration of GA_4 was zero and GA_1 did not reach 0.01 ng g^{-1}. The hormones with the highest levels were JA, SA and AIA, with concentrations of 11.59, 5.85 and 7.74 ng g^{-1} in December, respectively (Fig. 2.5).

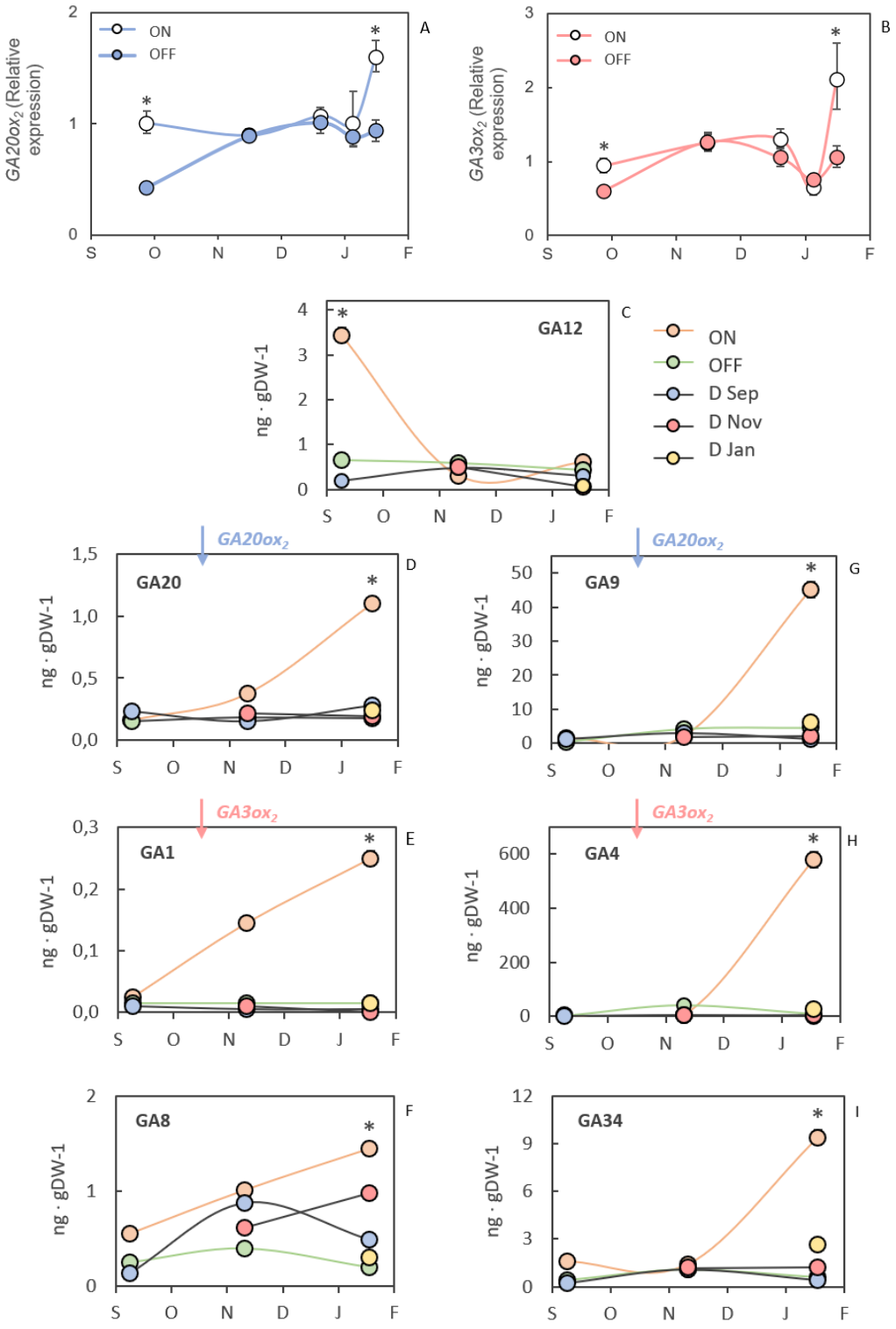


***Figure 2.2**. Endogenous gibberellin concentration of the non-13-hydroxylation pathway (GA_{12}, GA_9, GA_4, and catabolite GA_{34}) and 13-hydroxylation pathway (GA_{20}, GA_1, and catabolite, GA_8) in OFF, ON and defruited shoots of 'Nadorcott' mandarin buds. Relative expression of gibberellin biosynthesis enzymes, GA20ox2 (A) and GA3ox2 (B), also was assessed in OFF and ON buds. Shoots were defruited on 7th September, 10th November and 11th January. Each value is the mean of 3 biological bud replicates (4 shoots; 20 buds per replicate). Asterisks indicate significative differences.*

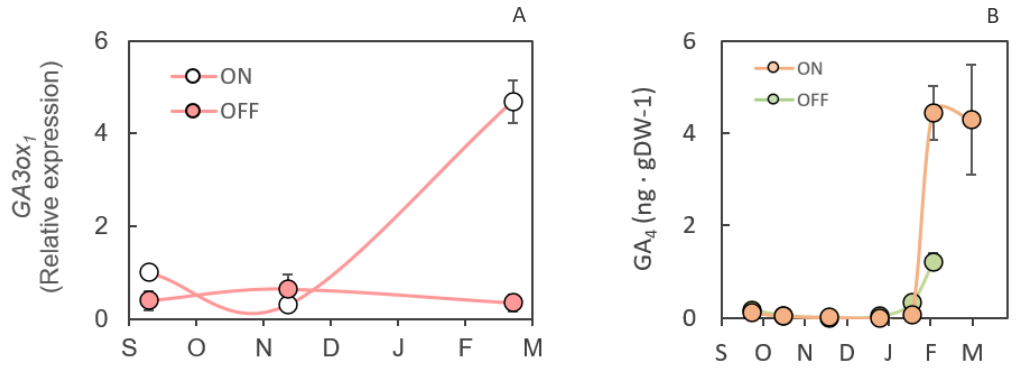


***Figure 2.3**. Endogenous gibberellin concentration of the active metabolite GA4 in OFF and ON shoots of 'Moncada' mandarin buds (B). Relative expression of the gibberellin biosynthesis enzyme GA3ox2 (A) was also assessed in OFF and ON buds. Each value is the mean of 3 biological bud replicates (4 shoots; 20 buds per replicate). Asterisks indicate significant differences. Standard errors (SE) are given as vertical bars; in some cases, the SE is smaller than the symbol size.*

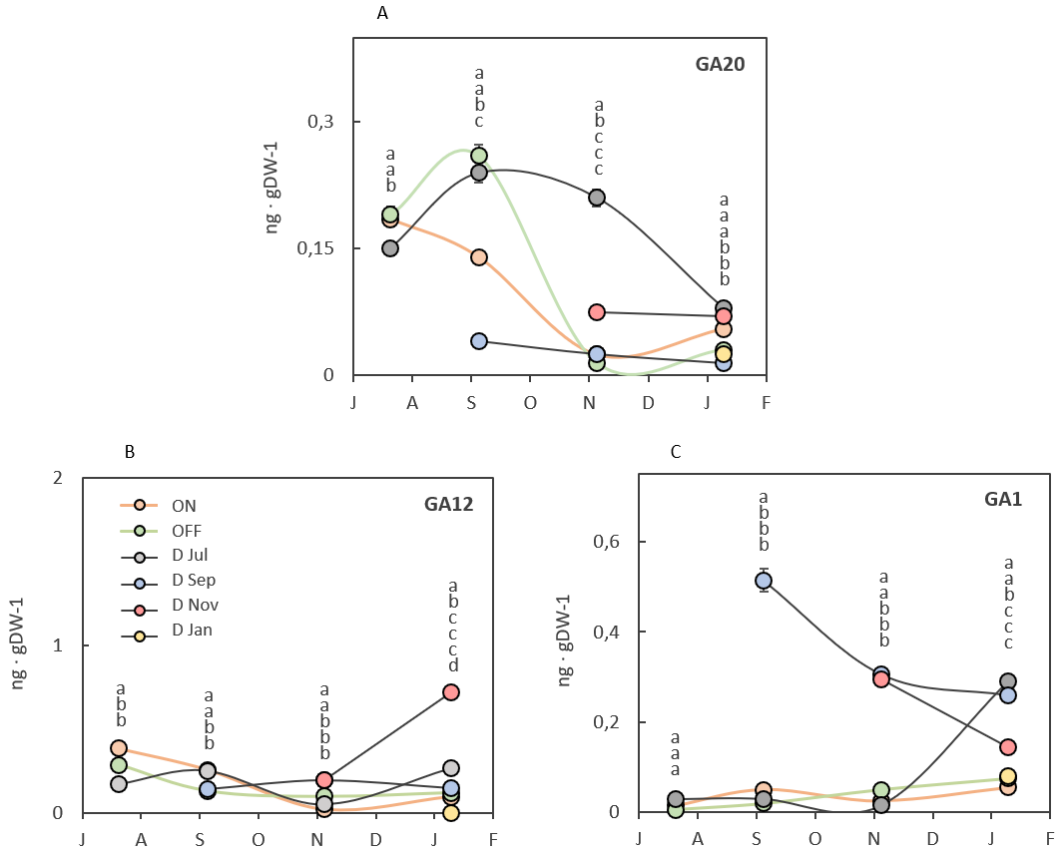


***Figure 2.4**. Endogenous gibberellin concentration of the non-13-hydroxylation pathway (GA12) and 13-hydroxylation pathway (GA20, GA1), in OFF, ON and Defruited shoots of 'Nadorcott' mandarin phloem tissues. Shoots were defruited on 7th Sep., 10th Nov. and 11th Jan. Each value is the mean of 3 biological bud replicates (4 shoots; 20 buds per replicate). Different letters indicate significative differences.*

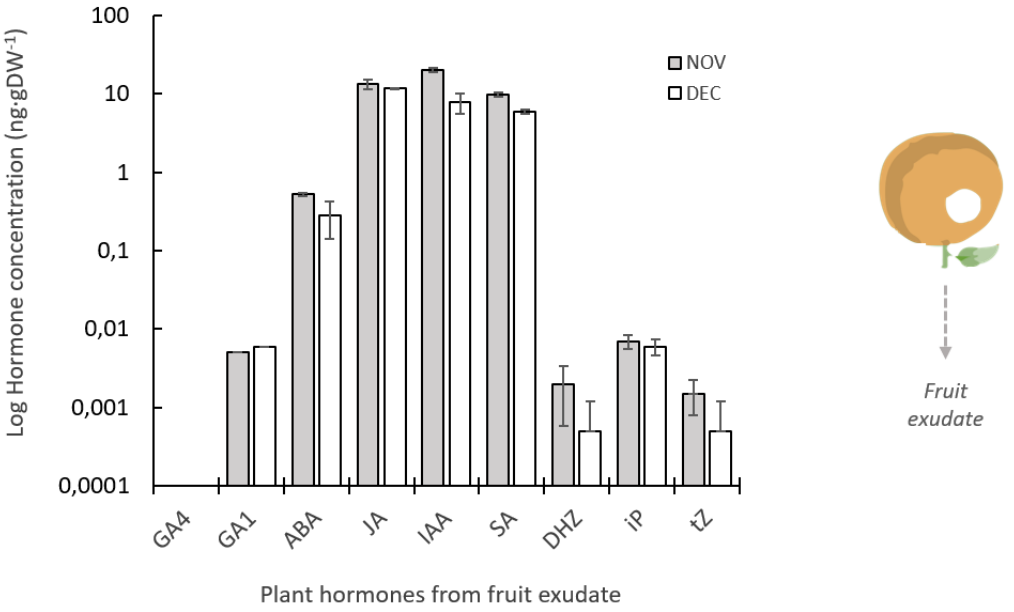


Figure 2.5. *Endogenous hormone concentration in fruit exudate of 'Nadorcott' mandarin during induction (November) and differentiation (December) periods: gibberellins (GA_4, GA_1); abscisic acid (ABA), jasmonic acid (JA), indoleacetic acid (IAA), salicylic acid (SA), cytokinins (DHZ, iP, tZ). Each value is the mean of 3 exudate replicates (3 fruits per replicate from 3 different ON shoots).*

In conclusion, in ON trees there was a peak of GAs (high concentration and synthesis) in the buds, coinciding with the period of floral differentiation. Conversely, the opposite occurred in fruitless trees (OFF) and in shoots thinned in September, November and January, where the concentration and synthesis of GAs were almost absent and remained stable throughout the period, with no significant differences between them. Phloem transport of GAs did not correlate with the peak of endogenous GAs in ON trees.

2.2. *Endogenous Control of Gibberellin Biosynthesis at the Time of Floral Differentiation: ON vs. OFF*

In sweet orange '*Lane late*', *LFY* and *DELLA* significantly increased their expression in the buds of OFF trees starting in December, maintained this trend until January and reached up to four times their relative value compared to ON trees (Fig. 2.6A, D). In contrast, the expression of the gibberellin receptor *GIDB1* was significantly lower in the

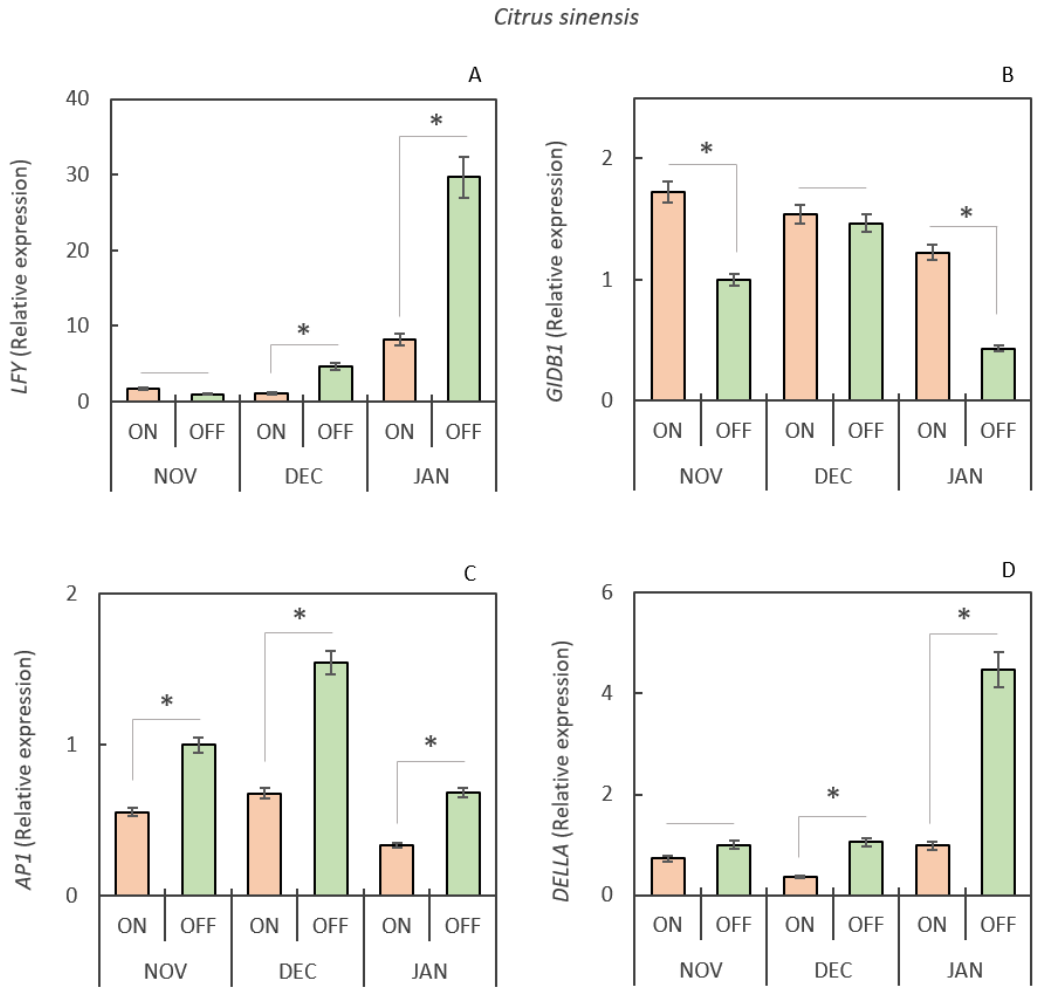


***Figure 2.6**. Nature behaver of genes related in flowering differentiation and gibberellin response on 'Lane late' sweet orange ON and OFF shoots. A, C: LFY and AP1 genes involved in floral differentiation. B: gibberellin receptor GIDB1. D: Response to gibberellin, DELLA protein. Samples (12 shoots per treatment) were taken once a month, between November and January. Each value is the mean of 3 biological replicates of 20 buds (4 shoots per replicate). The PCR reference gene was Lycopene (LYC). Asterisks indicate significative differences.*

buds of OFF trees in November and January (Fig. 2.6B). All this would be related to the lower concentration of GA_4 in the OFF shoots (0 ng g^{-1}) compared to the ON shoots (579.71 ng g^{-1}) (Fig. 2.2H).

The behaviour in other species was similar. In '*Nadorcott*' mandarin, *LFY* expression was consistently higher in OFF trees, twice as high as in ON trees (Fig. 2.7A), which correlated negatively with GA content in the buds (Fig. 2.2) and allowed the accumulation of *DELLA* proteins in OFF trees (Fig. 2.7C). Finally, an inverse relationship was observed between the duration of fruit presence on the plant and the expression of *LFY* ($r = 0.7232$, $p < 0.01$) and *DELLA* ($r = 0.7166$, $p < 0.01$) (Fig. 2.7B, D).

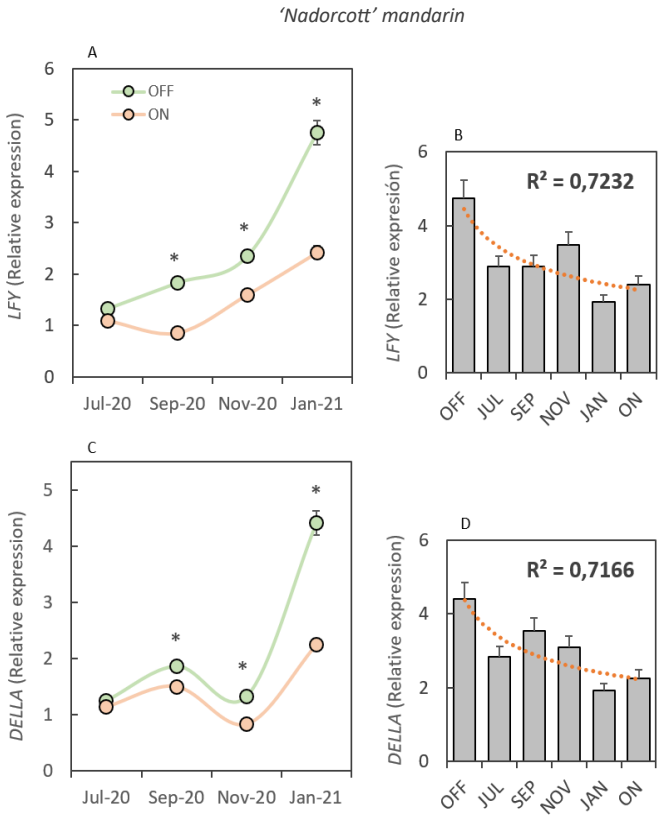


***Figure 2.7**. Time course of the relative expression of the differentiation gene LFY (A) and the DELLA protein coding gene (B) and the relationship between these genes with the time of fruit presence on the brunch (B & D) on 'Nadorcott' mandarin. Sampling started in summer (July) and ended in winter (January), taking 12 shoots per treatment every two months. In addition, another 12 shoots were collected 48h after thinning in JUL, SEP, NOV and JAN (B & D). Each value is the mean of 3 biological replicates of 20 buds (4 shoots per replicate). The PCR reference gene was lycopene (LYC). The r^2 calculation is statistically significant at $p<0.01$. Asterisks indicate significant differences.*

The more detailed study of the expression of *LFY* in comparison with the expression of the genes *CYCB2* (cell division) and *TFL1* and *CEN* (vegetative meristem) in '*Nadorcott*' mandarin and sweet orange '*Lane late*' shows a direct relationship with the expression of the *CYCB2* gene (Fig. 2.8C) and an inverse relationship with the expression of *TFL1* (Fig. 2.8F). In both species, *CEN* increased its expression at the end of the study

period, after the maximum expression of LFY (Fig. 2.8B, E). Its expression was significantly higher in ON trees, which only develop vegetative shoots, but it also increased in the buds of OFF trees (Fig. 2.1). In '*Nadorcott*' mandarin, another time point of increased *CEN* expression was detected, coinciding with the autumn bud break (Fig. 2.8B).

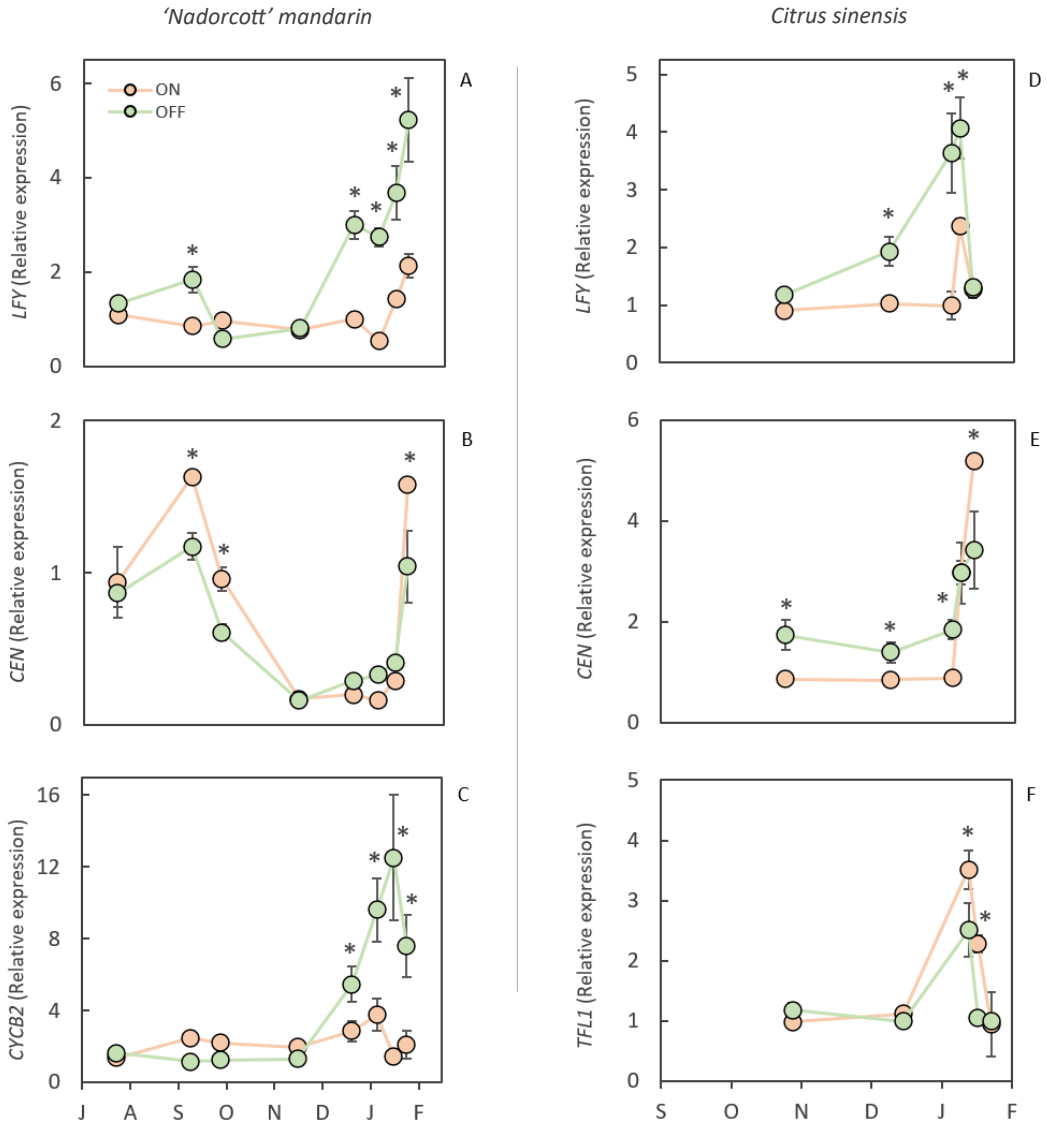


***Figure 2.8**. Time course of genes expression involved in flowering differentiation (LFY; A & D), cell division cycle (CYCB2; C) and meristem flowering repressors (CEN and TFL1; B, E & F) in the buds of 'Nadorcott' mandarin and 'Lane late' sweet orange during the induction and differentiation period. Sampling was carried out approximately monthly, but more intensively during the differentiation period (December and January). Each value is the mean of 3 biological replicates of 20 buds (4 shoots per replicate). The PCR reference gene was lycopene (LYC). Asterisks indicate significative differences.*

To establish a cause-effect relationship between GAs and the stimulation of *CEN* expression (and the repression of *LFY*), the temporal (before, during and after the floral induction period) and spatial (tree, leaf, bud) influence of gibberellic acid (GA_3) application on the expression of these genes in OFF trees was determined.

In September, two months before floral induction, *LFY* gene expression showed no significant differences between OFF, ON, or those treated with GA_3, regardless of application site (Fig. 2.9A-A). This lack of difference can be attributed to the absence of *FT* and *LFY* transcription at this early stage (Fig. 1.8A, Chapter I). However, *CEN* expression was higher in GA_3 treatments applied locally to the bud and the entire tree (Fig. 2.9A-B), but not in treatments applied locally to the leaf. This behaviour is similar to that of ON trees at the time of floral differentiation, meaning that when the GA content increases in the buds, vegetative development also increases (see Figs. 2.2 and 2.8B, E).

On the other hand, the continuous application of GA_3 to OFF trees from the floral induction period to the floral differentiation period (1st November – 15th January) significantly reduced *LFY* expression in December and halved it in January (Fig. 2.9A-C). The *AP1* gene showed a similar trend (Fig. 2.9B-A). In contrast, *CEN* doubled its expression in December and maintained this level in January. Treatments with PBZ on OFF trees did not increase the expression levels of the *LFY* and *AP1* genes and had no effect on *CEN*. As in ON trees, *LFY* expression was consistently lower than in OFF trees and remained at non-inductive levels. It is noteworthy that even in the presence of fruit, GA_3 treatments slightly reduced *LFY* expression in January (Fig. 2.9A-C) and increased *CEN* expression, which reached levels similar to those of treated OFF trees (Fig. 2.9A-D).

As a result, continuous applications of GA_3 enhanced vegetative development through *CEN* overexpression. Therefore, these sequential treatments modified the phenotype by increasing the length of leafless reproductive shoots (+136%), mixed shoots (+137%) and vegetative shoots (+125%) compared to untreated OFF tree shoots (schematic Fig. 2.10E). On the other hand, PBZ treatments significantly reduced the length of vegetative shoots (-165%); the length of leafless reproductive shoots (-92%) and leafy reproductive shoots (-64%) also decreased, but in this case without statistical differences (Fig. 2.10A). The effect of treatments on internode length was similar to that

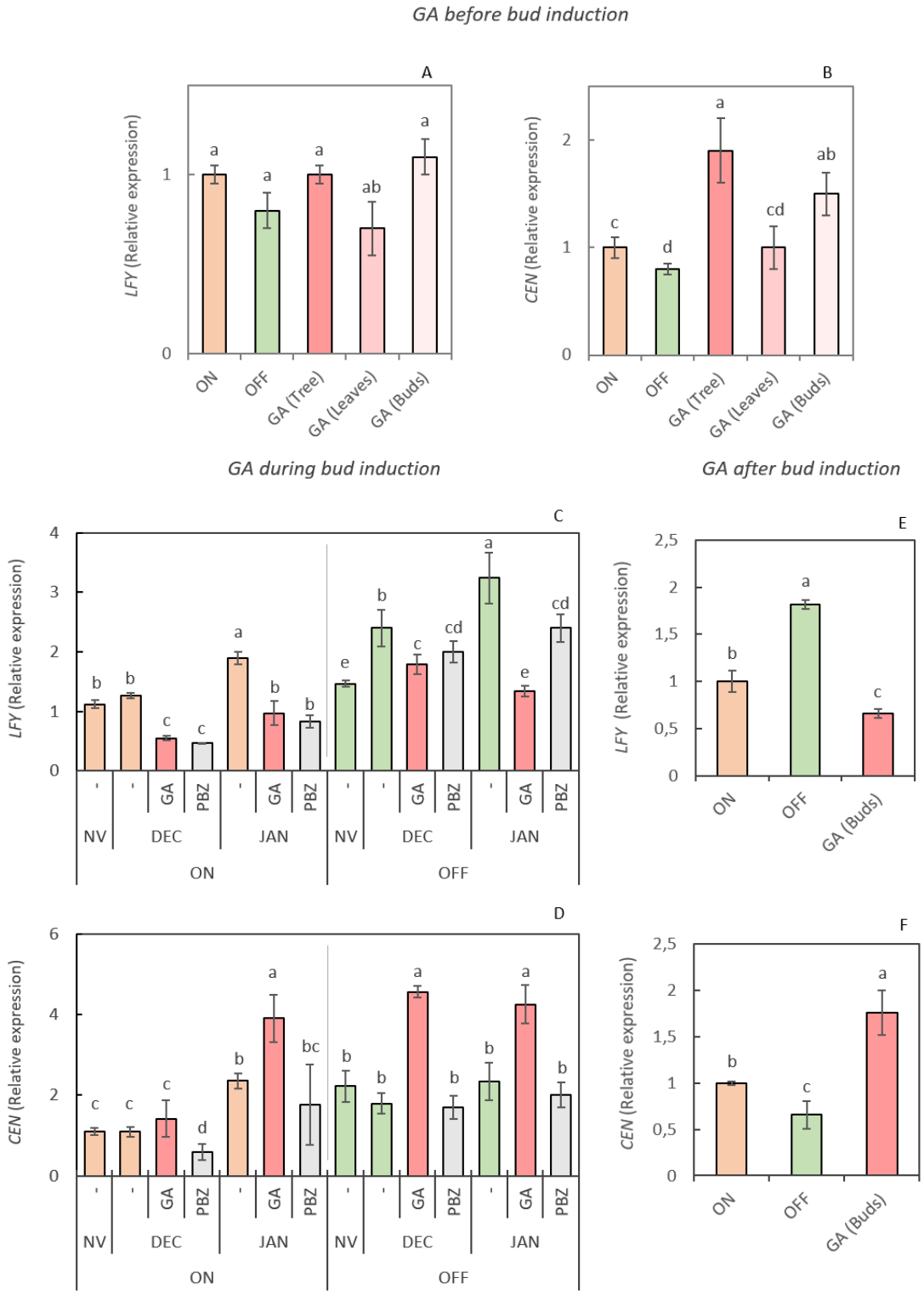


***Figure 2.9A**. Effect of gibberellic acid (GA, 25 mg l^{-1}) and paclobutrazol (PBZ, 1000 mg l^{-1}) treatments: before, during and after bud induction on differentiation flowering gene LFY and regulator of vegetative growth gene CEN, applied to 'Lane late' sweet orange. A, B: treatment carried out 26th of November, sampling 48h after application. C, D: 9 continuous GA and PBZ treatments between 1st of November and 19th of January (1 application per week). E, F: localised treatments on buds on 15th of January sampling 10 days after application. Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was lycopene (LYC). Different letter indicates statistical differences. ON: shoot with an apical fruit; OFF: nonfruiting shoot; GA (Tree): OFF trees treated with GA_3; GA (Leaves): Leaves from OFF shoots treated with GA_3; GA (Buds): Buds from OFF shoots treated with GA_3.*

on shoot length (Fig. 2.10B). Furthermore, continuous GA_3 applications also affected leaf development, significantly increasing leaf length in vegetative shoots (+38%) and mixed shoots (+44%) compared to untreated OFF shoots. The width of these leaves was also modified in both vegetative shoots (+37%) and mixed shoots (+55%). In contrast, in the PBZ treatments, leaf length and width decreased significantly only in vegetative shoots (-40% and -36%, respectively; Fig. 2.10C, D).

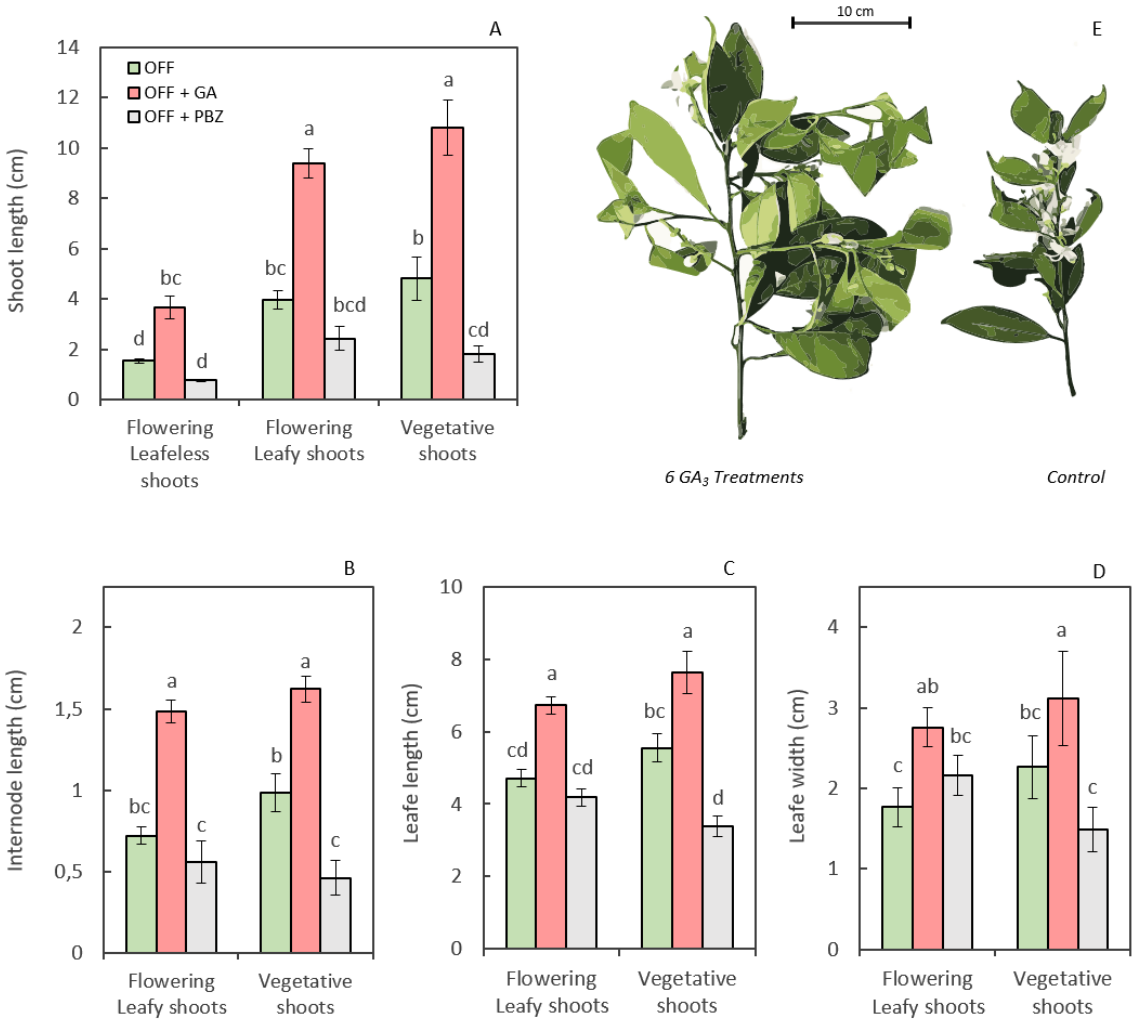


***Figure 2.10**. Morphological effect of 9 continuous gibberellic acid (GA, 25 mg l^{-1}) and paclobutrazol (PBZ, 1000 mg l^{-1}) treatments on next spring shoots. Shoot length (A), internode development (B) and leave length (C) and width (D). E: schema of treatments effect. Each value is the mean of 10 shoots measures and in the leaves each value is the mean of 10 leaves of different shoots. Different letters indicate statistical differences.*

Finally, in the GA_3 treatments, localised in the buds of OFF trees at the end of the floral differentiation period, the behaviour was similar to the continuous treatments described above; *LFY* and *AP1* expression significantly decreased compared to OFF (Fig. 2.9A-E and Fig. 2.9B-B), and *CEN* expression significantly increased compared to OFF and ON (Fig. 2.9A-F).

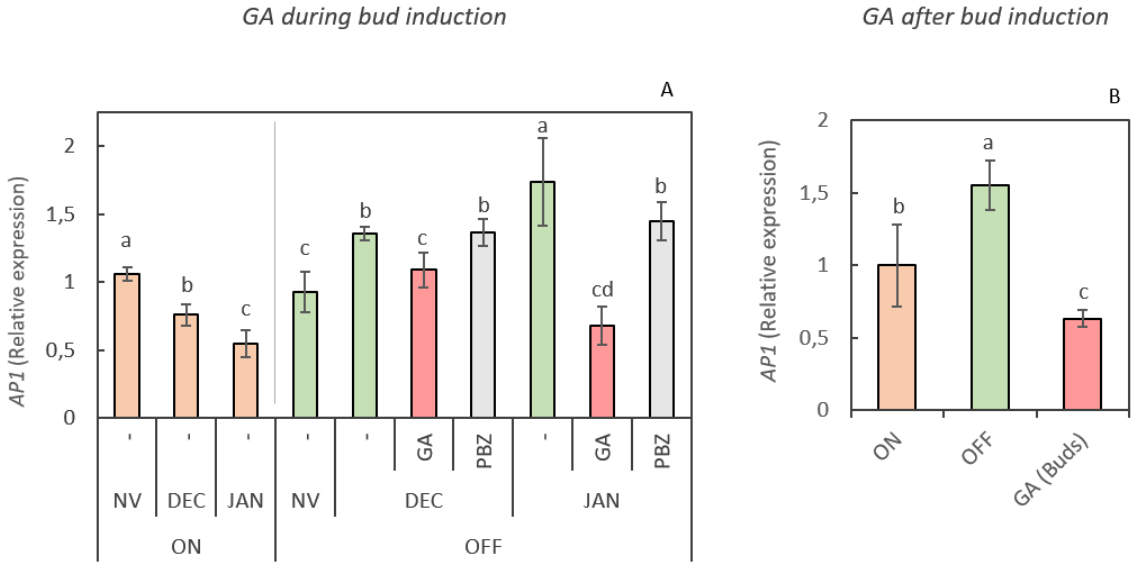


***Figure 2.9B**. Effect of gibberellic acid (GA, 25 mg l^{-1}) and paclobutrazol (PBZ, 1000 mg l^{-1}) treatments: during and after bud induction on differentiation flowering gene AP1 applied to 'Lane late' sweet orange. A: 9 continuous GA and PBZ treatments between 1st of November and 19th of January (1 application per week). B: localised treatments on buds on 15th of January sampling 10 days after application. Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was lycopene (LYC). Different letter indicates statistical differences.*

2.3. Anatomical Localization of CENTRORADIALIS (CEN) Expression in the Bud

Recent studies have revealed the regulatory role of CEN during thorn formation in citrus (Zhang et al., 2019). However, its role in axillary meristems to control vegetative development at the expense of reproductive development is still unknown.

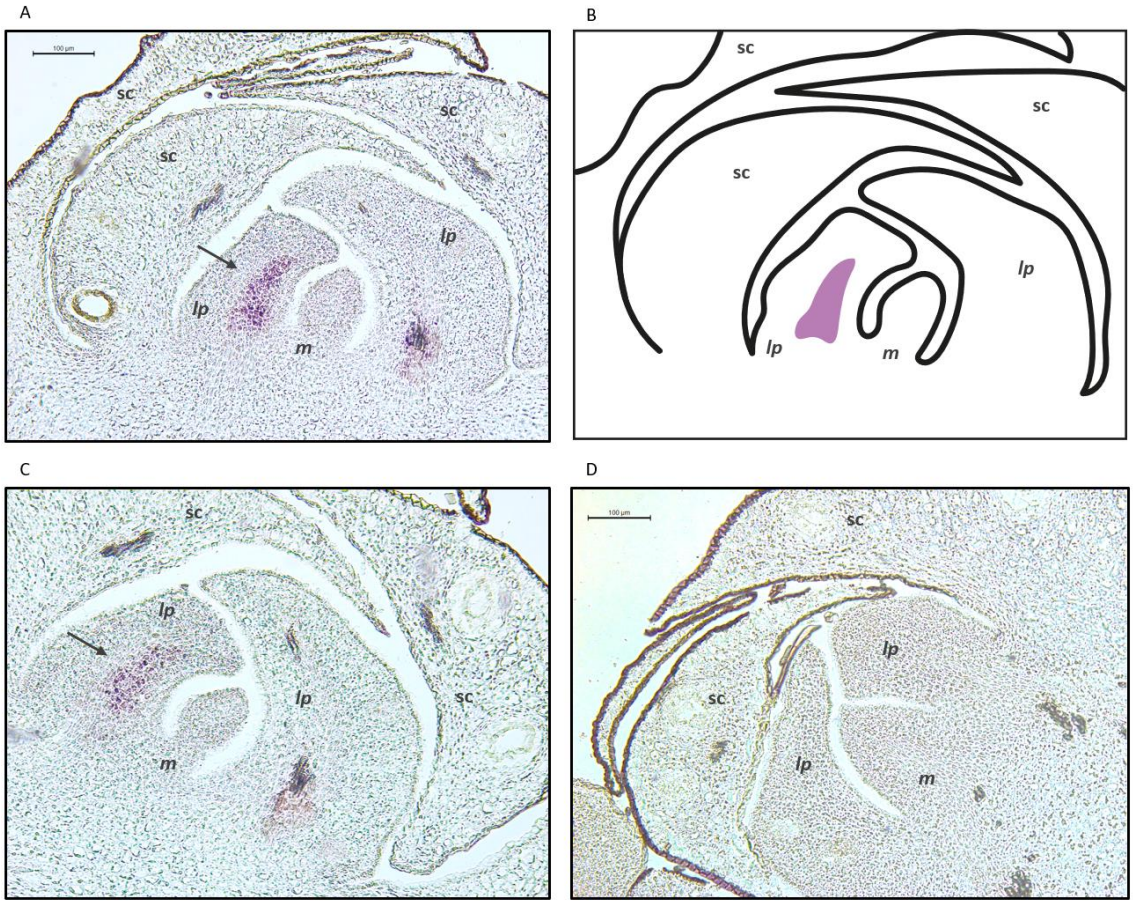


Figure 2.11*. In situ hybridization of CEN mRNA in the axillary buds of ON shoots in 'Lane late' sweet orange in September. Samples were from the closest buds to the fruit. (A) CEN expression was detected in the incipient leave primordium (lp), but not in the scales (sc) or meristem (m), as represented in the diagram (B), for clarity. (C) A deeper section of the same apex. (D) Sence probe as a control, without CEN expression. Arrows marks CEN expression.*

In situ hybridisation studies of the *CEN* gene, carried out when its relative expression was high (Fig. 2.8B), showed that it was anatomically located in the leaf primordia of latent buds in ON trees, *i.e.,* outside the main axillary bud meristem. The localisation of *CEN* expression correlates with the phenotype of GA3-treated OFF trees,

where shoot length and leaf size are greater than in untreated trees (Fig. 2.11). All this suggests that CEN expression is not responsible for maintaining an indeterminate meristem, but rather regulates leafy vegetative development in citrus, with a secondary role in controlling floral identity.

Chapter III. *The role of Auxin in Citrus flowering*

The results obtained by analysing the exudate from the fruit at flowering induction show that the concentration of IAA is high in both November and December, in comparison with the other hormones ((Fig. 2.5) - Chapter II). Indeed, previous studies have demonstrated 1) the inhibitory effect of auxins on citrus flowering (García-Luis et al., 1986) and 2) the correlation between the presence of fruits, auxin metabolism and flowering inhibition (Shalom et al., 2014; Haim et al., 2021). However, their mode of action in leaves (regarding flower induction) and buds (regarding flower differentiation) is still unknown. Therefore, in this chapter, we investigate the relationship between auxins, the presence of fruits, and the expression of flowering genes in leaves and buds.

3.1. Exogenous control of flowering with 2,4-D

The application of 2,4-D (12 mg l-1) during the floral induction period significantly reduced the flowering intensity by -52.6% in '*Navelina*' sweet orange (130 flowers per 100 nodes) compared to untreated trees (230 flowers per 100 nodes) (Fig. 3.1). This effect was not species-specific, as the treatment also reduced the number of flowers in '*Eureka*' lemon trees by -52.6% and in '*Oronules*' clementine mandarin trees by -36.1% when compared to the control (Fig 3.1). The treatment reduced the number of reproductive shoots and increased the number of vegetative shoots in all three species, with a 40.4% increase in '*Eureka*' lemon and a 36% increase in '*Oronules*' clementine mandarin (Fig 3.2).

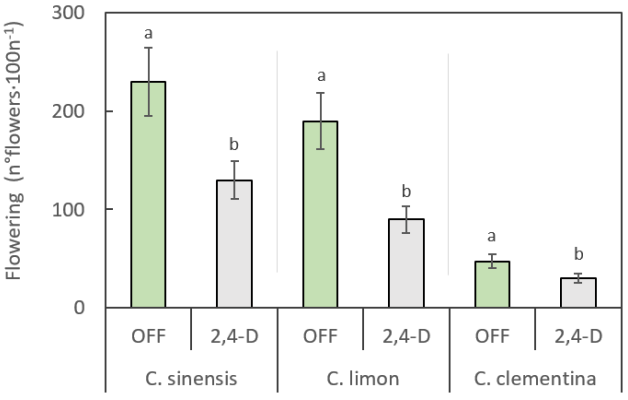


Figure 3.1*. Effect of 2,4D (12 mg l^{-1}) on the intensity of flowering in Citrus sinensis, Citrus limon and Citrus clementina. The treatment was carried out during the induction period (15th of November). The evaluation of flowering was conducted the following Spring. Different letters indicate significant differences (P<0.05).*

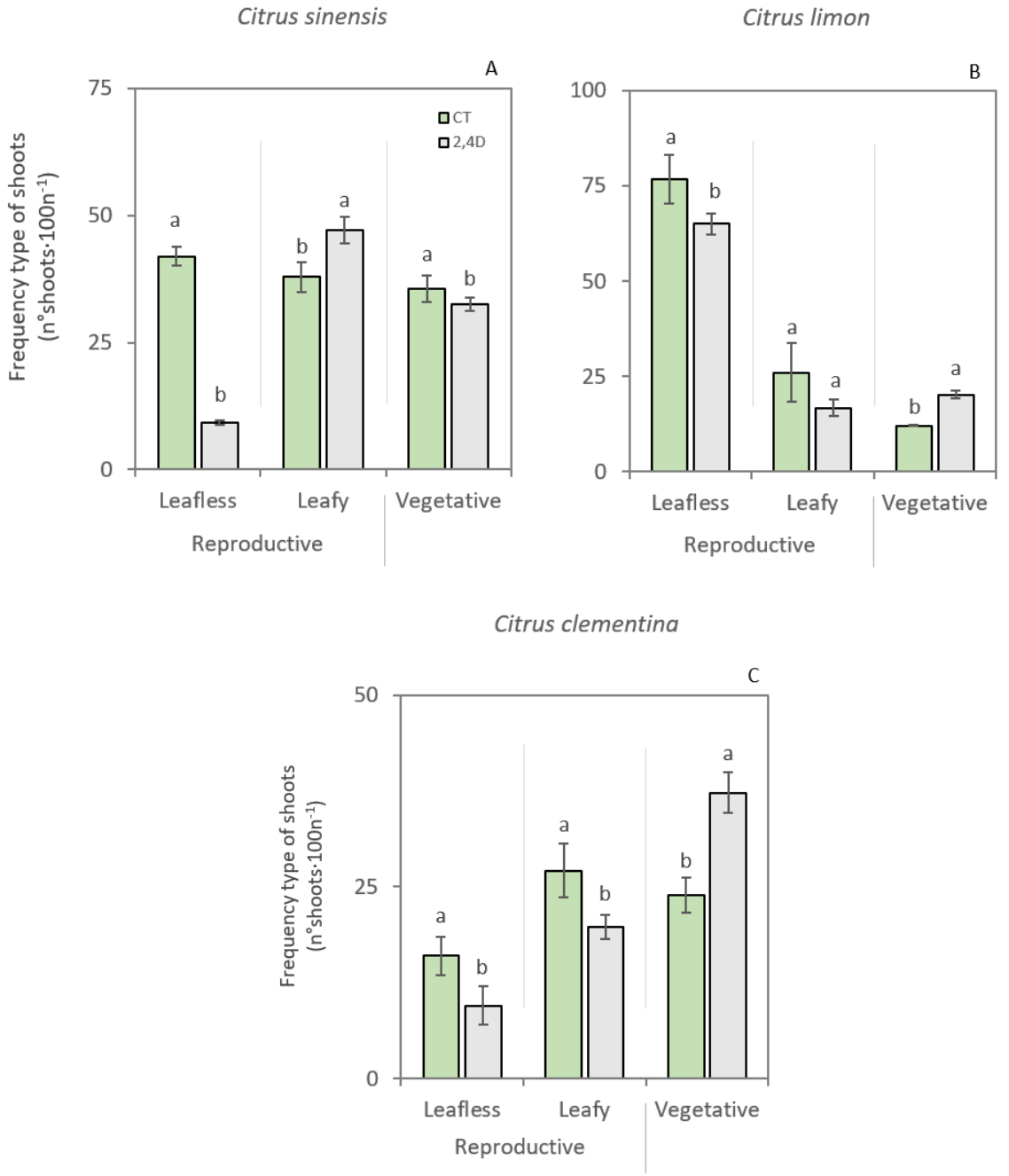


***Figure 3.2**. Effect of 2,4D (12 mg l^{-1}) on the quantity of reproductive and vegetative shoots developed in the upcoming Spring of Citrus sinensis (A), Citrus limon (B) and Citrus clementina (C). The treatment was carried out during the induction period (15th of November). Different letters indicate significant differences (P<0.05).*

In order to maintain a constant concentration of auxins in the shoot, mimicking the presence of fruit, four continuous applications of 2,4-D (12 mg l^{-1}, one every 20 days) were conducted on OFF trees between October 31st and January 13th. During the same period, TIBA (an auxin transport inhibitor) was also applied locally to the bud at a concentration of 1 g l^{-1}. This treatment served as a positive control since meristems need to reduce auxin concentration to initiate budding. The continuous applications of 2,4-D significantly reduced the number of flowers from 249.5 per 100 nodes in treated trees to 145.2 in untreated trees, but they did not achieve the same level of inhibitory effect as the presence of fruit (Fig. 3.3A). In addition, the 2,4-D treatment increased the number of leaves from 1.05 per 100 nodes in untreated trees to 20 in treated trees (Fig 3.3B). The localized applications of TIBA to the bud did not alter the number of flowers (Fig 3.3A) but increased the number of leaves compared to untreated trees (+97.5%) (Fig 3.3B).

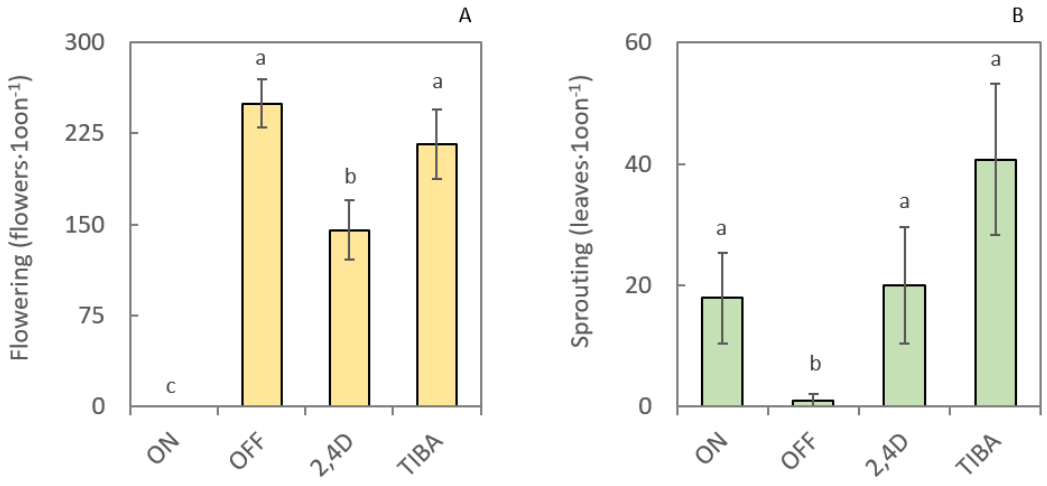


***Figure 3.3**. Effect of 2,4-D and TIBA on flowering (A) and sprouting (B) intensity of 'Valencia Late' sweet orange. ON (with yield), OFF (without yield), 2,4-D (12 mg l^{-1} of 2, 4-dichlorophenoxyacetic acid; synthetic auxin) and TIBA (1 g l^{-1} of 2,3,5-triiodobenzoic acid; auxin transport inhibitor). The applications were started at the end of October (31/10/2022) on 70 OFF shoots per treatment. Treatments were repeated every 20 days approximately (for a total of 4 applications) concluding in January (13/01/2023). Each value is the mean of 30 shoots per treatment. The experiment was carried out in Palermo, Italy. Different letters indicate significative differences (P<0.05).*

3.2. Effect of 2,4-D treatments on the expression of flowering genes

3.2.1. 2,4-D effect in the leaf

Treatment with 2,4-D did not show a consistent result in terms of relative expression of the *CiFT3* gene. In '*Navelina*' sweet orange, the relative expression of treated trees decreased significantly from 0.91 to 0.67 17 days after treatment (DAT) and then returned to a level similar to untreated trees from December (Fig. 3.4A). On the other hand, 'Eureka' lemon and '*Oronules*' clementine mandarin responded later to 2,4-D treatment, reducing *CiFT3* expression at 30 and 45 DAT, respectively (Fig. 3.4B and C).

Similarly, the effect of 2,4-D treatment on the expression of the inhibitory gene *CcMADS19* was inconsistent. While it did not alter its expression in '*Navelina'* sweet orange compared to untreated trees (Fig. 3.4D), it increased its expression in 'Eureka' lemon (Fig. 3.4E) and reduced it in '*Oronules*' clementine mandarin (Fig. 3.4F).

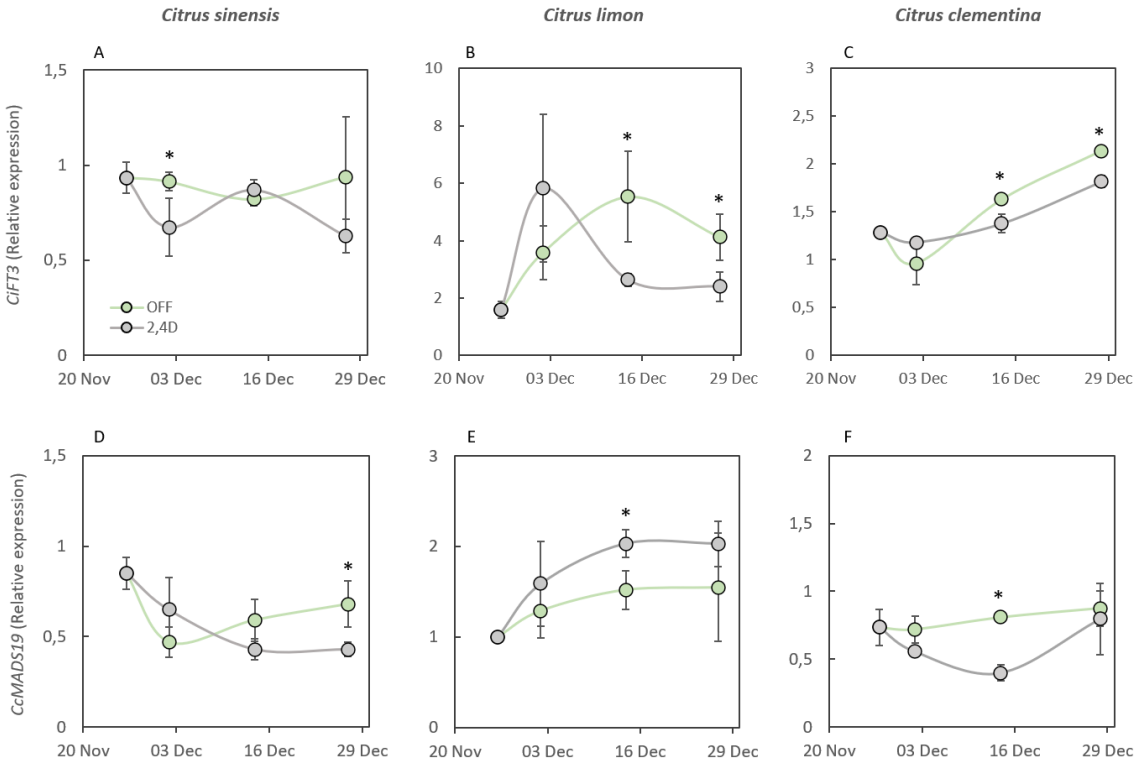


***Figure 3.4**. Impact of 2,4D (12 mg l^{-1}) on the expression of CiFT3 and CcMADS19 in Citrus sinensis (A, D), Citrus limon (B, E) and Citrus clementina (C, F). The treatment was applied at the floral inductive period (15th of November) on OFF trees. Each value is the mean of 3 mature leaves. The PCR reference gene was Actin. Asterisk indicates significant differences (P<0.05).*

3.2.2. 2,4-D effect in the bud

The expression of the *LFY* and *AP1* genes in the buds of OFF trees increased significantly between late November and mid-December and then stabilised in January. The *CEN* gene also increased significantly in expression from mid-December to January (Fig. 3.5). The application of 2,4-D did not immediately change the relative expression of the *LFY* and *AP1* genes. The effect was delayed by 30 days, so that 2,4-D blocked the observed increase in control buds, significantly reducing the expression of the *LFY* gene from 14.4 to 6.9 (Fig. 3.5A) and the *AP1* gene from 3.3 to 2.5 (Fig. 3.5B).

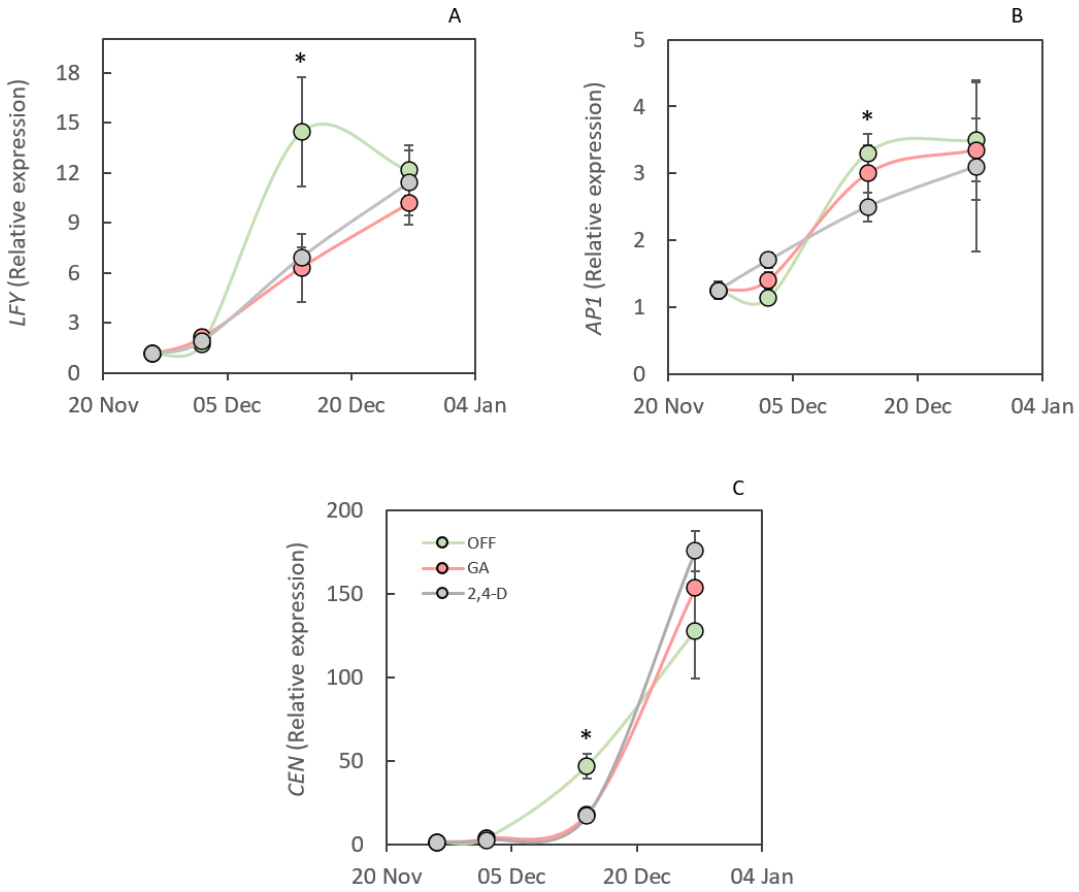


Figure 3.5. *Effect of 2,4-D (12 mg l-1) and GA_3 (25 mg l-1) on the expression of genes LFY (A), AP1 (B) and CEN (C). Treatments were applied during the floral inductive period (15th of November) to 20 'Oronules' clementine mandarin trees (10 with 2,4-D and the other 10 with GA_3). Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Asterisks indicate significant differences (P<0.05).*

This effect of 2,4-D was compared with the inhibitory effect shown by the application of GA_3 (Chapter II). The results show that the relative expression of the *LFY* and AP1 genes did not differ significantly between trees treated with GA3 and those treated with 2,4-D (Fig. 3.5A and B). In addition, 30 days after treatment (DAT), both treatments reduced the expression of the *CEN* gene compared to the control trees, from 46.9 to 17 relative units, and increased it in January, surpassing that of the control trees (Fig. 3.5C), which would explain the greater number of vegetative shoots formed as a result of the treatment (Figs. 3.2 and 3.3).

It's worth highlighting the inhibitory effect of 2,4-D on the biosynthesis of GAs (gibberellins), as evidenced by the significant reduction in the expression of the enzymes *GA20ox2* (-47.35%; Fig. 3.6A) and *GA3ox2* (-40.65%; Fig. 3.6B).

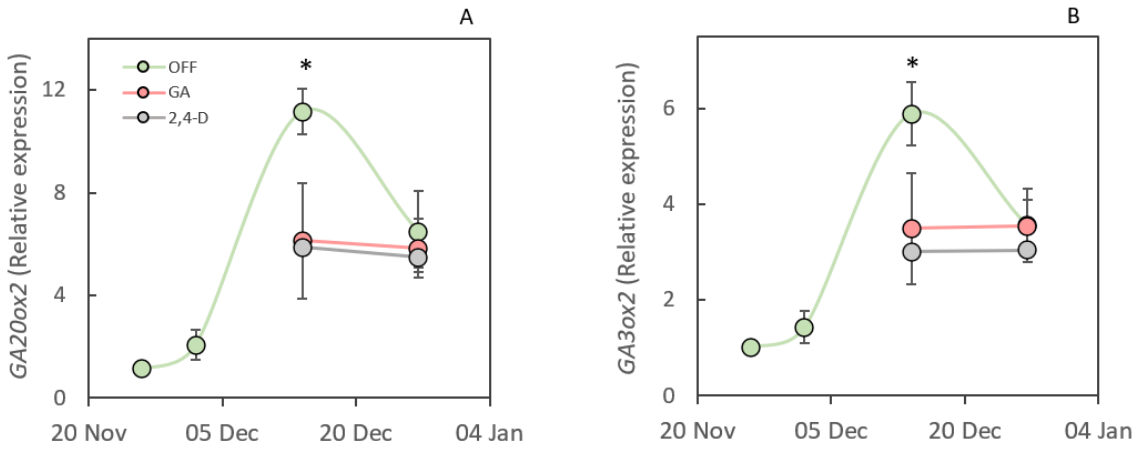


Figure 3.6. *Effect of 2,4-D (12 mg l-1) and GA_3 (25 mg l-1) on the expression of genes GA20ox2 (A) and GA3ox2 (B). Treatments were applied during the floral inductive period (15th of November) to 20 'Oronules' clementine mandarin trees (10 with 2,4-D and the other 10 with GA_3). Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Asterisks indicate significant differences (P<0.05).*

Furthermore, the effect of both treatments on the expression of auxin synthesis and transport genes was studied. Both 2,4-D and GA_3 significantly reduced auxin synthesis in the buds by decreasing the expression of *TRN2* (*TORNADO2*) from 10.9 to 4.6 (Fig. 3.7A) and *YUC4* (*YUCCA4*) from 6.6 to 2.8 (Fig. 3.7B) compared to untreated OFF trees. Additionally, polar auxin transport in the buds also decreased due to the effects of

both treatments. The expression of the genes *LAX3* (influx carrier; -54%; Fig. 3.7C) and *PIN3* (efflux carrier; -44%; Fig. 3.7D) was significantly reduced by the applications of 2,4-D and GA_3.

Finally, cell division in the axillary buds, determined by the expression of *CYCB2*, was also reduced by both treatments, from 18.5 to 7.2 relative units compared to control trees (Fig. 3.7E).

As fruit exerts a dominant effect on the supporting shoot, similar to apical dominance, the study aimed to investigate whether the variation in endogenous auxin

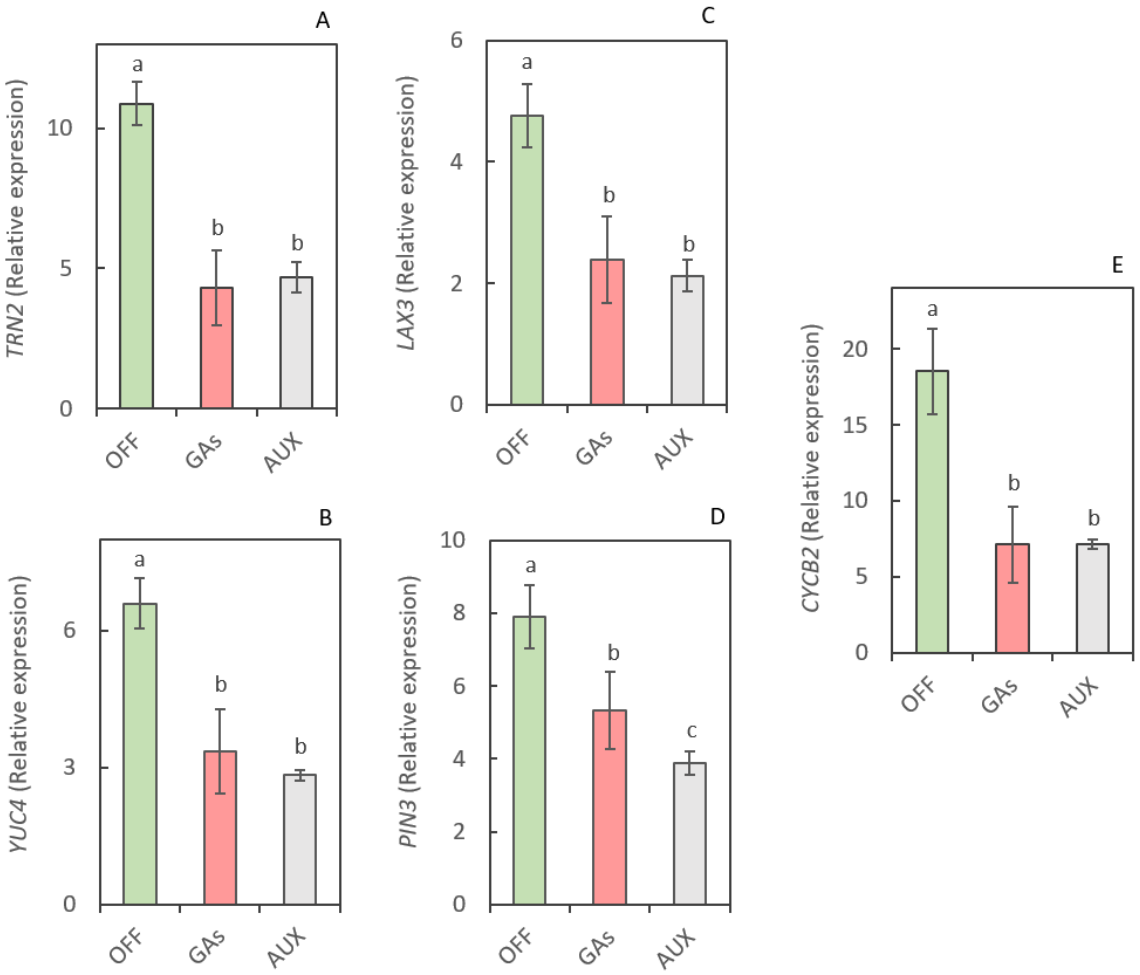


Figure 3.7. *Effect of 2,4-D (12 mg l-1) and GA_3 (25 mg l-1) on the expression of genes TRN2 (A), YUC4 (B), LAX3 (C), PIN3 (D) and CYCB2 (A). Treatment was applied to 'Oronules' clementine mandarin trees at the floral inductive period (15th of November) and buds were sampled one month later (14th od December). Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Asterisks indicate significant differences (P<0.05).*

content due to the presence of fruit is related to the regulatory mechanism of flowering in citrus.

3.3. The effect of fruit in auxin synthesis, transport and concentration

The high concentration of IAA in the fruit exudate during flower induction (Fig. 2.6 - Chapter II) suggests the importance of this hormone in the inhibition process. Indeed, the concentration of IAA in the leaves of branches with fruit (ON) and without fruit (OFF) increased significantly from July to September, but it was more pronounced in the ON branches and reached statistical significance. During the autumn-winter period, the concentration of IAA in the leaves of ON branches decreased but remained significantly higher than in the leaves of OFF branches at all times (Fig. 3.8A).

The increase in leaf IAA content could be due to increased transport from the fruit or increased synthesis in the leaf. The primary pathway for IAA synthesis in plants is via the amino acid tryptophan. The evolution of tryptophan concentration in mature leaves (7 months old) of OFF and ON branches increased during the periods of flower induction and differentiation, but it was six to seven times higher in OFF branches, showing an inverse correlation with IAA concentration. Conversely, the higher level of IAA in ON branches correlated with a lower relative concentration of its precursor (Fig. 3.8B).

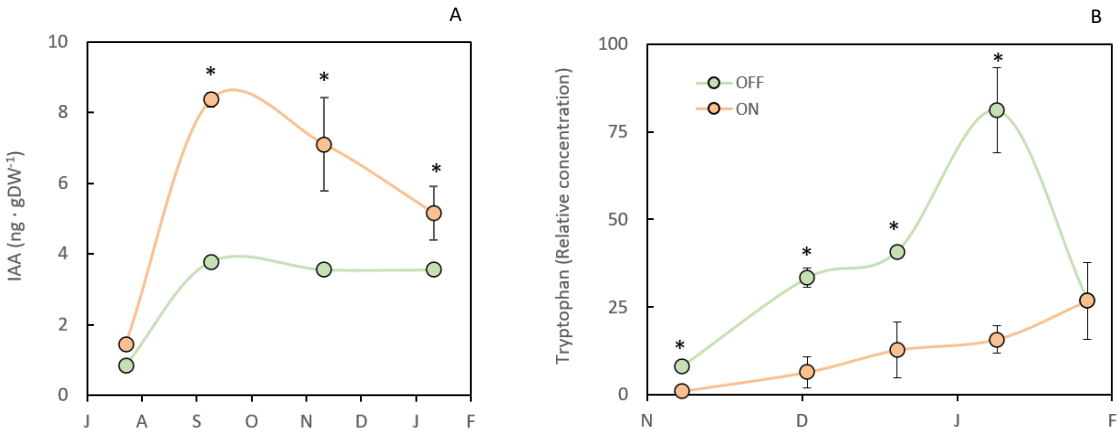


Figure 3.8. *Time-course of indole acetic acid (IAA) concentration (A) and tryptophan relative concentration (B) in leaves of ON and OFF 'Nadorcott' Mandarin trees. Each value is the mean of 3 mature leaves. Asterisk indicates significant differences (P<0.05).*

The presence of fruit also altered the content, synthesis, and transport of IAA in the buds. The IAA content was significantly higher in the buds of ON branches (60 ng gDW^{-1}) than in the buds of OFF branches (27.7 ng gDW^{-1}) in the month of November (Fig. 3.9A). The high concentration of IAA in ON branch buds was not due to increased synthesis, as assessed by the relative expression of *TRN2* (Fig. 3.9B) and *YUC4* (Fig. 3.9C). On the contrary, the expression of *TRN2* and *YUC4* was significantly lower in ON buds, showing a reduction of 77.4% and 31.8%, respectively. Additionally, the expression of the *PIN3* gene was also significantly lower in the buds of ON branches than in those of OFF branches (Fig. 3.9D), suggesting that the higher IAA concentration in the bud of ON

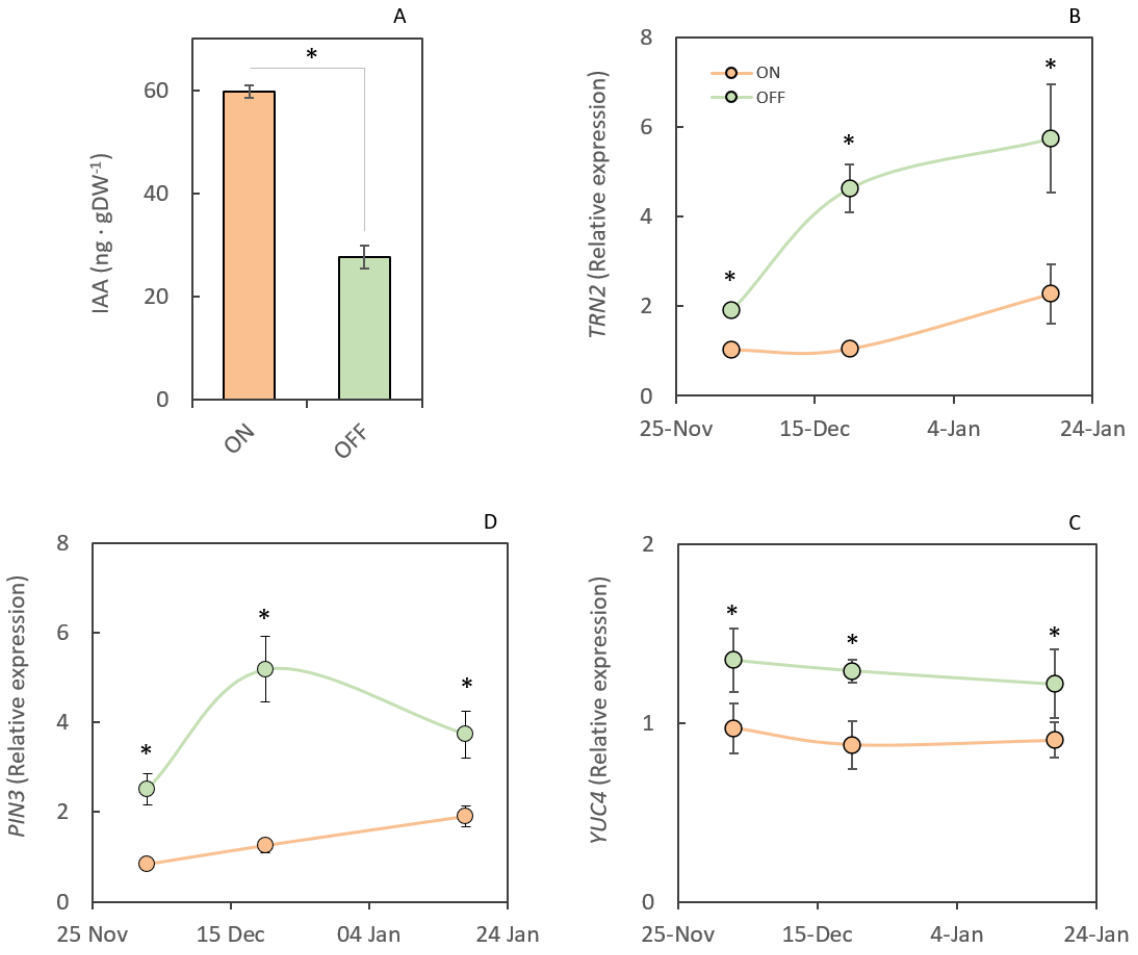


Figure 3.9. *A: Indole acetic acid (IAA) concentration determined the 20th of December in ON and OFF buds. Time-course of the expression of genes related with auxin synthesis, TRN2 (B) and YUC4 (C) and auxin transport, PIN3 (D) in buds of ON and OFF 'Nadorcott' Mandarin trees. Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Asterisks indicate significant differences (P<0.05).*

branches may be due to reduced transport from the bud, possibly because of the higher IAA concentration in the branch originating from the fruit.

In an additional experiment, in order to establish a cause-and-effect relationship between fruit, IAA, and flowering, fruit thinning was carried out in July, September, and November. The increase in flowering due to thinning depended on the timing of thinning, such that if the fruit remained on the branch until September, the flowering intensity of the OFF branches was not regained (Fig. 2.1, Chapter II), confirming the results of Martínez-Fuentes et al., (2010). Thinning in July and September reduced leaf IAA concentration in September compared to ON branches, but not in November (Fig. 3.10A). Thinning in November did not reduce the IAA concentration. In the phloem, IAA concentration was high in ON branches in September, higher than in OFF branches, and low in November and January. Thinning in July and September reduced the IAA concentration 48 hours after treatment. However, in the case of the July treatments, the IAA concentration in the phloem increased again, coinciding with autumn budding (data not shown). In the buds, thinning in September consistently reduced the IAA concentration compared to the buds of ON branches (Fig. 3.10C).

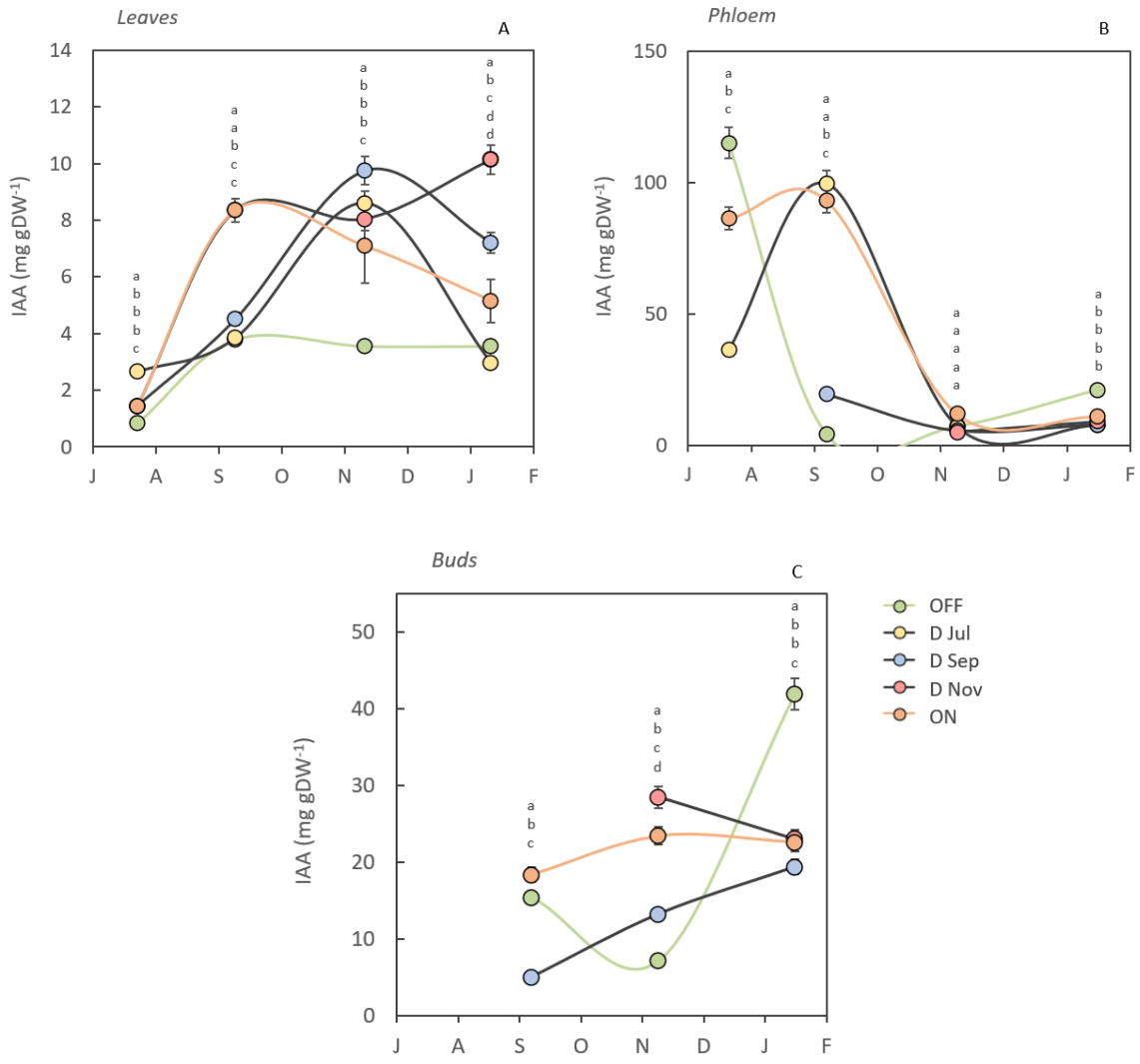


Figure 3.10. *Effect of thinning dates on the concentration of IAA in 'Nadorcott' mandarine leaves (A), phloem (B) and axillary buds (C). Defruiting of ON shoots was carried out on the 21st July, 7th September and 10th November. Each value is the mean of 3 biological replicates, consisting of four shoots from which 3 leaves, 2 g phloem or 20 buds per replicate were selected. Different letters indicate significative differences (p<0.05). Standard errors (SE) are given as vertical bars; in some cases, the SE is smaller than the symbol size.*

Chapter IV. *Isolating axillary buds from fruit inhibitory effect: Girdling and Double Girdling*

The previous chapters have shown that the presence of fruits alters the hormonal balance in the buds, including the alteration of gibberellin biosynthesis (Chapter II) and the disruption of auxin homeostasis during floral differentiation (Chapter III). However, no consistent relationship was found between the hormones (Chapters I and III) and the increased expression of the *CcMADS19* gene in leaves. Therefore, the signal(s) that trigger(s) the activation of the inhibitory mechanism of flower induction by the effect of the fruit, through the stimulation of *CcMADS19* gene expression, is/are unknown.

In the following chapter, the transmission of the inhibitory signal(s) between the fruit and the leaves and buds will be modified using the technique of branch girdling. By varying the timing and location of the technique, the effect of interrupting phloem transport between the fruit and the buds on flowering and its control during the stages of flower induction and differentiation will be studied. In addition, girdling allows further investigation of the nutritional hypothesis suggesting that carbohydrate depletion by the growing fruit is related to flowering inhibition (Goldschmidt and Sadka, 2021).

The aim of the present chapter is to determine the effect of girdling on three main aspects: 1) on the flowering stimulus in shoots with an apical fruit, 2) on the expression of the *CiFT3* gene in leaves and *CsLFY* in buds, and 3) on the hormonal and nutrient balance in leaves and buds.

4.1. The effect of girdling on flowering depends on the place where it is carried out

To modify the transmission of inhibitory signal(s) between the fruit and the leaves and buds to stimulate flowering, the localised effects of shoot apical girdling (A-G), close to the fruit, and basal girdling (B-G), at the base of the shoot, were studied. In addition, the combined effects of both types of girdling, apical and basal (AB-G), were also studied in order to completely isolate the buds from the influence of the fruit and the rest of the plant. In addition, all treatments were applied to all trees used in the experiment to

ensure that the effect of the treatment was independent of the nutritional status of the tree.

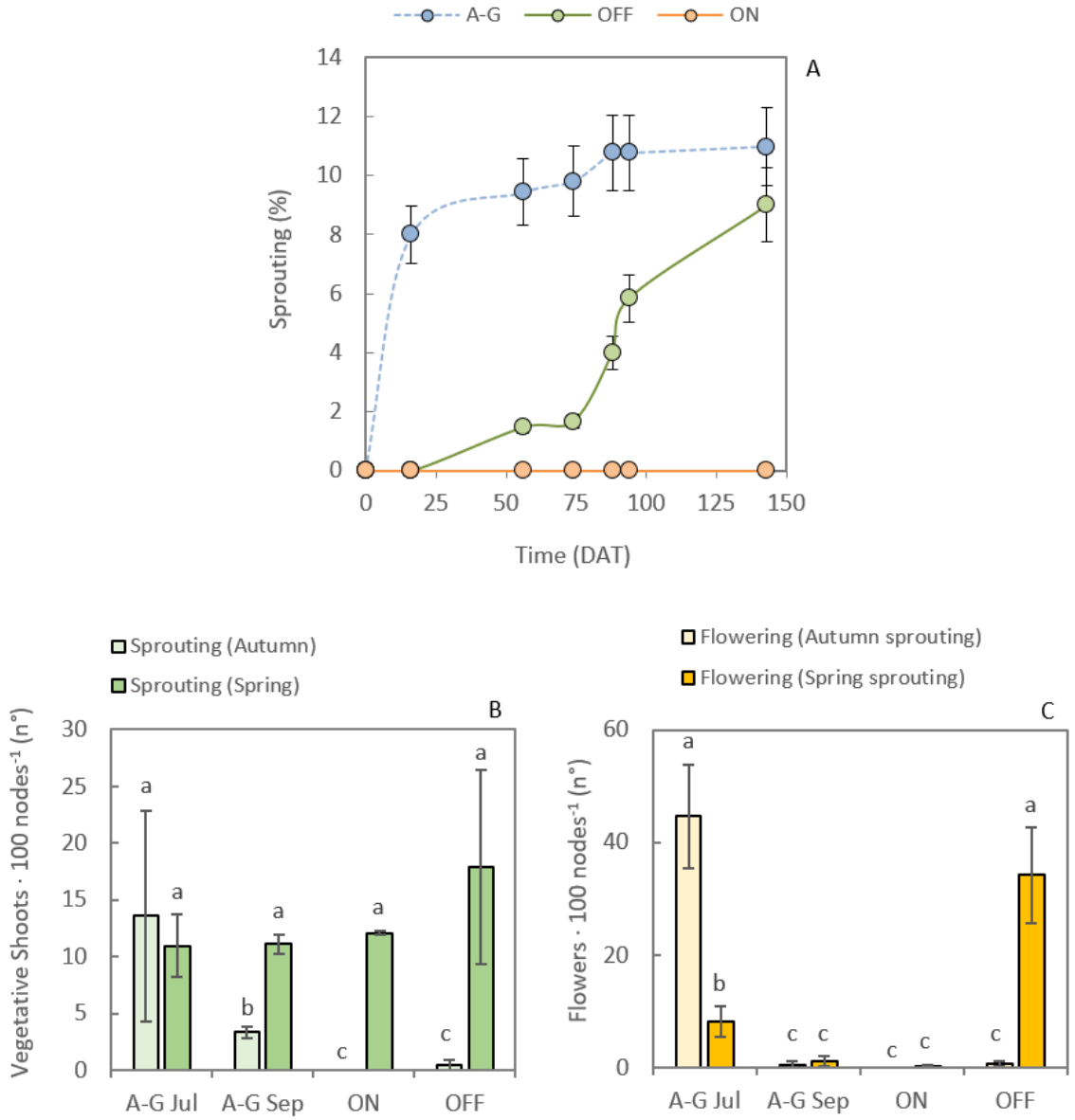


Figure 4.1. *Effect of Apical Girdling (A-G) in bud sprouting and flowering. Treatments were applied to 'Nadorcott' shoots with a fruit in apical position (ON). Shoots with fruit (ON) and without fruit (OFF) were used as control unable and able to flower, respectively. A: Time-course of vegetative development per treatment (A-G was made the 3rd of July 2017. B and C: Average number of vegetative shoots and flowers generated after A-G (3rd of July and 3rd of September, 2017). Each value is the mean of 60 shoots. Different letters indicate significant differences.*

In the first experiment, the A-G girdling performed in July stimulated the sprouting of approximately 8% of the axillary buds 16 days after treatment (Fig. 4.1A). Although it did not reach statistical significance, the percentage of buds sprouting increased slightly to about 10% nine days later (Fig. 4.1A). However, the buds on the shoots with fruit (ON) and without fruit (OFF) did not sprout (Fig. 4.4A). The buds on the ON shoots remained dormant almost two months after the start of the experiment, whereas the buds on the OFF shoots started to sprout at the end of the summer, reaching a value of almost 8% (Fig. 4.4A). The spontaneous increase in OFF sprouting in mid-September, 74 days after treatment, was due to the decrease in summer temperatures. The A-G treatment carried out in early September was much less effective in stimulating autumn sprouting, resulting in 3.8 vegetative shoots per 100 nodes (Fig. 4.1B).

In the following spring, the flowering of the A-G Jul treatment primarily occurred on the autumn vegetative shoots (45 flowers · 100 nodes^{-1}), reaching values similar to those produced by the OFF controls (38 flowers · 100 nodes^{-1}) on the spring shoots (Fig. 4.4C). In other words, the treatment advanced the restart of vegetative development by one cycle compared to the OFF branches, which is a characteristic requirement for flowering in polycarpic species. The axillary meristems of fruit-bearing shoots that did not sprout in autumn did not flower in the spring.

Delaying the A-G to late September or changing the type of girdling (B-G or AB-G) had no effect on autumn sprouting due to the temperature drop. However, in the spring, the total number of vegetative shoots significantly differed between the single girdling treatments (A-G and B-G) (3.4 vegetative shoots · shoot^{-1}) compared to the ON shoots (1.9 vegetative shoots · shoot^{-1}) (Fig. 4.2A). The sprouting capacity depended on the position of the node on the shoot, with higher sprouting at the apical nodes compared to the basal ones (Fig. 4.2B). On the other hand, double girdling (AB-G) prevented the formation of vegetative shoots.

The results obtained with apical and basal girdling were as expected and comparable to previous studies (Martínez-Montagud, 2015). In the case of A-G, the increase in sprouting is likely related to the loss of sink capacity and the apical dominance of the fruit due to the interruption of carbohydrate and auxin transport through the phloem.

For B-G, the axillary meristems are not isolated from the effect of the fruit, but they are isolated from the rest of the tree. Nevertheless, this treatment stimulated bud sprouting in the spring after harvest.

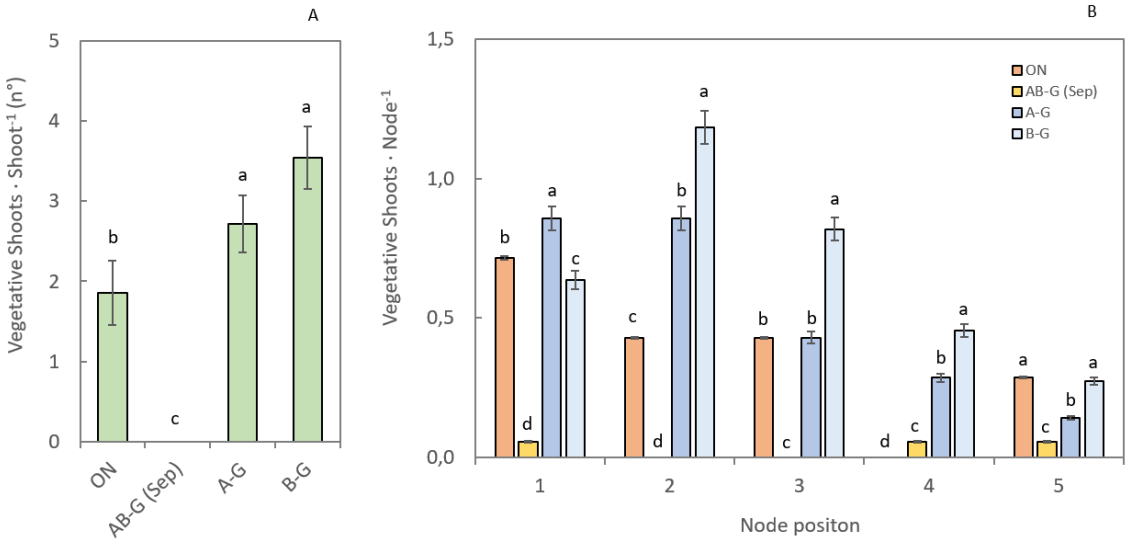


Figure 4.2. *Effect of Apical (A-G), Basal (B-G) and Double Girdling (AB-G Sep) in the development of new vegetative shoots during spring. Treatments were applied the 20th of September to 'Nadorcott' shoots with fruit in apical position (ON). A: Average of Vegetative Shoots per treatment. B: Average of Vegetative Shoots per node. Each value is the mean of 15 shoots per treatment. Different letters indicate significative differences in each node position.*

However, the most surprising result was obtained with double girdling, both apical and basal (AB-G). All axillary buds on the shoots transformed into reproductive buds, even in the presence of fruits (Fig. 4.3A, B), and none of them managed to develop vegetative shoots (Fig. 4.2B). In particular, the AB-G treatment achieved an average of 11 flowers per shoot (Fig. 4.3A) and 2 flowers per node, significantly different from the others (without flowering). Furthermore, the behaviour among nodes was identical (Fig. 4.3B), unlike what happened with the vegetative shoots.

As this was a new experimental model, the AB-G treatment was replicated in different plots in the same year, with the same result (Fig. 4.4A, D). In the following years, the experiment was repeated by modifying the timing of the double girdling, always avoiding the stimulation of autumn sprouting. When carried out in November (AB-G Nov), coinciding with the induction stage, and in December (AB-G Dec) during the floral

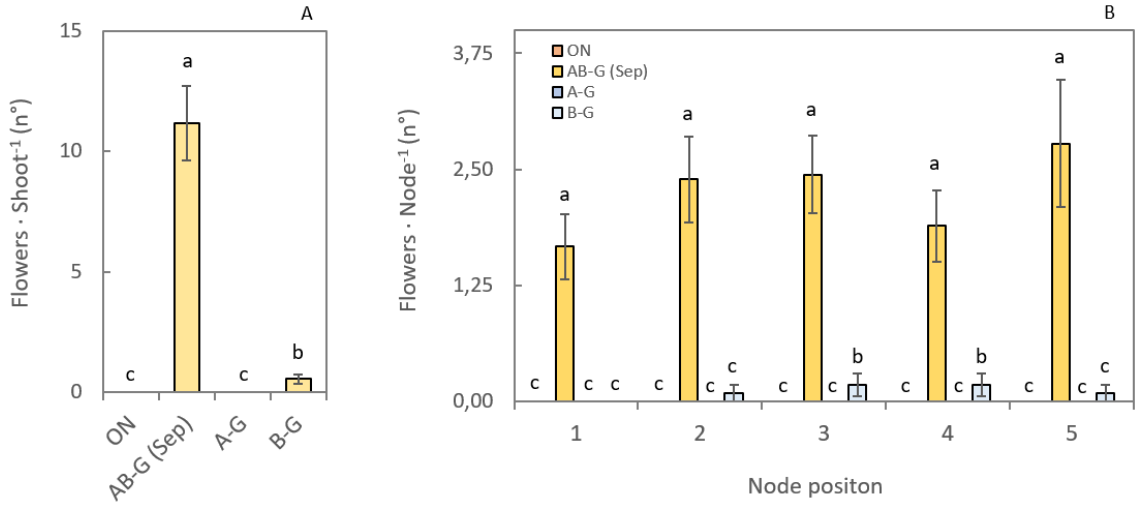


Figure 4.3. *Effect of Apical (A-G), Basal (B-G) and Double Girdling (AB-G Sep) in spring flowering. Treatments were applied the 20th of September to 'Nadorcott' mandarin shoots with fruit in apical position (ON). A: Average of flowers per treatment. B: Average of flowers per node. Each value is the mean of 15 shoots with fruit in apical position. Different letters indicate significative differences in each node position.*

differentiation period, the same result was obtained as with the AB-G conducted in September, before floral induction. Vegetative sprouting decreased (Fig. 4.4B, C), and the number of floral shoots increased significantly compared to the ON shoots (Fig. 4.4E, F). Consequently, the results suggest that: 1) the treatment is capable of activating the flowering potential of axillary buds on fruit-bearing shoots, and 2) the effect of AB-G is independent of the time of application.

The evaluation of flowering by shoot type (flowers or inflorescences with or without young leaves) showed a qualitative change due to the treatment. Comparing flowering in OFF shoots of 'Nadorcott' mandarins with flowering induced by AB-G, it was observed that the percentage of single flowers (SF) and inflorescences (I) without leaves increased by 23.11% and 9.85% respectively in the AB-G shoots, while the percentage of mixed shoots decreased significantly by -31.24%. The variation was less in the vegetative shoots with flower in apical position (L-SF; Table 1).

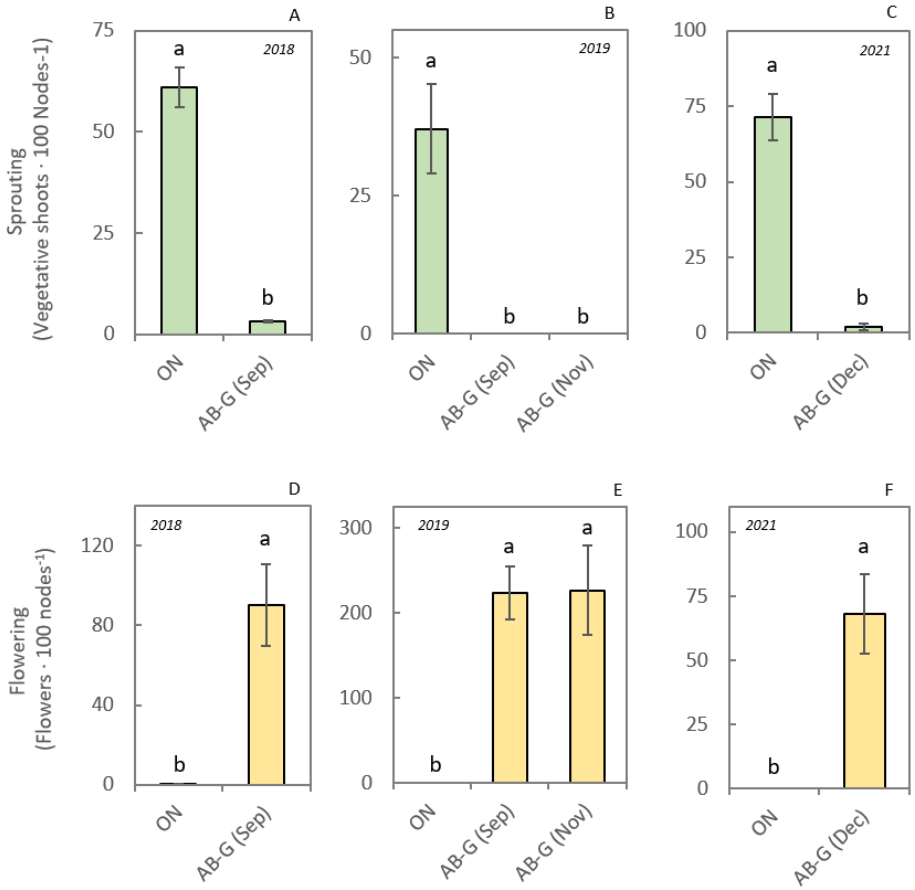
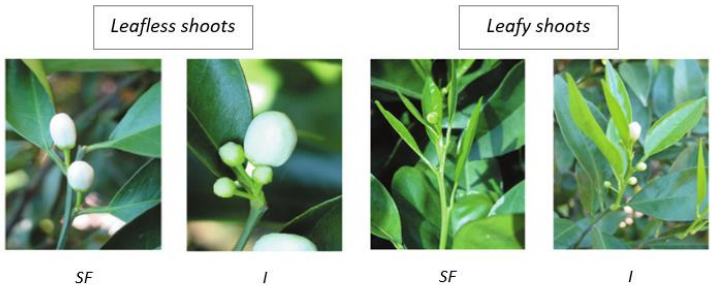


***Figure 4.4**. Effect of timing of Double Girdling (AB-G) before (September), during (November) and after (December) inductive period in 'Nadorcott' mandarin. A, B, C: vegetative shoots generated by Double Girdling in three different years. C, D, E: Flowering intensity induced by Double Girdling in three different years. Each value is the mean of 15 shoots. Different letters indicate significant differences.*

***Table 1**. Comparison of flowering distribution between OFF and AB-G, evaluated in spring 2019. The treatment was carried out before inductive period (September 2018) in 'Nadorcott' mandarin. Each value is the mean of 15 shoots. Reproductive shoots without leaves (I, inflorescence and SF, solitary flower); Mixed shoots (L-I, inflorescence with leaves and L-SF, vegetative shoot with solitary flower in apical position).*

	Leafless shoots				Leafy shoots			
	Single flowered		Inflorescence		Single flowered		Inflorescence	
	AB-G	OFF	AB-G	OFF	AB-G	OFF	AB-G	OFF
Flowers (n°)	101	57	60	42	9	13	31	98
%	50,25	27,14	29,85	20,00	4,48	6,19	15,42	46,67
AB-G/OFF	23,11		9,85		-1,71		-31,24	



In summary: 1) A-G stimulates sprouting, whereas AB-G stimulates flowering; 2) If A-G is carried out before the temperature drops, it stimulates autumn sprouting, which is capable of flowering. This raises three questions, that will be developed throughout in this chapter:

1. How is the ability to flower reactivated in autumn vegetative shoots?
2. What mechanism allows double girdling to activate the flowering potential in the axillary meristems of a fruit-bearing shoot?
3. Is this mechanism similar to that of fruitless shoots, that can flower due to low temperatures?

The expression of *CcMADS19* increased in the leaves of ON shoots but not in the leaves of OFF shoots or the new leaves of A-G Jul shoots (Fig. 4.5A). Consequently, the expression of *CiFT3* remained low in the leaves of ON shoots compared to OFF shoots and A-G Jul shoots (Fig. 4.5B).

These results suggest that *CcMADS19* is silenced in A-G Jul shoots. Since its activation is associated with changes in histone methylation, trimethylation of H3K27 (associated with gene silencing) was examined at two points in the promoter region of the *CcMADS19* locus. The leaves of new autumn shoots generated by the A-G treatment showed a higher accumulation of H3K27me3 than the leaves of ON shoots in November (Figure 4.5C). The expression of the methyltransferase *CcATX1* (homologous to Arabidopsis *TRITHORAX1*), which catalyses the deposition of the mark H3K4me3 (Pien et al., 2008), was not increased by the treatment. However, the young leaves of A-G Jul shoots showed a reduction in the expression of *CcELF6* (*EARLY FLOWERING 6*) (Fig. 4.5D). The Arabidopsis ELF6 protein is an H3K27me3 demethylase involved in the reactivation of *FLC* after vernalization (Crevillen et al., 2014). The Arabidopsis proteins CLF (CURLY LEAF) and VIN3 (VERNALIZATION INSENSITIVE3) are part of the Polycomb Repressive Complex 2 (PRC2), which activates *FLC* repression during Arabidopsis vernalization. The expression of *CcVIN3* increased in young leaves of A-G Jul shoots (Fig. 4.5D), correlating with the increase in H3K27me3 and the silencing of *CcMADS19*.

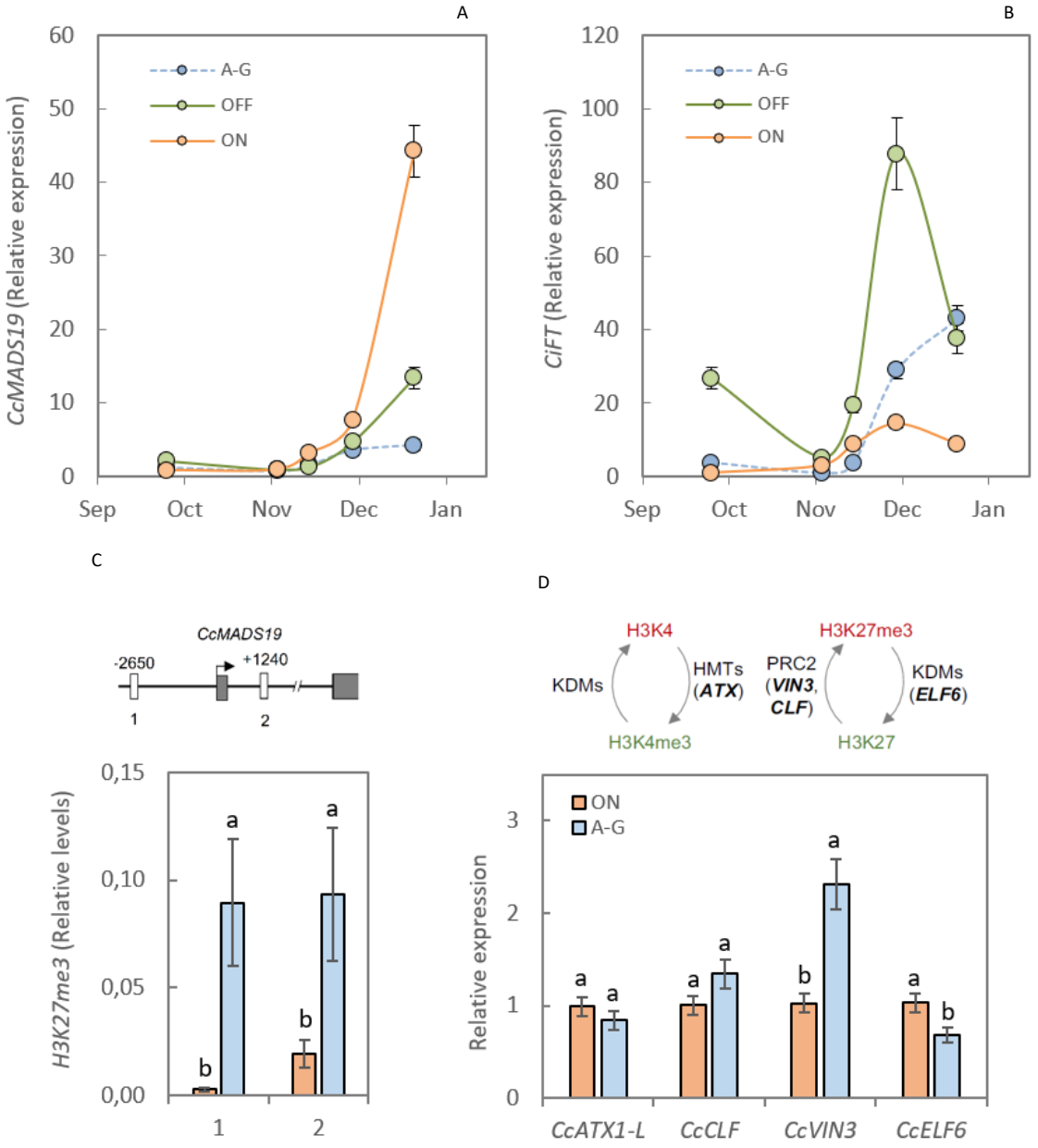


Figure 4.5. *CcMADS19 active/repressed state correlates with changes in histone methylation. (A) and (B) Relative expression of CcMADS19 and CiFT3 in adult leaves from ON and OFF shoots, and young leave from A-G shoots. (C) H3K27me3 levels in the same leaves determined by ChIP of two regions located on the promoter of the CcMADS-box19 locus. (D) Relative expression in adult leaves from ON shoots and young leaves from A-G shoots of CcELF6, CcATX1-L, CcCLF, CcVIN3 genes determined by RT-qPCR. Data correspond to leaves of tangor 'Nadorcott' (Citrus clementina x Citrus sinensis) sampled at early autumn before the inductive period (1 October). Different letters indicate statistical significance. (Mesejo et al., 2021).*

4.2. RNA Sequencing: common pathways which are activated/repressed in Double Girdling, Small fruit, and OFF-trees to promote flowering

While the double girdling technique promotes the flowering of all axillary buds on the shoot, similar to what happens in an OFF shoot, its mechanism of action may differ significantly from the floral induction process for two reasons: 1) the wound caused by the interruption of phloem transport; 2) the presence of the fruit during the induction period. Therefore, to obtain a comprehensive view of the metabolic pathways that AB-G modifies to promote flowering, excluding other effects specific to the technique, RNA-seq was performed during the floral induction period with samples from 4 phenotypes (1 non-flowering, 3 flowering), which were compared as follows: 1) ON shoot with a spring fruit, incapable of flowering in the following spring; 2) OFF shoot without fruit, which naturally flowers (14 flowers · shoot^{-1}) (Fig. 4.6); 3) ON shoot girdled (AB-G Sep) that flowers like the OFF shoot; and 4) autumn shoot with small fruit from a late summer flowering (SF), which flowers in spring but with less intensity (Fig. 4.6).

4.2.1. What happens in natural conditions? Differentially expressed genes (DEGs) in ON vs OFF trees

The presence of fruit during the floral induction period promotes significant changes ($P<0.01$) in the expression of 753 genes in the leaves and 11,615 genes in the buds (Fig. 4.7A). However, to narrow down the study to the most quantitatively important DEGs, only sequences with a minimum twofold difference in expression between the ON and OFF samples were analyzed (log2 Fold Change (FC) ≥ 1). This involved the analysis of 235 DEGs in the leaves and 3,254 DEGs in the buds (Fig. 4.7B). Out of these, a total of 153 and 1,084 were overexpressed, while 82 and 2,170 were repressed in the leaves and buds, respectively (Fig. 4.7E). Figures 4.7C and 4.7D show the Volcano Plots of the DEGs in the leaves and buds, respectively, with FC ≥ 1. DEGs that are highly repressed/overexpressed are separated from the value 0 on the X-axis, and those showing higher statistical significance are separated from the value 0 on the Y-axis. For instance, in the buds, *Ciclev10012089mg* (*ASYMMETRIC LEAVES 1; AS1*), a transcription

factor related to the adaxial/abaxial polarity of leaf development (Li et al., 2016), is shown to be repressed in the ON tree (Fig. 4.7D). In the leaves, some of the most repressed sequences in the ON tree are of unknown proteins (*Ciclev10022521mg, Ciclev10006384mg, Ciclev10029484mg*) according to the NCBI database.

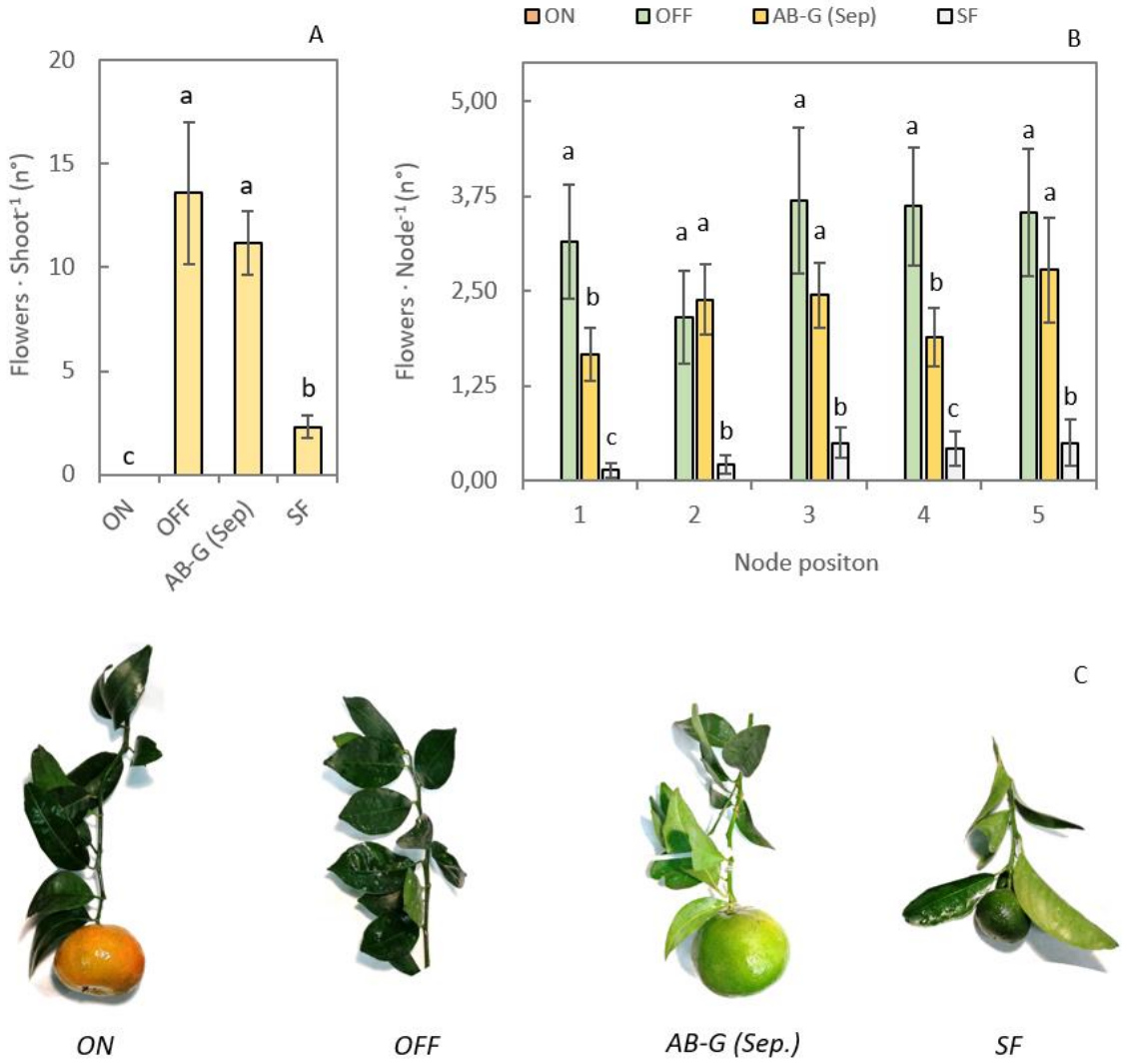


Figure 4.6. *Floral phenotype in 'Nadorcott' mandarin of the treatments involved in the RNA Sequencing analyse. A: Average flowering per treatment. B: Average flowering per Node. C: Treatments involved in the RNA-Sequencing analyse. ON: shoot with fruit in apical position, OFF: vegetative shoot, AB-G (Sep.): Double Girdling in shoots with fruit in apical position, SF: small fruit generated in an extemporaneous flowering in summer. Each value is the mean of 15 shoots. Different letters indicate significative differences in each node position.*

The results suggest that the presence of fruit has a significant impact on genetic regulation in the buds, where the percentage of genes with differential expression is 93.5% higher than in the leaves.

Functional characterization of the 235 DEGs in the leaves, using Gene Ontology (GO) annotations (www.geneontology.org), reveals that these genes are classified in the generic category 'Biological Process' (Fig. 4.8). The most enriched GO-terms (Fold enrichment > 5), albeit with a small number of genes, were related to secondary metabolism, response to jasmonic acid (5 genes up- and 2 downregulated), and response to karrikins (3 genes upregulated and 3 repressed). Jasmonic acid is a hormone associated with abiotic stress and widely studied in citrus (Ollas et al., 2012; Mahmoud et al., 2021). Karrikins are compounds related to seed germination and seedling growth. Mutants with altered response to karrikins exhibit seeds with increased latency, altered photomorphogenesis of seedlings, and modified leaf shape (Waters et al., 2014). Additionally, there were categories related to biotic defense responses with a Fold-enrichment of around 3 but a larger number of genes. Furthermore, significant categories included protein phosphorylation and response to external stimuli (Fig. 4.8).

On the other hand, the functional classification of genes that are overexpressed or repressed in the buds of ON trees compared to OFF trees (Fig. 4.9) is related to key biological processes like the cell cycle process, primary metabolism, or hormonal response (Fig. 4.9A). These genes are also related to molecular functions such as microtubule and cytoskeleton formation, glycosyltransferase activity, and transcriptional regulation (Fig. 4.9C), which influence cellular composition (Fig. 4.9B).

What happens in natural conditions? (ON vs OFF)

Differential gene expression – DGEs

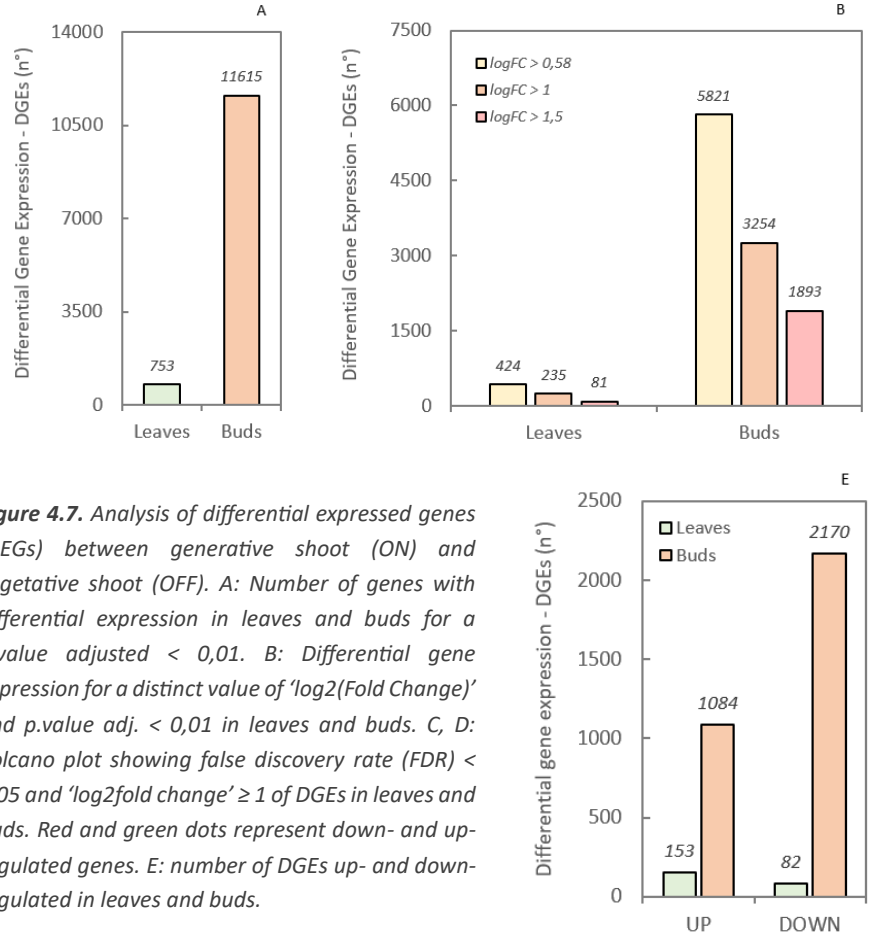
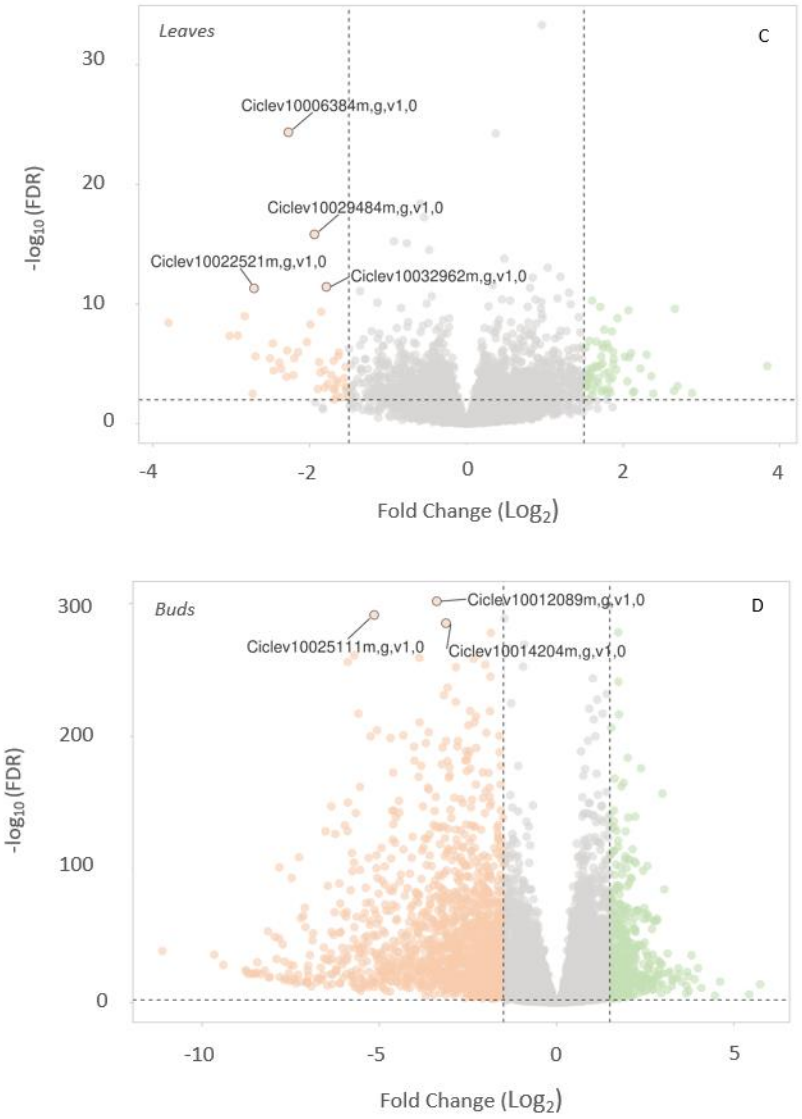


Figure 4.7. *Analysis of differential expressed genes (DEGs) between generative shoot (ON) and vegetative shoot (OFF). A: Number of genes with differential expression in leaves and buds for a p.value adjusted < 0,01. B: Differential gene expression for a distinct value of 'log2(Fold Change)' and p.value adj. < 0,01 in leaves and buds. C, D: Volcano plot showing false discovery rate (FDR) < 0,05 and 'log2fold change' ≥ 1 of DGEs in leaves and buds. Red and green dots represent down- and up-regulated genes. E: number of DGEs up- and down-regulated in leaves and buds.*



Gene expression constitutive differences between ON and OFF leaves.

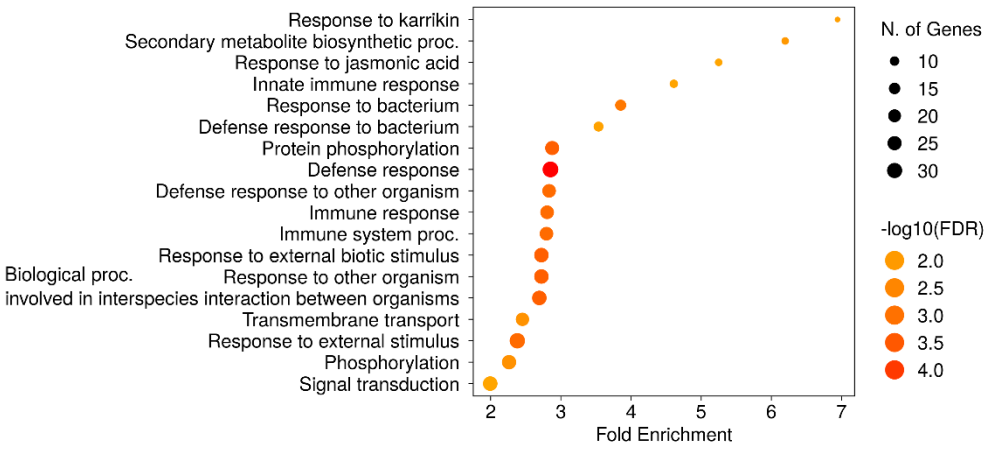


***Figure 4.8**. Gene Ontology (GO) overrepresentation test for the 235 DEGs between ON (with fruit) and OFF (without fruit) 'Nadorcott' mandarin leaves. The test only shows the category 'Biological Process'. Test made wtih ShinyGO 0.77 software. Restriction values: log2(Fold Change) ≥ +1 or ≤-1 and p.value adj. < 0,01. Fold Enrichment is given by the ratio between the relative frequency of genes belonging to a given process in the library and in the reference genome (Citrus clementina).*

Looking at the major 'Biological Process' categories, the most abundant category of repressed genes in ON trees compared to OFF trees is the 'Cell cycle process'. Within this group, there are 124 genes with differential expression, of which 116 are downregulated. This group includes key cyclins and kinase-dependent cyclins. Only 8 genes are upregulated, and these are related to membrane synthesis (data not shown).

Additionally, two other gene categories, 'Response to hormone' and 'Carbohydrate metabolic process,' were highly enriched in ON buds. Both categories contain the largest groups of genes, with 257 and 201 DEGs, respectively (Fig. 4.9A).

Gene expression constitutive differences between ON and OFF buds.

***Figure 4.9**. Gene Ontology (GO) overrepresentation test for the 3254 DEGs between ON (with fruit) and OFF (without fruit) 'Nadorcott' mandarin buds. DEGs were classified in three categories: (A) Biological Process, (B) Cellular Component (C) Melecular Function, using ShinyGO 0.77 software. Restriction values: 'log2(Fold Change)' ≥ 1 or ≤ -1 and p.value adj. < 0,01. Fold Enrichment is given by the ratio between the relative frequency of genes belonging to a given process in the library and in the reference genome (Citrus clementina).*

To further our understanding of the first category, 'Response to hormone', the DEGs were further classified according to the type of hormone they interact with, resulting in four new subgroups: response to auxins, ethylene, jasmonic acid, and gibberellins. In some cases, due to the interaction between these hormones, certain DEGs were placed in more than one subgroup. The largest subgroup, composed of 37 DEGs, included genes involved in the auxin response pathway, with 78.4% of them being downregulated. In particular, both auxin receptors (*Auxins Response Factors; ARF2* and *ARF3*), genes responsible for regulating hormone homeostasis (*TORNADO2; TRN2*), as well as the influx (*LAX2* and *LAX3*) and efflux (*PIN1, PIN4*, and *PIN6*) transporters were repressed in ON fruit-bearing shoots (Fig. 4.10A).

The second subgroup includes genes related to the ethylene response, with a total of 36 DEGs, showing both up- and down-regulation. The top 5 most upregulated DEGs in this subgroup included 4 ethylene response factors (ETRs) and a gene associated with oxidative stress response (*SAG21*). Among the most downregulated genes were *TOE2* and *SAUR78*, which act as inhibitors of ETRs (Fig. 4.10B).

Another significant group consists of genes related to the jasmonic acid response, 20 of which were upregulated in ON shoots. Prominently upregulated genes in this group include *GRXC9* and *WRKY70*, which are associated with the response to external stress and defense against external agents, and *JAR1*, an enzyme involved in the activation of jasmonic acid through conjugation to jasmonoyl-L-isoleucine (Fonseca et al., 2009) (Fig. 4.10C).

Finally, thereare a total of 14 genes related to the response to gibberellins that are highly repressed in ON trees. Among these genes, *GASA4* and *GASA14* are regulated by GAs and are related to floral meristem identity (Roxrud et al., 2007). *SNEEZY (SNE)* is involved in GA response signaling (Ariizumi and Steber, 2011), and *AtEXPA1* is an expansin related to cell elongation (Zhong et al., 2015). Additionally, the enzyme Kaureno oxidasa, which is involved in the initiation of the GA synthesis pathway (Davies, 2010), was upregulated in ON tree buds (Fig. 4.10D).

The second category, 'Carbohydrate metabolic process,' contained four groups of DEGs (Fig. 4.11A). The most enriched group (Fold enrichment >15) was related to sucrose

and starch metabolism and contained 22 DEGs. Of these, 77.3% were downregulated in ON shoots, with prominent repression of sucrose invertases (*SUS2* and *SUS6)*, cell wall invertase (*CWINV1*), and starch hydrolysis (*AMY2*) (Fig. 4.11B). Metabolism of secondary metabolites also showed a high number of affected regulatory genes, with repression levels surpassing those of upregulation (Fig. 4.11C). Lastly, processes such as 'Glycolysis' (Fig. 4.11D) and galactose metabolism (Fig. 4.11E) were also affected in fruit-bearing shoots.

Figure 4.12 summarizes the most significant changes induced by the presence of fruit in the hormonal and nutritional metabolic pathways of the buds, based on KEGG pathway database.

In summary, the results of the RNA-seq analysis of leaves and buds of ON and OFF trees during the floral induction period provide a comprehensive overview of the most significant differences at that specific time. The results are qualitative consistent with those obtained in previous chapters while expanding the information and avenues for future study.

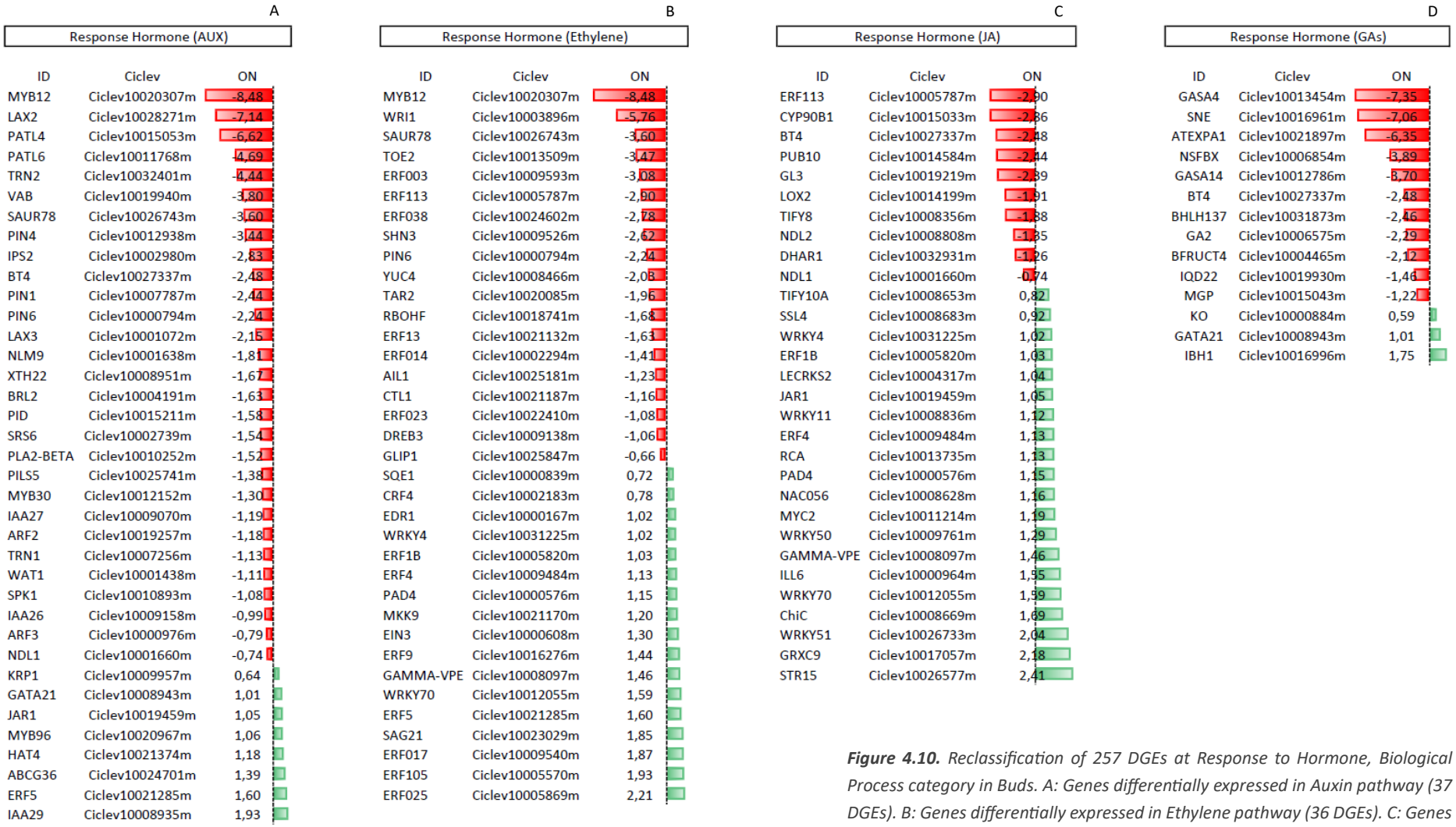


Figure 4.10. *Reclassification of 257 DGEs at Response to Hormone, Biological Process category in Buds. A: Genes differentially expressed in Auxin pathway (37 DGEs). B: Genes differentially expressed in Ethylene pathway (36 DGEs). C: Genes differentially expressed in Jasmonic Acid pathway (30 DGEs). D: Genes differentially expressed in Gibberellin pathway (14 DGEs).*

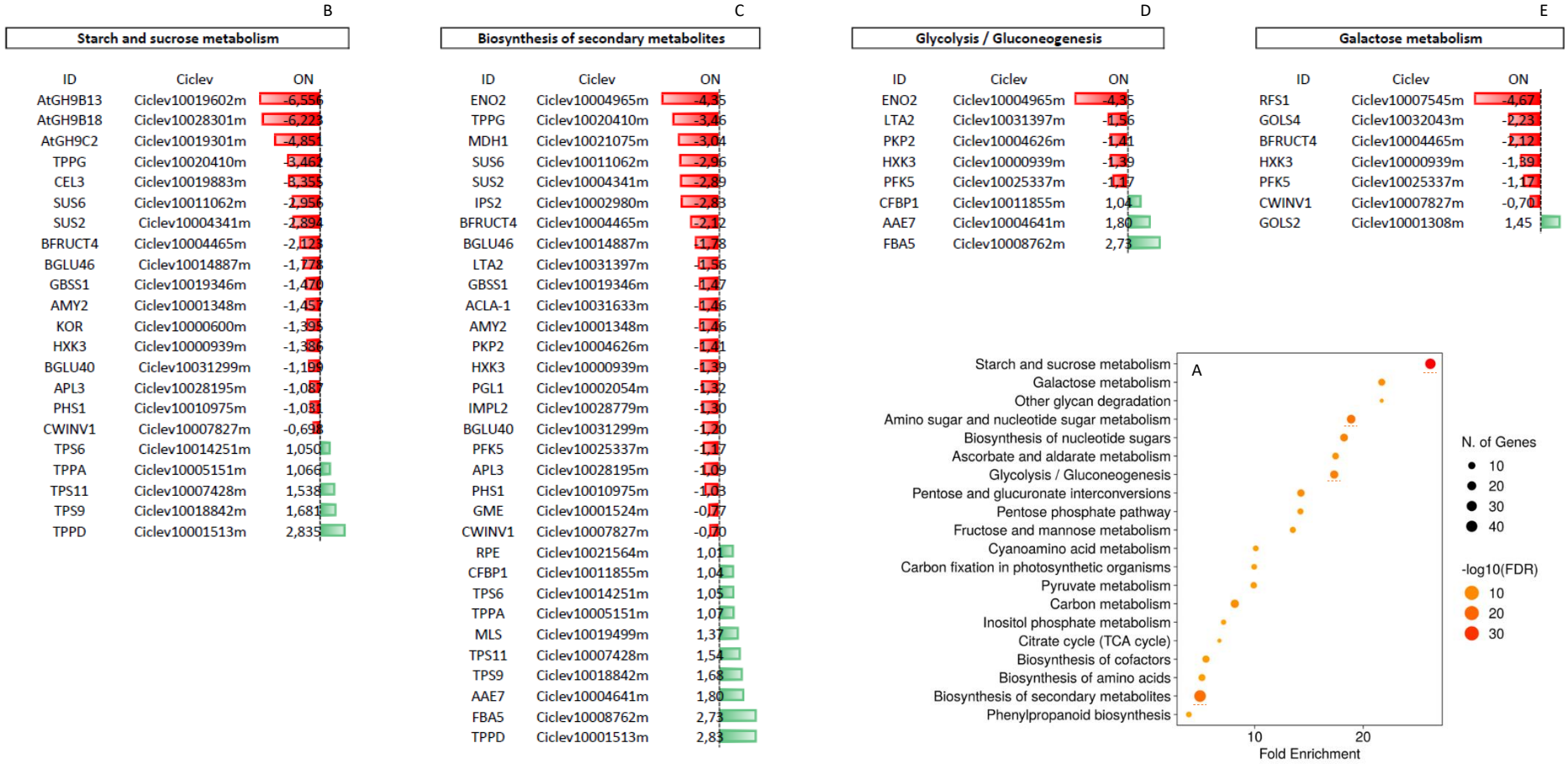
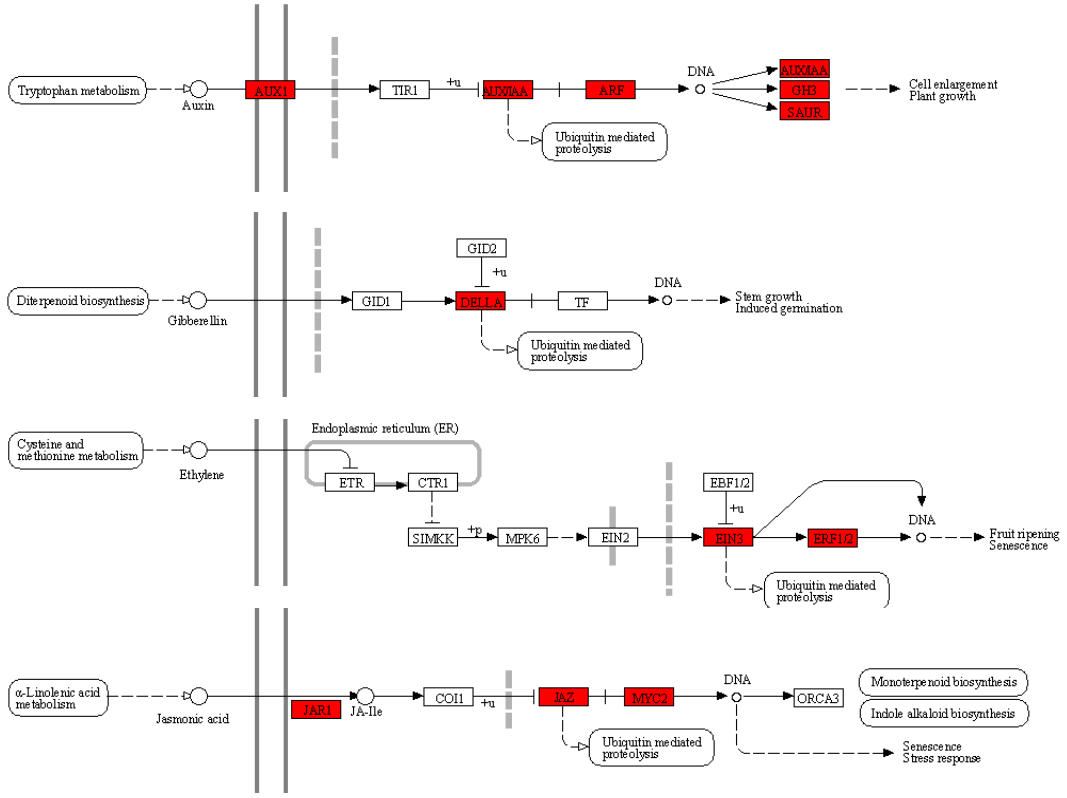


Figure 4.11. *Reclassification of 201 DGEs at Carbohydrate metabolic process, A: KEGGs pathways in Buds. B: Genes differentially expressed in Starch and Sucrose metabolism (22 DGEs). C: Genes differentially expressed in Biosynthesis of secondary metabolites (32 DGEs). D: Genes differentially expressed in Glycolysis/Gluconeogenesis (8 DGEs). D: Genes differentially expressed in Galactose metabolism (7 DGEs).*

Plant Hormone – Signal Transduction

A



Starch and Sucrose Metabolism

B

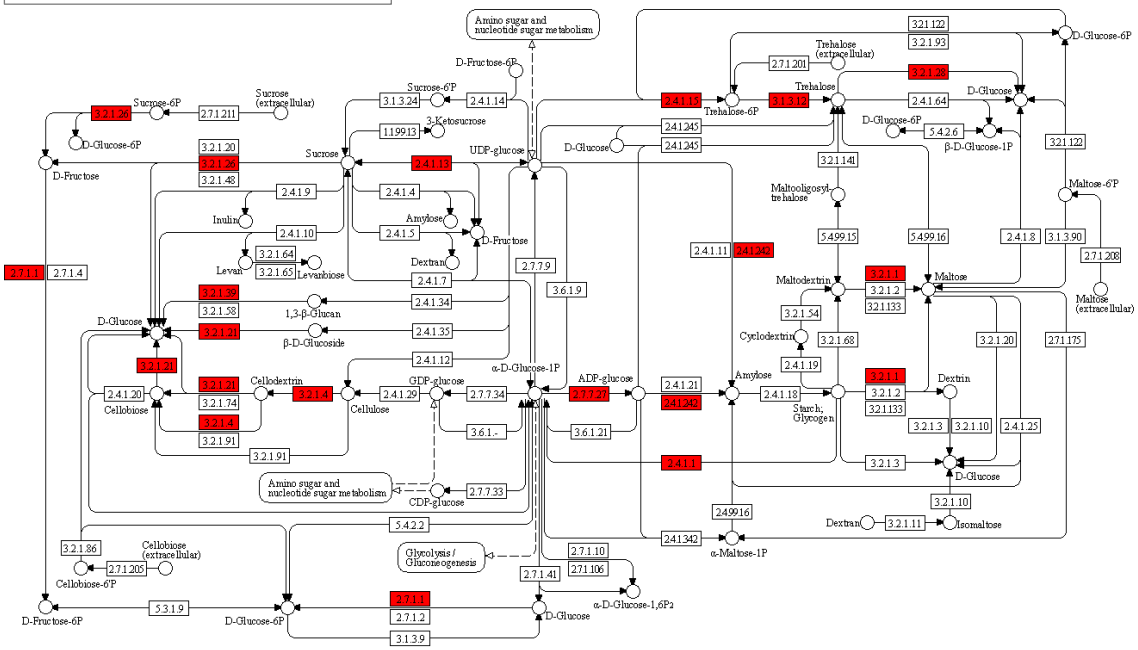


***Figure 4.12**. KEEGs pathways analyse of two reclassification categories in Buds. A: Response to Hormones: Auxin, Gibberellin, Ethylene and Jasmonic Acid. B: Starch and Sucrose Metabolism. Squares in red indicate alterations, up- or down- regulation of enzymes in the pathway.*

Among the main results from the RNA-seq analysis which are consistent with previous approaches (GA and AUX treatments, their antagonists PBZ and TIBA, presence or absence of fruit), the following stand out:

1. In the buds of ON trees, cell division is virtually absent throughout the flowering induction period compared to OFF shoots (Chapter II; Fig. 2.8C).
2. Buds of ON trees show lower expression of auxin transporters (*PIN*) and synthesis genes (*TRN2* and *YUC4*) (Chapter III; Fig. 3.9).
3. Similarly, in the case of gibberellins, their synthesis in ON bud is not activated until January, during the differentiation period (Chapter II; Fig. 2.2A and B). An increase in kaureno oxidase expression, the enzyme responsible for the initial stage of GA synthesis, was detected during the induction period (Chapter IV; Fig. 4.10D).
4. Finally, jasmonic acid (JA) was the hormone with the highest concentration in fruit exudate (Chapter II; Fig. 2.5), and the level of overexpression of JA response genes is higher in buds of fruit-bearing trees (Fig. 4.10B).

Therefore, the results suggest that the hormonal balances in the buds of ON shoots is altered during the flowering induction stage, leading to reduced meristematic activity compared to OFF shoots. Consequently, the buds of fruit-bearing shoots remain in a state of basal metabolism, reducing energy acquisition processes such as glycolysis, starch hydrolysis, and sucrose transport (Fig. 4.12B)

4.2.1.1. Prediction of potential Transcription Factors regulating ON vs OFF DEGs

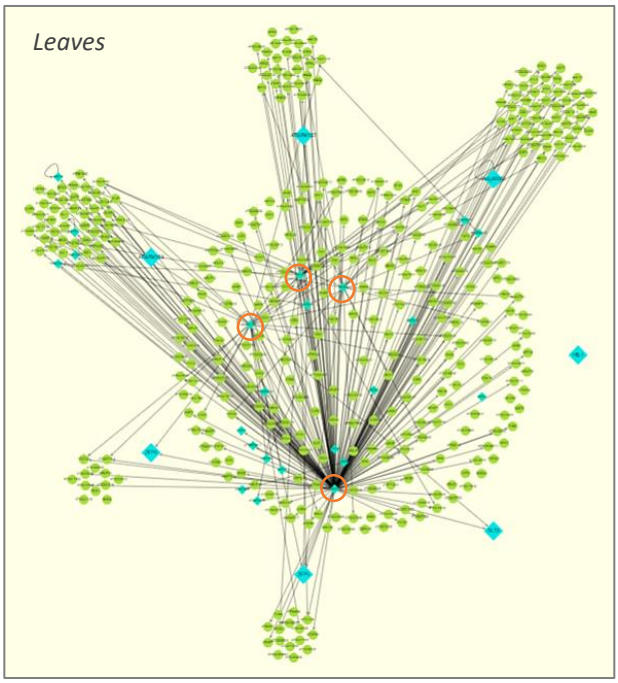
Transcription factors (TFs) are proteins that control cellular processes by regulating the expression of target genes. Thus, TFs play a key role in signalling and phytohormone biosynthesis, as well as in cell growth and differentiation. Using the *TF2Network bioinformatics* package (with Arabidopsis as a reference genome), the TF network associated with the identified DEGs in leaves and buds was studied. This required translating the *Citrus* gene IDs into the nomenclature of their counterparts in the Arabidopsis genome.

The green nodes represent the 753 DEGs identified in the leaves, and the blue diamonds represent the TFs proposed by the program (Fig. 4.13A). Among them, the high interaction of *AT5G04760/DIV2* with the other genes stands out (Fig. 4.13B). This gene modulates ABA signaling, and its importance in processes related to water and salt stress has been demonstrated (Fang et al., 2018). The activation of stress response in leaves due to the presence of fruit was previously described by Muñoz-Fambuena et al. (2013) through proteomics. Additionally, three other TFs showed high interaction, including *AKS1* and *AGL2*, which are related to the regulation of flowering time (Sung et al., 2000; Zhao et al., 2001), and *EIN3*, which triggers the ethylene response (Dolgikh et al., 2019) (Fig. 4.13C).

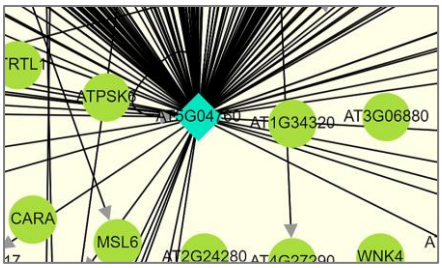
In the buds, the number of detected genes was much higher, with 3254 DEGs (Fig. 4.7B). As a result, the level of interaction increased (Fig. 4.14A), revealing a greater number of TFs compared to the leaves. The TFs proposed by the bioinformatics analysis were classified into three groups: 1) those related to ABA and abiotic stress signalling (*WRKY33*, *HB7*, *ANAC072*, *DIV2*, *HB6*), 2) hormone regulators (*EIN3* and *GL3*), and 3) flowering time regulators (*AGL7*, *AGL20*, and *AP2*) (Fig. 4.14B). *AGL7* and *AGL20* are counterparts to *AP1* and *SOC1*, respectively (Mandel et al., 1992; Na et al., 2013).

The prediction of these transcription factors based on the RNA-seq analysis between ON and OFF trees provides new insights into the inhibitory effect of fruit during floral induction. It is worth noting that this prediction is based on gene-TF interaction annotations from the model plant Arabidopsis. Therefore, there may be other relationships with these or other TFs in citrus. An obvious example is one of the predicted TFs, *AKS1*, which is related to flowering induction by photoperiod through its interaction with *CONSTANS (CO)*. Whereas Arabidopsis is a short-day plant and responds to this stimulus, citrus species are day-neutral.

A



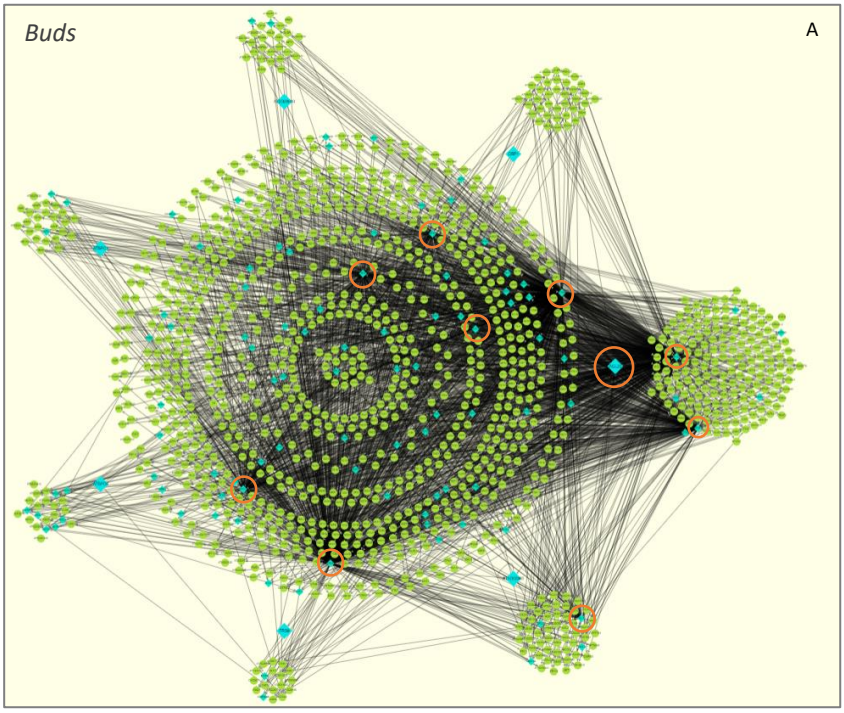
B



C

	Gene ID	Symbol	Description
ABA and stress	AT5G04760	DIV2	Protein which plays negative roles in salt stress and is required for ABA signalling in Arabidopsis.
Hormones	AT3G20770	EIN3	A nuclear transcription factor that initiates downstream transcriptional cascades for ethylene responses
Flowering time	AT1G51140	AKS1	Involved in photoperiodism flowering. Binds to the E-box cis-element in the *CONSTANS* (*CO*) promoter to regulate flowering.
	AT2G45660	AGL20 (SOC1)	Controls flowering and is required for *CO* to promote flowering. It acts downstream of *FT*. *AGL20* upregulates expression of *AGL24* in response to GA

Figure 4.13*. Transcription factors predicted in 'Nadorcott' mandarin leaves throughout 753 DGEs analysed using TF2Network software. A: Transcription factors predicted (blue diamonds), genes in interaction with transcription factors predicted (green dots). Black lines indicate an interaction protein-DNA. B: AT5G04760, transcription factor with the huge interaction. C: All transcription factors predicted.*



B

	Gene ID	Symbol	Description
ABA and stress	AT2G22430	HB6	Encodes a homeodomain leucine zipper class I (HD-Zip I) protein that is a target of the protein phosphatase ABI1 and regulates ABA responses in Arabidopsis.
	AT2G38470	WRKY33	Member of the plant WRKY transcription factor family. Located in nucleus. Involved in response to various abiotic stresses - especially salt stress. Regulates cytochrome P450 gene CYP94B1 to control apoplastic barrier formation in roots to confer salt tolerance.
	AT2G46680	HB7	HOMEOBOX 7 encodes a putative transcription factor which is detected in all tissues examined. Is transcriptionally regulated in an ABA-dependent manner and may act in a signal transduction pathway which mediates a drought response.
	AT4G27410	ANAC072	Encodes a NAC transcription factor induced in response to desiccation. It is localized to the nucleus and acts as a transcriptional activator in ABA-mediated dehydration response.
	AT5G04760	DIV2	Protein which plays negative roles in salt stress and is required for ABA signalling in Arabidopsis.
Hormones	AT3G20770	EIN3	A nuclear transcription factor that initiates downstream transcriptional cascades for ethylene responses.
	AT5G41315	GL3	Encodes a basic helix loop helix domain protein that interacts with GL1 in trichome development. GL3 interacts with JAZ and DELLA proteins to regulate trichome initiation.
Flowering time	AT1G69120	AGL7 (AP1)	Floral homeotic gene encoding a MADS domain protein homologous to SRF transcription factors. Interacts with LEAFY. Binds to promoter and regulates the expression of flowering time genes SVP, SOC1 and AGL24.
	AT4G36920	AP2	Encodes a floral homeotic gene, it is involved in the specification of floral organ identity and establishment of floral meristem identity.
	AT2G45660	AGL20 (SOC1)	Controls flowering and is required for CO to promote flowering. It acts downstream of FT. AGL20 upregulates expression of AGL24 in response to GA.

***Figure 4.14**. Transcription factors predicted in 'Nadorcott' mandarin buds throughout 3254 DGEs analysed using TF2Network software. A: Transcription factors predicted (blue diamonds), genes in interaction with transcription factors predicted (green dots). Black lines indicate an interaction protein-DNA. B: All transcription factors predicted.*

4.2.2. What do the flowering phenotypes have in common? ON vs OFF, AB-G & SF

To determine which pathways are shared among the three flowering phenotypes, AB-G, SF, and OFF, in contrast to the non-flowering phenotype (ON), a new analysis was conducted with all four phenotypes.

In the leaves, DEGs were selected based on a p-value adjusted < 0.01 and a Log2Fold Change ≥ ±0.58. In comparison to the leaves of the ON phenotype, significant changes were observed in 4,477 DEGs in the leaves of AB-G buds, 3,084 DEGs in the leaves of summer fruit (SF) buds, and 424 DEGs in the leaves of OFF buds (Fig. 4.15A), as opposed to the 235 DEGs analyzed using FC≥1 (Fig. 4.7B).

The Circos Diagram for multiple comparisons indicates a high interaction between DEGs of the AB-G and SF phenotypes (Fig. 4.15C), which are both capable of flowering while retaining the fruit. The Venn diagram analysis confirms the high overlap between AB-G and SF, with 1,143 shared DEGs (24.3% of the total). The overlap between the three flowering phenotypes, in contrast to the ON phenotype, was significantly lower, at 4.1% (195 DEGs). This overlap was greater than that between OFF and AB-G (101 DEGs) and OFF and SF (31 DEGs) (Fig. 4.15B).

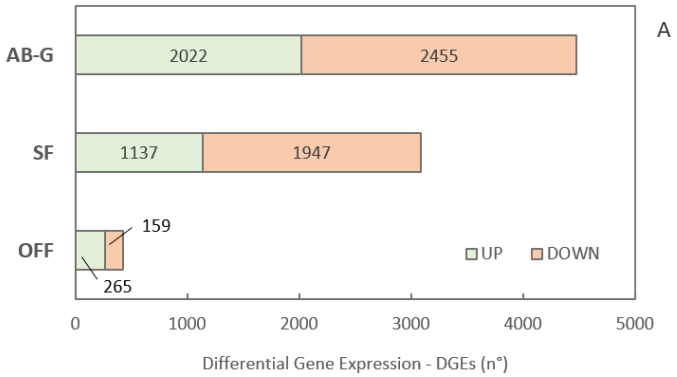
The functional classification of these 195 DEGs according to Gene Ontology (GO) shows that they are grouped under the general category 'Biological Process' (Fig. 4.16). The enriched subcategories were related to plant interaction with other organisms, activation of defense pathways, hormonal response, and response to external stimuli.

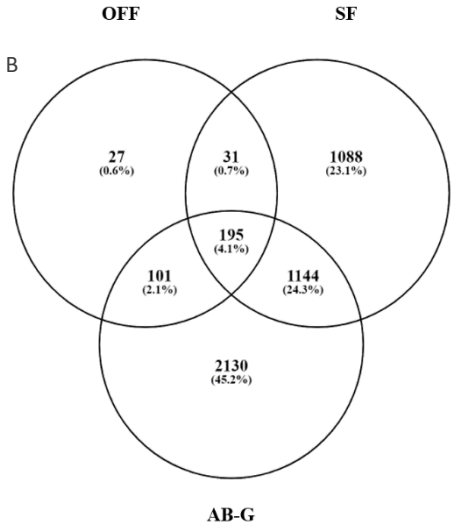
Finally, transcription factors (TFs) were predicted for these 195 DEGs. The initial number of genes for the analysis was limited, which reduced the number of TF-gene target interactions (Fig. 4.17A). As a result, the analysis highlighted the transcription factor *DIV2* (Fig. 4.17B), which is associated with ABA signaling pathways under water and salt stress conditions (Fang et al., 2018).

Identification of DEGs between ON-trees and three flowering phenotypes. (ON vs OFF / ON vs AB-G / ON vs SF)

Leaves

D*ifferentially Expressed Genes (DGEs).*





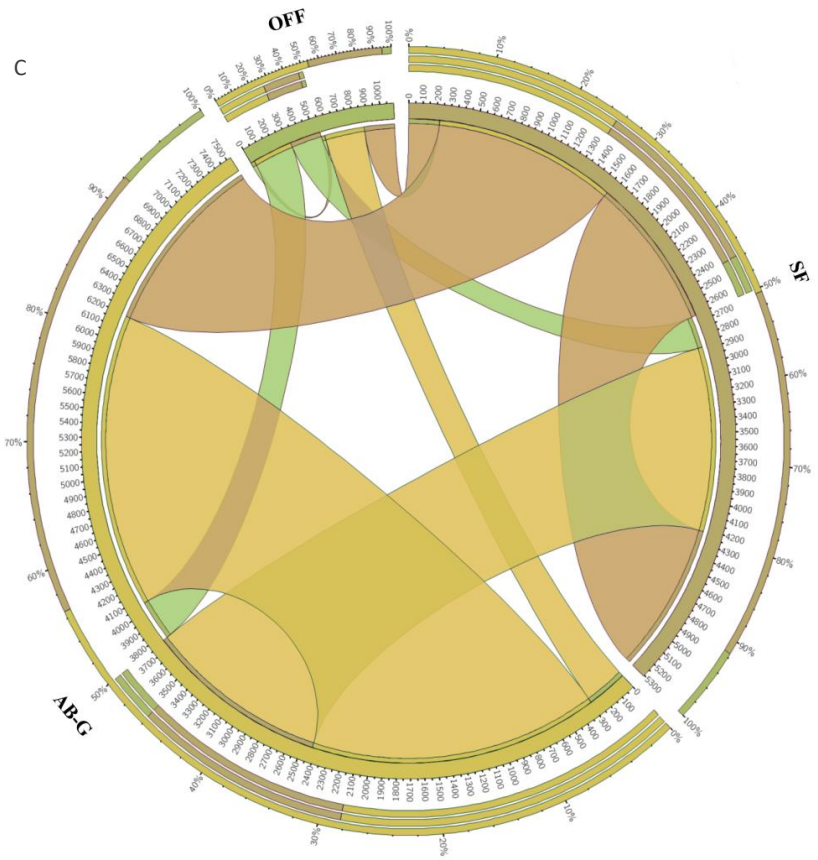


Figure 4.15. *Identification of DEGs between ON-trees and three flowering phenotypes (ON vs OFF / ON vs AB-G / ON vs SF) in 'Nadorcott' mandarin leaves. A: Number of DEGs. (P-value adj. < 0,01 and genes with the regulation ratio log2 ≥ 0,58 or ≤ -0,58 were selected). B: Venn Diagram showing the overlapping number of DEGs among the treatments (Venny 2.1 software). C: Circos Diagram showing overlapping and specific response of comparative DEGs of the treatments, (Circos Table Viewer v0.63 software); OFF: trees without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*

Leaves - Gene Ontology and Transcription Factors predicted.
(195 DGEs in common)

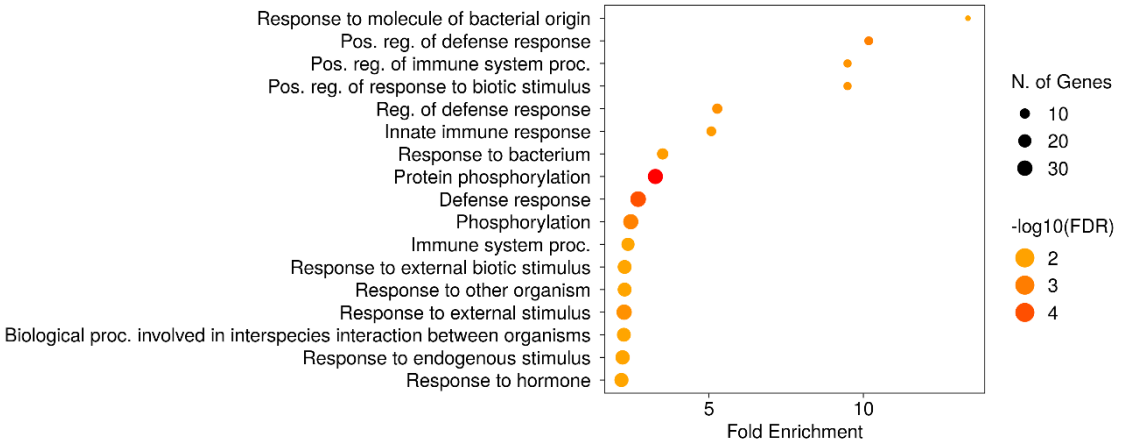
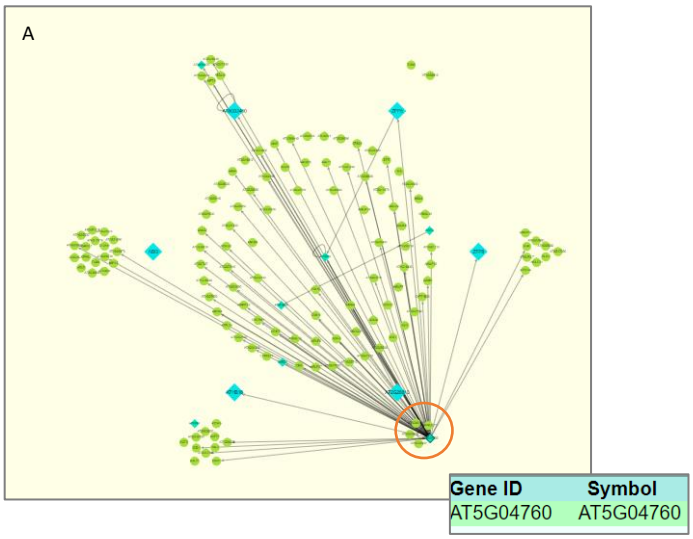


Figure 4.16. *Gene Ontology (GO) classification of the 195 DEGs in the leaves that have all the comparisons (ON-OFF; ON-ABG; ON-SF) in common. log2(Fold Change) ≥ 0,58. The DGEs were divided into three different categories: biological processes (BP), cellular component (CC) and molecular function (MF), using ShinyGO 0.77 software. Results only have been obtained for Biological Process, due to a low DEGs number. ON and OFF: trees with and without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*



B

	Gene ID	Symbol	Description
ABA and stress	AT5G04760	DIV2	Protein which plays negative roles in salt stress and is required for ABA signalling in Arabidopsis.

Figure 4.17*. Transcription factors predicted of the 195 DEGs in the leaves that have all the comparisons (ON-OFF; ON-ABG; ON-SF) in common, using TF2Network software. A: Transcription factors predicted (blue diamonds), genes in interaction with transcription factors predicted (green dots). Black lines indicate an interaction protein-DNA. B: Transcription factors predicted. ON and OFF: trees with and without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*

The analysis in the buds used more restrictive analysis values than in the leaves due to the high number of DEGs (Fold Change ≥ ±1). Nonetheless, the number of DEGs in the comparisons ON vs OFF, ON vs AB-G, and ON vs SF was high (3,254, 3,865, and 1,746 DEGs, respectively). In all three comparisons, approximately one-third of the genes were upregulated, and two-thirds were downregulated (Fig. 4.18A).

In contrast to the leaves, the interaction of DEGs between possible comparisons was higher between OFF and AB-G than between the other two, as seen in the Venn Diagram and Circos Diagram, indicated by the width of the bands (Fig. 4.18C). While OFF and AB-G (the phenotypes that flowered the most) shared 1,434 DEGs (22.9%), AB-G/SF and OFF/SF only shared 240 and 161 DEGs, respectively (Fig. 4.18B). Finally, the Venn diagram shows that 38.8% (2,429 DEGs) of the total DEGs are shared in all three comparisons between the buds of ON trees and those of the flowering phenotypes (Fig. 4.18B). This percentage of similarity is +34.7% higher than that found in leaves.

The functional classification of these 2,429 coincident DEGs, using Gene Ontology (GO) annotations, shows results for the categories 'Biological Process' (Fig. 4.19A), 'Cellular Component' (Fig. 4.19B), and 'Molecular Function' (Fig. 4.19C). The results from all three categories suggest that the meristems have already started the differentiation stage. In the 'Biological Process' category, the subcategories 'Carbohydrate metabolic process', 'Cell wall organization', and 'Biogenesis' stand out due to the high number of DEGs involved. In the 'Cellular Component' category, the enriched subcategories (x2.5) are related to the formation of new cells, particularly highlighting microtubule formation, the cytoskeleton, the cell wall, and organelles (Fig. 4.19B). In the 'Molecular Function' category, there is a high enrichment for microtubule binding activity, tubulin, and cytoskeletal proteins (Fig. 4.19C).

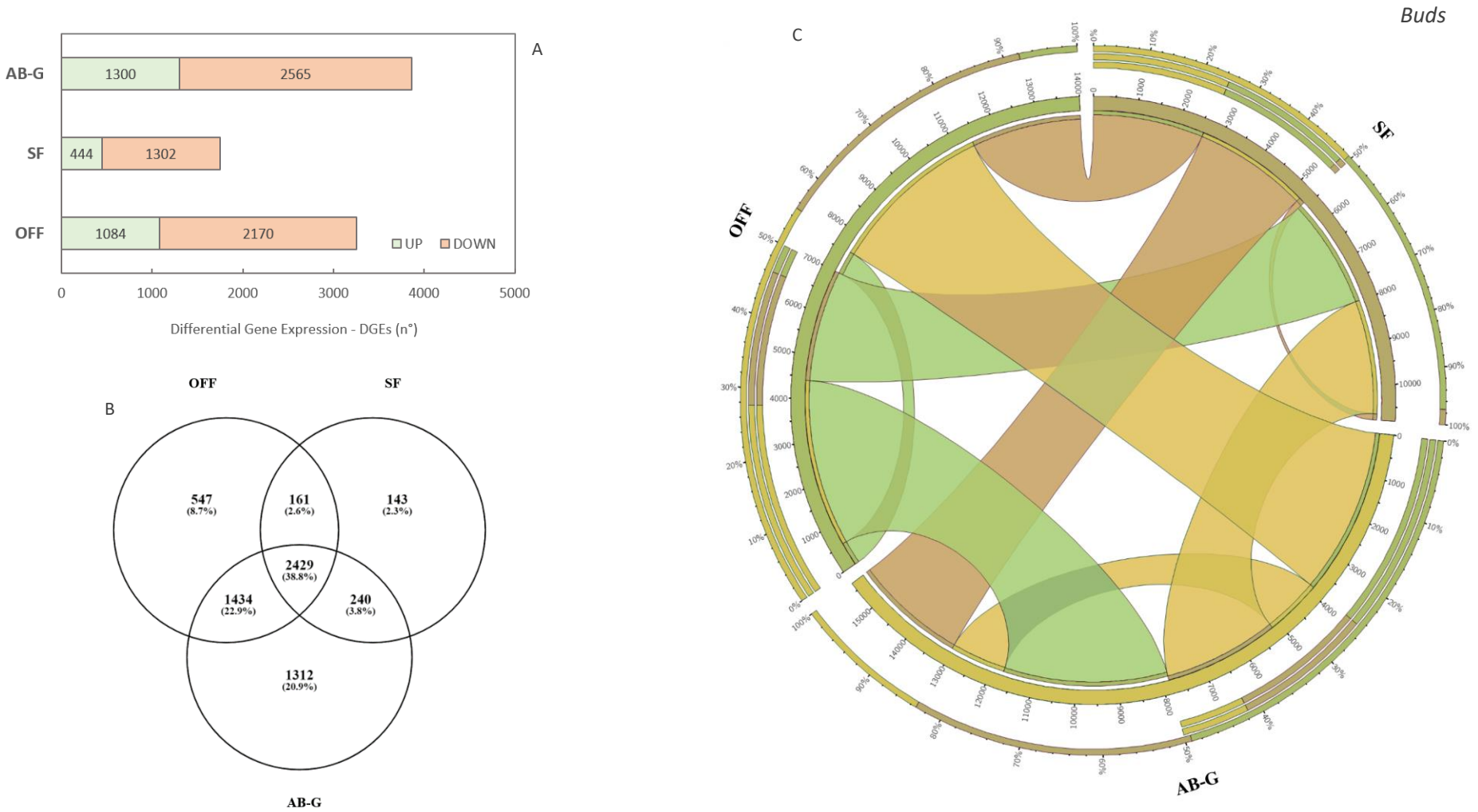


Figure 4.18. *Identification of DEGs between ON-trees and three flowering phenotypes (ON vs OFF / ON vs AB-G / ON vs SF) in 'Nadorcott' mandarin buds. A: Number of DEGs. (P-value adj. < 0,01 and genes with the regulation ratio log2 ≥ 1). B: Venn Diagram showing the overlapping number of DEGs among the treatments (Venny 2.1 software). C: Circos Diagram showing overlapping and specific response of comparative DEGs of the treatments, (Circos Table Viewer v0.63 software); OFF: trees without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*

Buds - Gene Ontology and Transcription Factors predicted. (2429 DGEs in common)

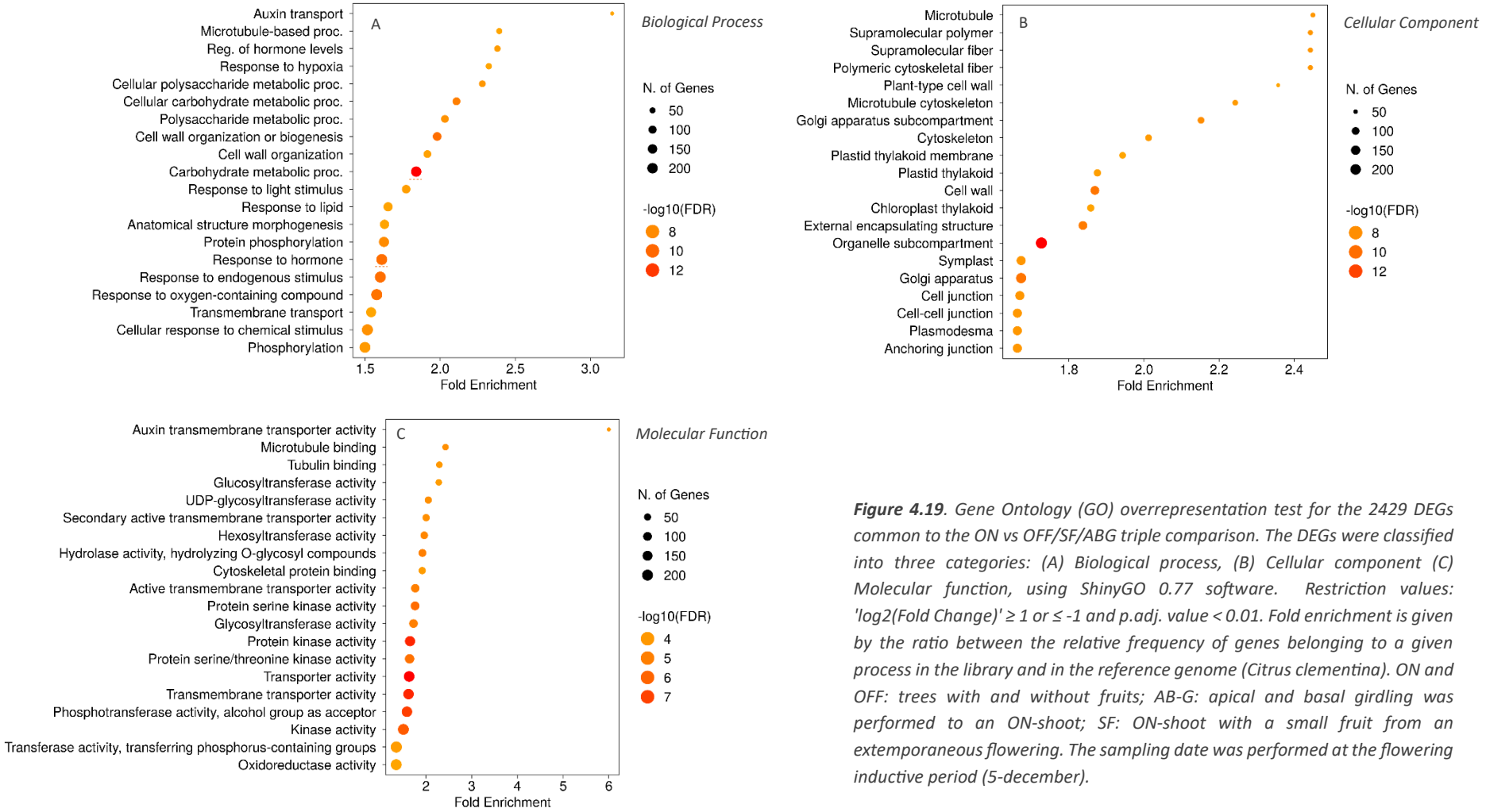


***Figure 4.19**. Gene Ontology (GO) overrepresentation test for the 2429 DEGs common to the ON vs OFF/SF/ABG triple comparison. The DEGs were classified into three categories: (A) Biological process, (B) Cellular component (C) Molecular function, using ShinyGO 0.77 software. Restriction values: 'log2(Fold Change)' ≥ 1 or ≤ -1 and p.adj. value < 0.01. Fold enrichment is given by the ratio between the relative frequency of genes belonging to a given process in the library and in the reference genome (Citrus clementina). ON and OFF: trees with and without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*

It's worth noting that the most enriched subcategories in 'Biological Process' (x3.5) and 'Molecular function' (x6) were related to auxin transport, although enrichment was also observed in the subcategories 'Response to hormone' and 'Response to endogenous stimulus' (Fig. 4.19A).

As a result, the findings suggest that in the axillary meristems of the three flowering phenotypes (2,429 DEGs), hormonal activity, particularly auxin transport, is modified during the initiation of floral differentiation, and processes related to cell division, such as microtubule formation, are activated. But among all the genes enriching these pathways, which ones are up- or downregulated to allow the three flowering phenotypes to initiate the process of floral differentiation?

A detailed analysis of the 'Response to Hormone' subcategory with 225 DEGs was conducted using a Heat Map (Fig. 4.20). The figure shows the overexpression (in green) or repression (in red) of genes related to the major hormones in the buds of the ON phenotype (which does not flower) compared to the phenotypes that do flower (OFF, SF, and AB-G).

The group of genes related to ABA was the largest, with 24 repressed and 27 overexpressed genes (Fig. 4.20A). Notably, there was a high level of repression (3x) of the *HVA22c* gene in the ON buds, which is related to previous findings (Chen et al., 2002) showing that the overexpression of this gene is localised to floral buds only.

The group of genes related to auxin response was also significant (35 DGEs). Among them, the *PATL6* gene, which is related to polar auxin transport (Zhou et al., 2019), was the most repressed in the ON buds. This is reinforced by the repression of the auxin influx (*LAX3*) and efflux (*PIN1, PIN4*, and *PIN6*) transporters in ON buds compared to the floral phenotypes. Overexpression of *IAA19* and *IAA29* was observed in ON buds. Aux/IAA proteins are short-lived transcription factors that function as repressors of early auxin response genes at low auxin concentrations. Furthermore, the auxin response factor *ARF16* was also overexpressed in ON buds. However, single mutants of *arf16* do not show phenotypic alterations (Guilfoyle & Hagen, 2007). Therefore, the results suggest that polar auxin transport is active in buds of the three

flowering phenotypes, in contrast to the buds of ON shoots, where auxin transport is almost halted.

The interaction between auxins and ethylene is shown by the presence of auxin-related genes among the ethylene response genes (*YUC4* and *PIN6* as repressed and *TIR1*, an auxin receptor, as overexpressed). However, the ethylene response results are not easy to interpret. Ethylene response factors (ERFs) show heterogeneous expression. *EIN3*, *ERF9*, and *ERF105* are overexpressed, while the transcription factor *TOE2*, which inhibits *EIN3* transcription, is strongly repressed in ON buds. On the other hand, *ERF014*, *ERF003*, *ERF038*, and *ERF113* were found to be repressed in ON buds (Fig. 4.20B).

The jasmonic acid (JA) signaling pathways were also disrupted with 21 DEGs, 9 repressed and 12 overexpressed. Among them, the overexpression of *WRKY* transcription factors (*WRKY4, 50, 51*, and *70*) is notable, as they play an important regulatory role in the response to environmental stress (Ruan et al., 2019).

Finally, some genes related to GAs and CKs also altered their expression. In the GAs group, the repression of *EXPA1*, a gene related to cell elongation (Fig. 4.20E), was notable, and in the case of CKs, *CYCD3.1*, a cyclin related to the cell cycle, was repressed (Fig. 4.20F).

Response to Hormone

***Figure 4.20**. Reclassification of the sub-category 'Response to Hormone' (225 DGEs) in 'Nadorcott' mandarin buds. Heat map of different response to hormones. Colour intensity (green: Up-regulated, red: Down-regulated) indicates level of gene expression. A: Heat map, response to Abscisic Acid (50 DGEs in this annotation). B: Heat map, response to Ethylene (24 DGEs in this annotation). C: Heat map, response to Jasmonic Acid (21 DGEs in this annotation). Red and green intensities indicate down- and up-regulation of the ON phenotype respectively. ON and OFF: trees with and without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*

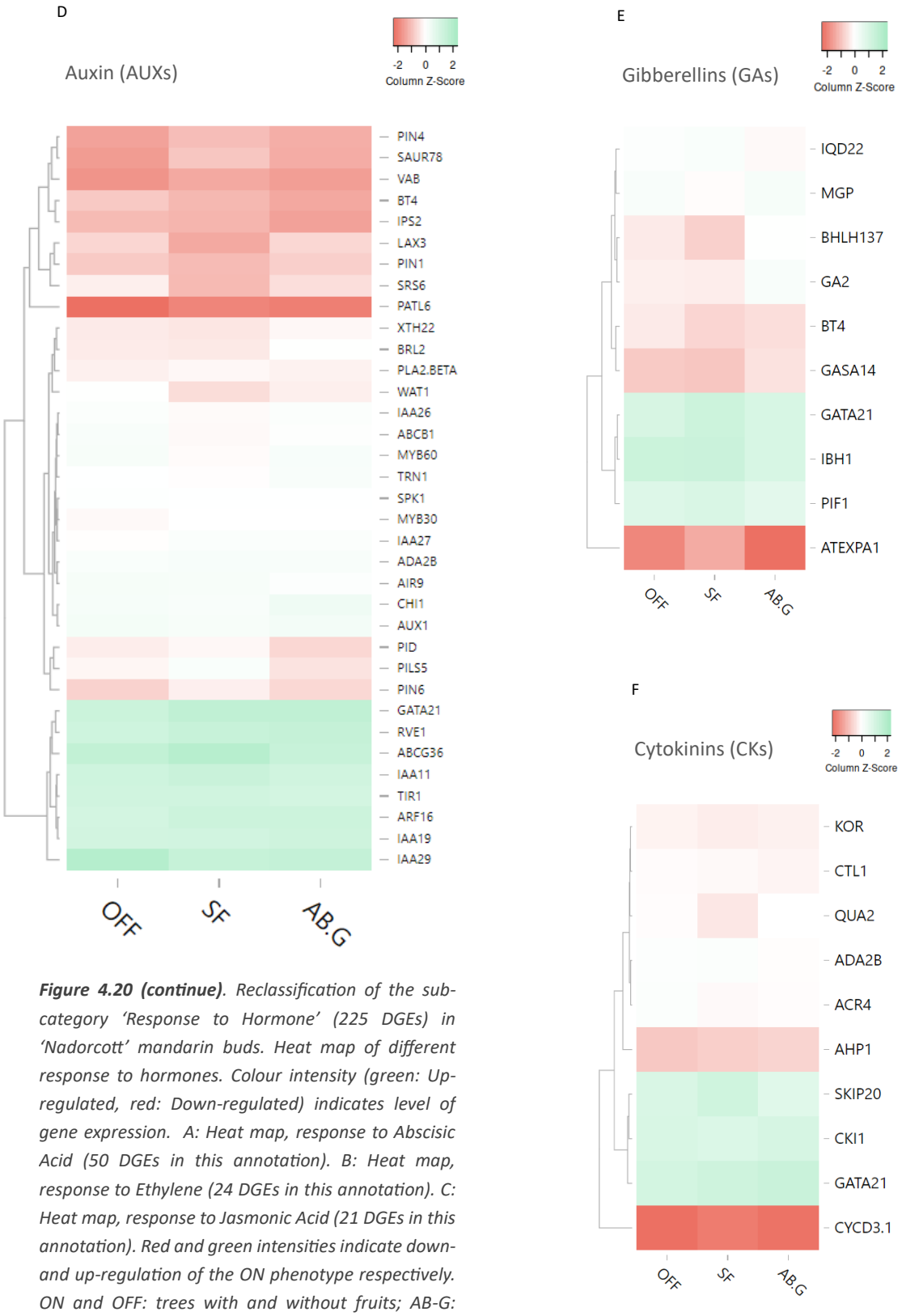


***Figure 4.20 (continue)**. Reclassification of the sub-category 'Response to Hormone' (225 DGEs) in 'Nadorcott' mandarin buds. Heat map of different response to hormones. Colour intensity (green: Up-regulated, red: Down-regulated) indicates level of gene expression. A: Heat map, response to Abscisic Acid (50 DGEs in this annotation). B: Heat map, response to Ethylene (24 DGEs in this annotation). C: Heat map, response to Jasmonic Acid (21 DGEs in this annotation). Red and green intensities indicate down- and up-regulation of the ON phenotype respectively. ON and OFF: trees with and without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. The sampling date was performed at the flowering inductive period (5-december).*

The 'Heat Map' analysis of the 193 DEGs in the 'Carbohydrate Metabolic Process' subcategory shows differences in starch and sucrose metabolism (Fig. 4.21A), secondary metabolite biosynthesis (Fig. 4.21B), glycolysis (Fig. 4.21C), or galactose metabolism (Fig. 4.21D) between the three flowering phenotypes and the ON shoots. Genes that regulate trehalose-6-phosphate metabolism (*TPS6, TPS9,* and *TPS11*; trehalose-6-phosphate synthase) were overexpressed in ON shoot buds, indicating a response to nutrient deficiency (Nunes et al., 2013) in fruit buds (Fig. 4.21A). Similarly, glycosyl hydrolase enzymes of the cell wall (*GH9C2*) are activated under conditions of high sugar consumption (Lee et al., 2007). Notably, there is overexpression of a fructose-1,6-bisphosphate aldolase (*FBA5*), involved in the synthesis of glyceraldehyde 3-phosphate, and repression of a hexokinase (*HXK3*), and the catalytic domain of the 2-oxoacid dehydrogenase acyltransferase enzyme, which catalyses the conversion of alpha-keto acids to acyl-CoA and CO_2. Finally, the galactose pathway, which is a substrate for cell wall polymers (Seifert et al., 2002), is predominantly repressed in ON buds, as shown by the enzyme *RSF1* (galactinol--sucrose galactosyltransferase). Figures 4.22 and 4.23 summarize the main differences between the fruiting phenotype and the three flowering phenotypes in the hormonal and nutritional metabolic pathways of the buds, according to the KEGG database.

The prediction of transcription factors (TFs) in the buds related to the 2,429 common DEGs in the three comparisons (ON vs. OFF, AB-G, and SF) increased the number of interactions compared to the prediction in the leaves (Fig. 4.24A), due to the larger number of genes involved. As in the simple ON-OFF comparison (Fig. 4.14), the analysis predicted TFs responsive to hormonal signaling. Among these, the analysis repeated the prediction of *HB6* and *DIV2* and added *ABF3*, which is related to responses to environmental stress mediated by abscisic acid (ABA). The prediction also repeated *EIN3*, responsible for the ethylene response, and *GL3*, which interacts with jasmonic acid (JA). The prediction of TFs directly regulating the flowering process was the same as in the simple comparison, confirming the importance of *AP1 (AGL7), SOC1*, or *AP2* in the flowering process (Fig. 4.24B).

Figure 4.21. *Heat map reclassification of the category 'Carbohydrate Metabolic Process' (193 DEGs) in 'Nadorcott' mandarin buds. Colour intensity (green: Up-regulated, red: Down-regulated) indicates level of gene expression. A: Starch and Sucrose Metabolism regulation (13 DEGs in this annotation). B: Biosynthesis of Secondary Metabolites (25 DEGs in this annotation). C: Glycolysis/ Gluconeogenesis process (8 DEGs in this annotation). D: Galactose Metabolism (6 DEGs in this annotation). ON and OFF: trees with and without fruits; AB-G: apical and basal girdling was performed to an ON-shoot; SF: ON-shoot with a small fruit from an extemporaneous flowering. (Sampling date on the 5th of December).*

Plant Hormone – Signal Transduction

A

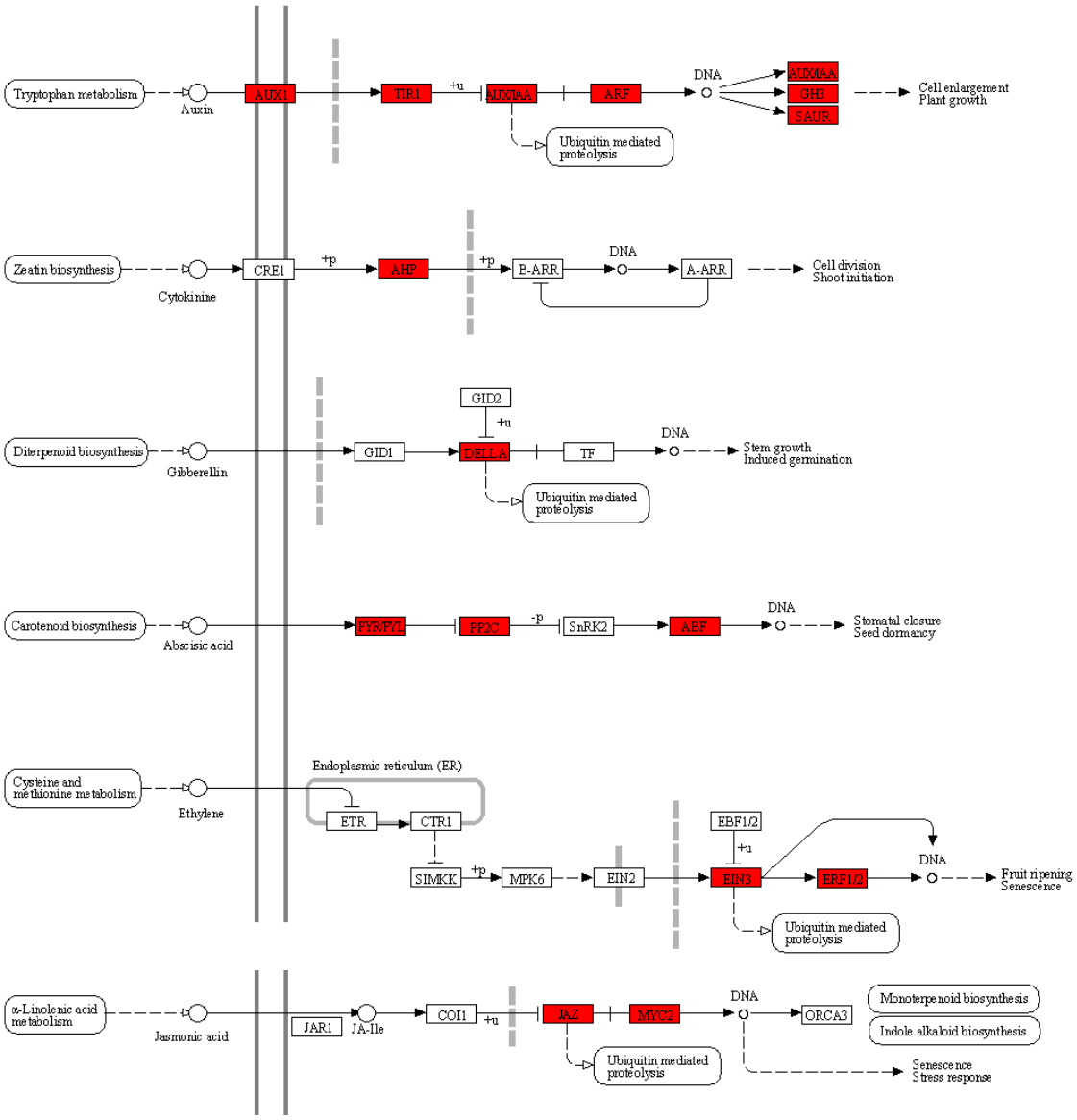
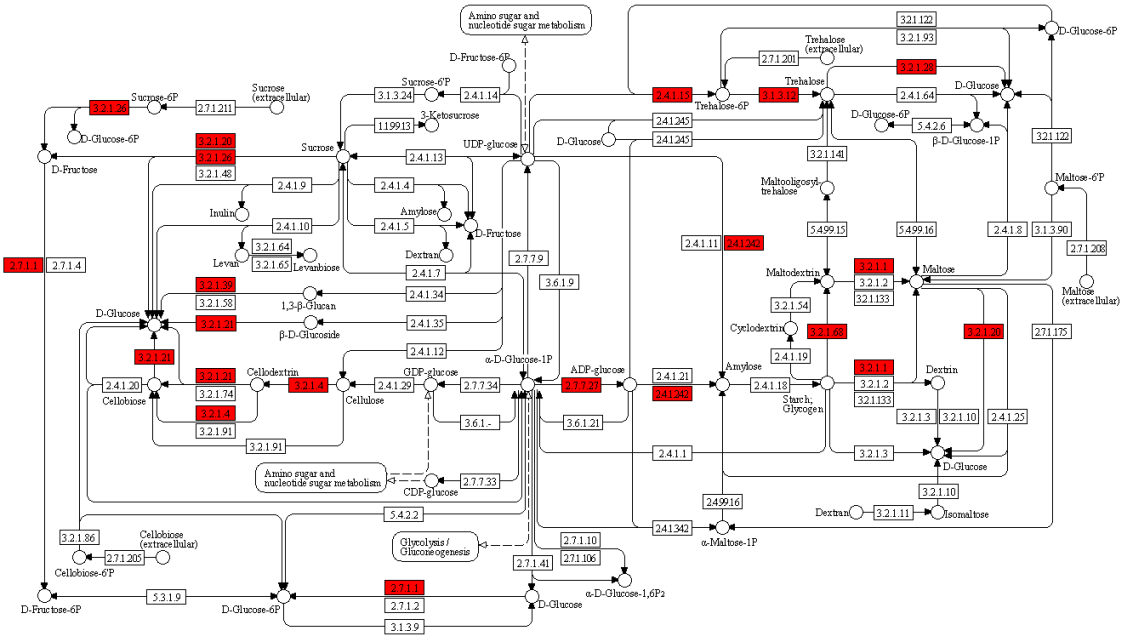


Figure 4.22. *KEEGs pathways analyse of Response to Hormones reclassification at Biological Process in Buds. Pathways altered: Response to Auxin, Cytokinin, Gibberellin, Abscisic Acid, Ethylene and Jasmonic Acid. Classification was done using ShinyGO v.0,77 software.*

Starch and Sucrose Metabolism

B



Galactose Metabolism

C

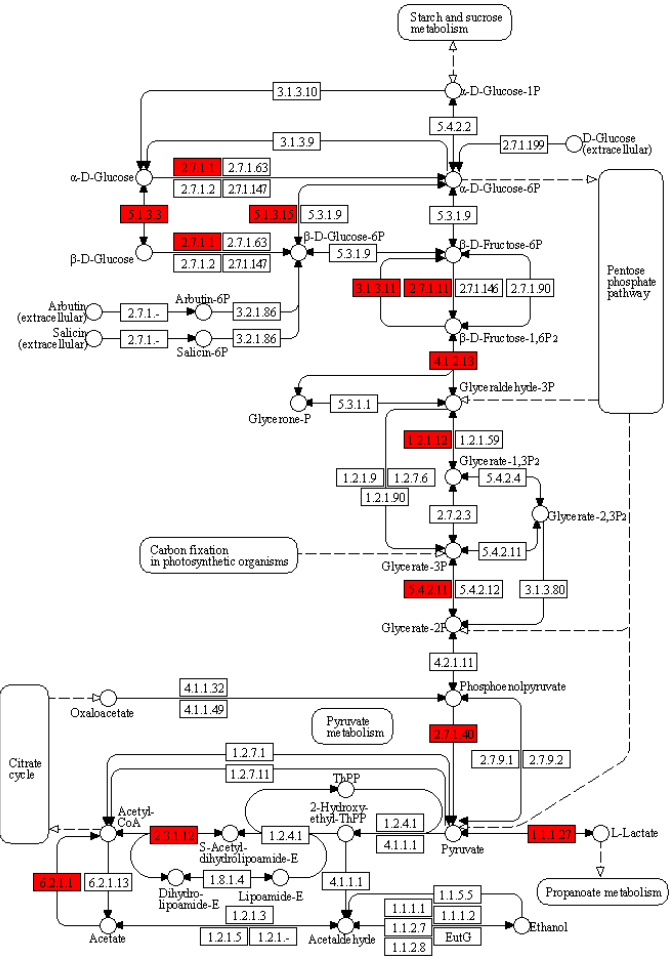
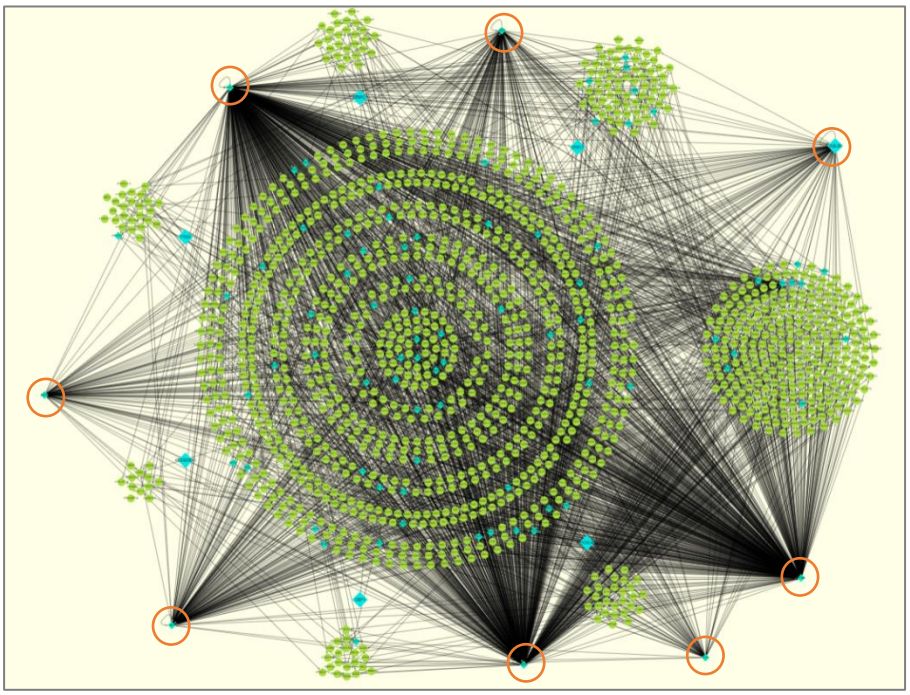


***Figure 4.23**. KEEGs pathways analyse of Carbohydrate Metabolic Process at Biological Process in Buds. Principal pathways altered: (A) Starch and Sucrose Metabolism & (B) Galactose Metabolism. Classification was done using ShinyGO v.0,77 software.*

A



B

	Gene ID	Symbol	Description
ABA and stress	AT4G34000	ABF3, PBF5	Expressed in response to stress and abscisic acid. Encodes an ABA-responsive element-binding protein
	AT5G04760	DIV2	Protein which plays negative roles in salt stress and is required for ABA signalling in Arabidopsis.
	AT2G22430	HB6	Negative regulation of abscisic acid-activated signaling pathway. Response to water deprivation.
Hormone	AT3G20770	EIN3	Ethylene-activated signaling pathway, sugar mediated signaling pathway
	AT5G41315	GL3, GLABRA 3, MYC6.2	GL3 interacts with JAZ and DELLA proteins to regulate trichome initiation.
Flowering time	AT1G69120	AGL7 (AP1)	Flower development, maintenance of floral meristem identity.
	AT2G45660	AGL20 (SOC1)	Positive regulation of flower development, response to cold.
	AT4G36920	AP2, FL1, FLO2	Cell differentiation, flower development, sexual reproduction, specification of floral organ identity.

***Figure 4.24**. Transcription factors predicted in 'Nadorcott' mandarin buds throughout 2429 DGEs analysed using TF2Network software. A: Transcription factors predicted (blue diamonds), genes in interaction with transcription factors predicted (green dots). Black lines indicate an interaction protein-DNA. B: All transcription factors predicted.*

In conclusion, the behaviour of the three flowering phenotypes differs qualitatively between leaves and buds. While there is more similarity between the two fruit phenotypes (AB-G and SF) in leaves, there is more similarity between the two most flowering phenotypes (OFF and AB-G) in buds. Additionally, the behaviour between leaves and buds also differs quantitatively, as the three phenotypes share 39% of the DEGs in the buds, while they only share 4.1% in the leaves (see Figs. 4.15 and 4.18).

All this suggests that the three phenotypes have different mechanisms for controlling flowering, especially for the floral induction process in the leaves. In this case, the three phenotypes only shared a transcription factor related to ABA and stress, and none related to flowering time, according to the TF prediction analysis. Therefore, the following section studies the influence of AB-G on the expression of the *CiFT3* gene.

In the buds, however, the three phenotypes shared important TFs for the flowering process, such as *AP1* and *SOC1*, as well as other TFs related to hormonal response. But the study of the 2,429 DEGs shared between the three comparisons revealed interesting results in the buds, indicating how the three flowering phenotypes (OFF, AB-G, SF) differ from the non-flowering phenotype (ON). Differences were found in cell division, some hormonal pathways, and the primary metabolism of the buds. All three processes showed a similar pattern of regulation between OFF, AB-G, and SF compared to fruit-bearing shoots. In the case of hormones, the main pathway affected was related to auxins (Fig. 4.22), showing changes in both homeostasis and, primarily, in polar transport. ABA and JA signalling pathways were also altered, as well as GAs and CKs to a lesser extent. Pathways for carbohydrate energy production and transport were also differentially expressed at several regulatory points, as was galactose metabolism (Figure 4.23).

Consequently, the results suggest that auxins, together with carbohydrate metabolism, play a fundamental role during the period of floral induction and differentiation of the buds. In ON shoots, the capacity for polar auxin transport is reduced, resulting in decreased meristematic activity compared to the buds of shoots that flower (OFF, AB-G, and SF). Furthermore, the buds of ON shoots remain in a state of basal metabolism, with reduced processes for energy production, such as glycolysis and starch hydrolysis, and sucrose transport.

However, considering that the RNA-seq only shows the effect of girdling at a single time point (floral induction), three months after the treatment, the next section delves deeper into the mechanism through which girdling activates floral induction, through hormonal and nutritional factors, by modifying the timing of the treatment and the analysis timepoints, studying both short-term and long-term responses.

4.3. Effect of double-girdling (AB-G) on the expression of flowering time and floral identity genes

Flower induction in citrus trees in Mediterranean climates occurs from November onwards, triggered by cold temperatures that activate the expression of the *CiFT3* gene in the leaves. Flower induction is quantitative and depends on the duration and intensity of the cold. For example, in Satsuma mandarins, it takes 1.5 months at 15°C for the plant to flower (Nishikawa et al. 2007). From 10 days into the experiment (20th September), the minimum temperature remained below 15°C (Fig. 4.25A). Although it is unknown whether a temperature below 15°C has a greater effect on *CiFT3*, the accumulation of chilling hours (hours below 7.2°C) was calculated for reference. Accumulation of chilling hours started 40 days after treatment (end of October) and by the end of December (100 days after treatment), chilling hours accumulated linearly and remained constant until the end of the experiment (Fig. 4.25A).

Vegetative shoots without fruit (OFF) responded naturally to chilling by increasing the relative expression of *CiFT3* from 100 days after treatment (DAT), reaching a level 15 times higher than that of fruit-bearing shoots (ON) at 130 DAT (Fig. 4.25C). The application of AB-G, carried out 2 months before the induction period, stimulated the expression of *CiFT3* with both quantitative and qualitative differences: 1) the relative expression increased up to 250 times compared to ON shoots (Fig. 4.25C), and 2) the activation of *CiFT3* expression occurred 60 days earlier, with a significant increase (13 times that of ON) observed at the first sampling at 49 DAT (Fig. 4.25C). At this time, the relative expression of *CiFT3* in the leaves of AB-G shoots had already reached the final value observed in OFF shoots at the end of the cycle (15 times that of ON shoots) (Fig. 4.25B).

To confirm the effect of the early expression of *CiFT3* in AB-G shoots, the experiment was repeated the following year with AB-G treatment on 1st October and sampling 8 days after treatment (DAT). The results confirm that the increase in *CiFT3* expression due to AB-G occurs in advance, before the temperature drop (Fig. 4.25D).

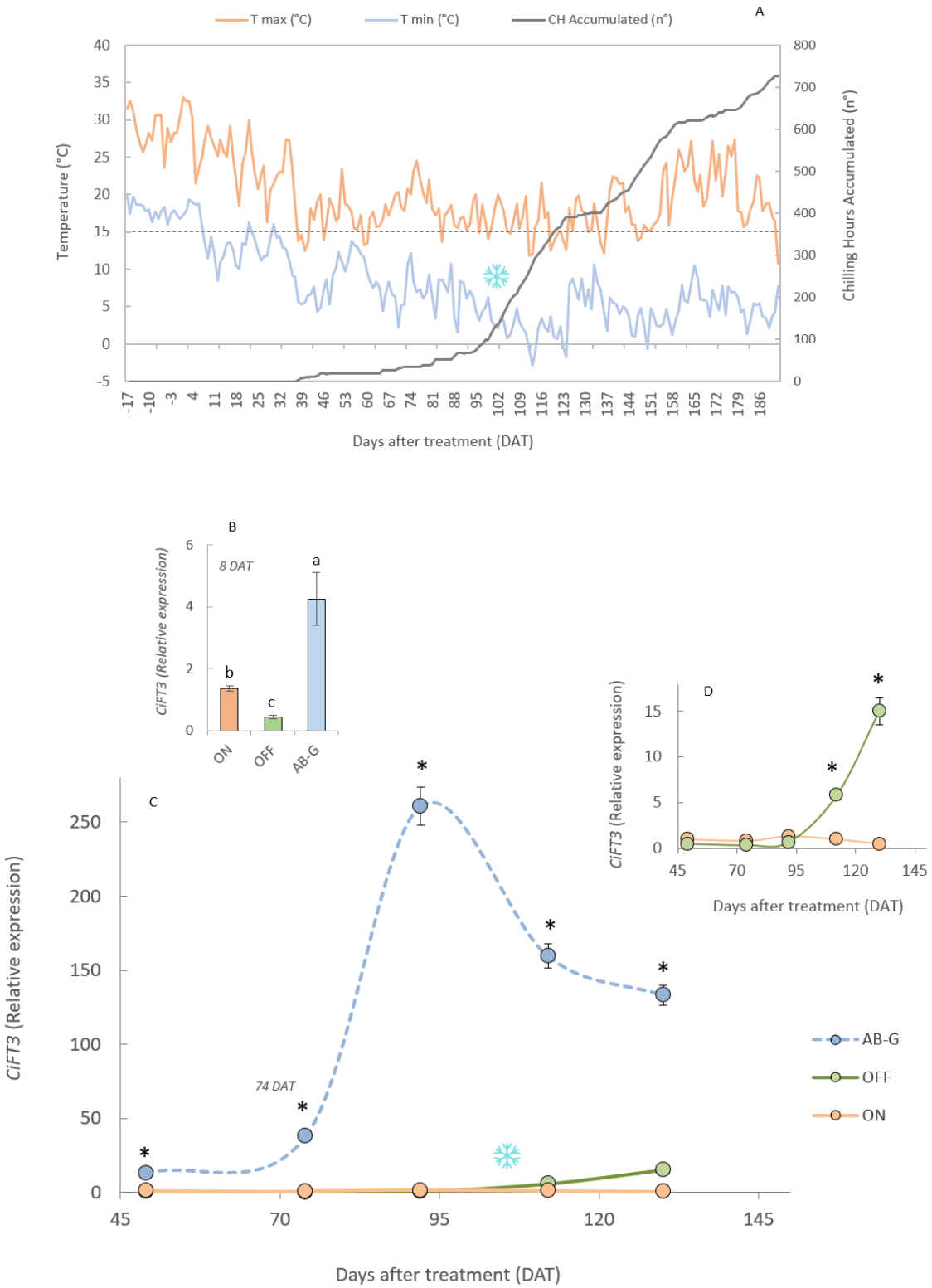


***Figure 4.25**. A: Time-course of minimum and maximum daily temperature and chill hours (<7,2°C) accumulated along the experiment (year 2018). B: Relative expression of gene CiFT3 in leaves, 8 days apical-basal girdling. Treatment 1st of October (year 2020). C: Time-course of the relative expression of gene CiFT3 in mature leaves of 'Nadorcott' mandarin. Treatment 20th of September (year 2018). D: A detail of the CiFT3 expression for treatments ON and OFF. Each value is the mean of 3 leaves. AB-G: Double Girdling, OFF: vegetative shoot, ON shoot with fruit in apical position. The PCR reference gene was Actin. Asterisk or letters indicate significative differences (P<0.05).*

On the other hand, AB-G treatment did not reduce the expression of the repressor gene *CcMADS19* in leaves compared to ON shoots, as observed in leaves of OFF shoots (Fig. 4.26). This result was also observed in the RNA-seq analysis, where the ON/AB-G comparison showed a log_2FC of -0.45 for the *CcMADS19* gene (adjusted P-value 0.0002). The results therefore suggest that: 1) the expression of the inhibitory gene *CcMADS19* is constitutive in the presence of fruit; 2) the mechanism of action of AB-G treatment on flower induction is significantly different from the epigenetic reset observed in vegetative shoots (Fig. 5), as indicated by the limited number of differentially expressed genes (DEGs) shared between OFF and AB-G in leaves (2.1%; Fig. 15), as also shown by RNA-seq analysis; 3) therefore, the isolation of buds caused by AB-G should directly promote floral induction in leaves, given its short-term effect. But does it also promote immediate floral differentiation in the buds? And if so, is this effect independent of temperature?

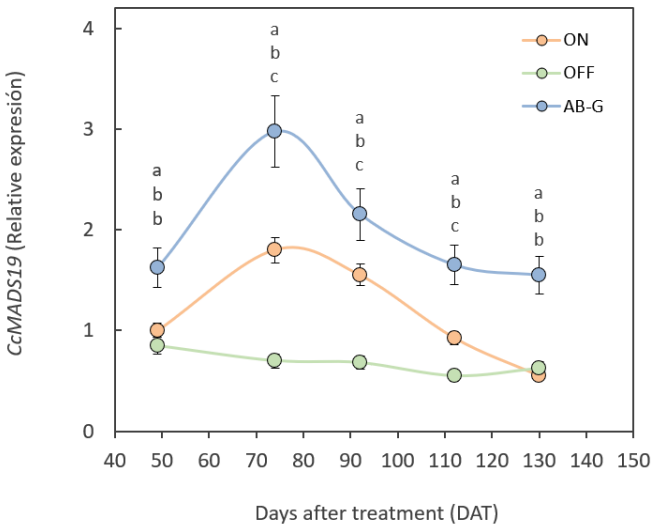


Figure 4.26. A: Effect of double girdling (AB-G) on the relative expression of the CcMADS19 gene in mature leave of 'Nadorcott' mandarin. Treatment was carried out 20th of September. Each value is the mean of 3 leaves. OFF: vegetative shoot, ON shoot with fruit in apical position. The PCR reference gene was Actin. The PCR reference gene was Actin. Letters indicate significative differences (P<0.05).

The AB-G treatment, when applied before the floral induction period, is able to initiate the inductive process in the leaves in the short term, as observed at 8 days after treatment (8 DAT) (Fig. 4.25B). However, it does not activate the reproductive state of the meristem. Therefore, the expression of the genes responsible for the floral identity of the meristem, *LFY* and *AP1* (Fig. 4.27A, B), do not show a significant change in relative expression at 8 DAT compared to the ON condition due to the treatment. Nevertheless, genes responsible for the vegetative state of the meristem, such as *CEN* (Fig. 4.27E) and

TFL1 (Fig. 4.27D), significantly reduce their expression in response to treatment. In addition, the treatment significantly increases the expression of the cell cycle cyclin *CYCB2* (Fig. 4.27C).

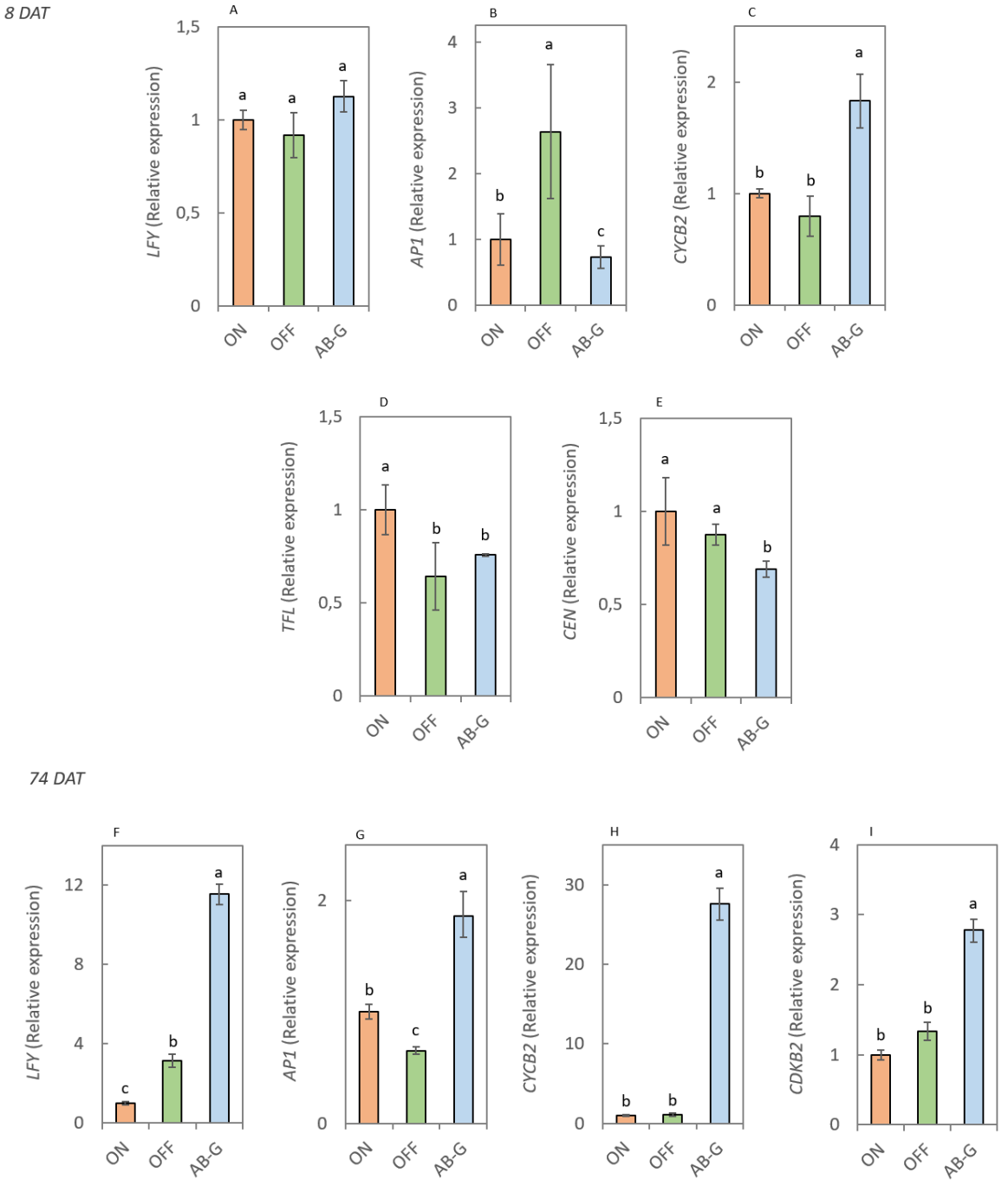


***Figure 4.27**. Short term (8DAT) and long term (74 DAT) effect of double girdling (AB-G) on the expression of floral identity genes (A, B, F, G), cell division genes (C, H, I) and floral repressors (D, E) in 'Nadorcott' mandarin. Each value is the mean of 3 biological replicates (20 buds per replicate). Short term effect: Treatment 1st of October and sampling 8th of October 2020. Long-term effect: Treatment 20th of September and sampling 3rd of December 2018. The PCR reference gene was Lycopene (LYC). Different letters indicate significative differences.*

However, the long-term study of the effect of AB-G at 74 days after treatment (DAT), just before the floral induction period of the OFF control, shows that *LFY* increases its expression 12 times, while *AP1* doubles in the buds of AB-G shoots compared to ON and OFF shoots (Fig. 4.27F, G). Additionally, *CYCB2* shows a relative increase in expression of up to 28 times compared to the buds of ON and OFF shoots (Fig. 4.27H), and *CDKB2* (*cyclin-dependent kinase*) expression is also increased by AB-G treatment (Fig. 4.27I).

When the AB-G treatment was applied during the induction period at the end of November (29/11), it also increased the expression of *CiFT3* at 21 DAT, multiplying it by 5 compared to ON shoots and by 13 at 50 DAT (Fig. 4.28A). The expression of *LFY* showed

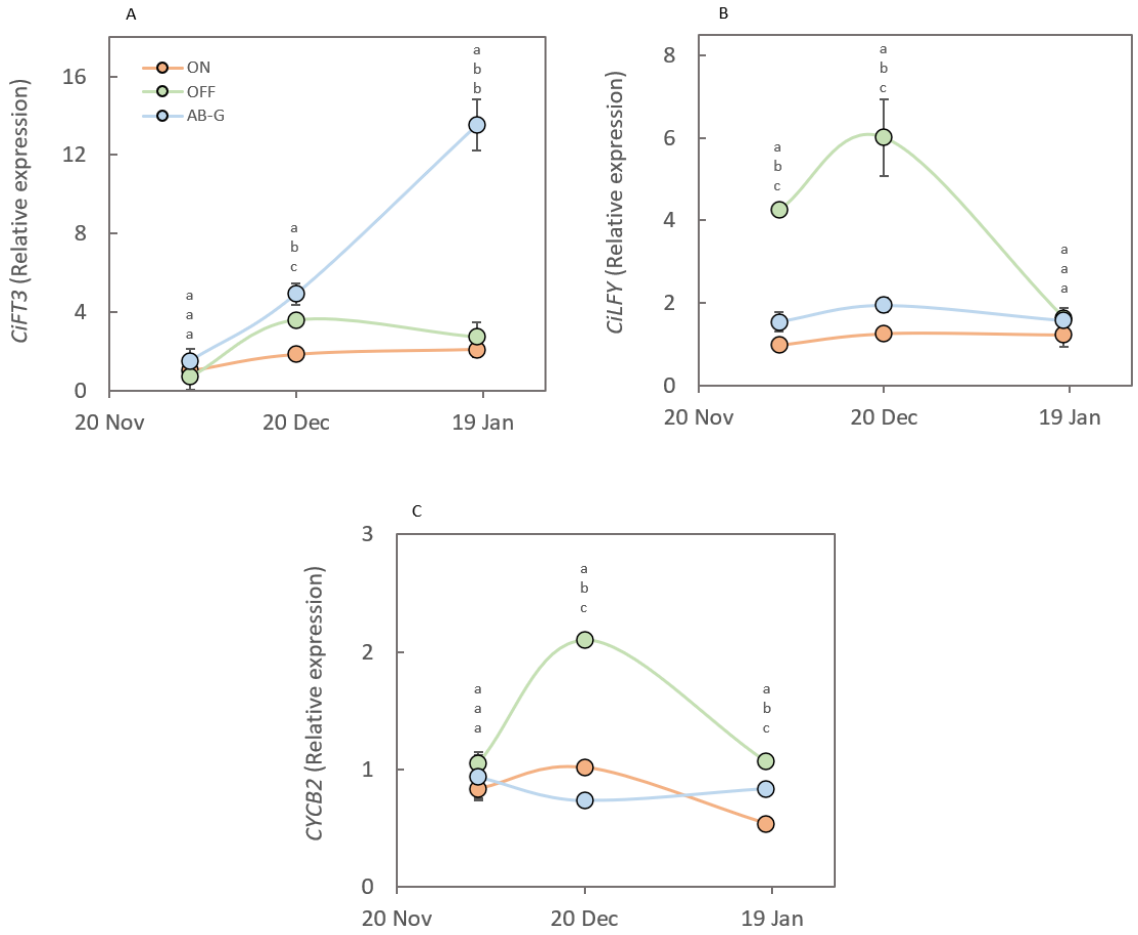


Figure 4.28. *Effect of AB-G after starting chilling hours accumulation, beginning of differentiation period in 'Nadorcott' mandarin. A: Relative expression of gene CiFT3 in leaves. Each value is the mean of 3 mature leaves. The PCR reference gene in leaves was Actin. B: Flowering differentiation gene LFY. C: Cell division cycle gene, CYCB2. Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene for buds was Lycopene (LYC). Treatment 29th of November and 1st sampling 4 days after treatment. Different letters indicate statistical differences.*

a slight significant difference in the short term, at 4 and 21 DAT, between the buds of AB-G shoots and ON shoots (Fig. 4.28C), in contrast to the treatment carried out in September (Fig. 4.27A). The high expression of *LFY* in the buds of the OFF control indicates the start of floral differentiation. However, the expression of *CYCB2* did not show any significant variation during this period, probably due to the low temperature (average minimum of 4.7°C) (Fig. 4.28).

In conclusion, these results indicate that the double girdling treatment (AB-G) promotes flowering in citrus trees independently of environmental conditions and probably through endogenous regulatory signals. Performing the experiment under high temperature conditions could further develop this hypothesis. Hormonal signals and nutritional stress have already been proposed as pathways to induce flowering (Bernier & Périlleux, 2005; Wada and Takeno, 2010).

4.4. Effect of double-girdling (AB-G) on hormonal regulation

The interruption of transport by the double girdling (AB-G), carried out in September (20/9), altered the hormone concentration in the shoot cortex (phloem and cortex) both in the short term (8 DAT) and in the long term (74 DAT). During sampling, phloem tissue isolated between the two girdles (AB-G, buds) was distinguished from tissue remaining attached to the plant beyond the basal girdling (AB-G, tree).

The concentration of GA_{12}, a gibberellin precursor that acts as a long-distance growth signal (Regnault et al., 2015), showed a slight difference between AB-G and ON shoots (Figure 4.29B). However, although the concentration is similar to that of OFF shoots, this effect did not lead to an increased synthesis of the active gibberellin GA_1 (Fig. 4.29A). The situation with cytokinins was different. Both isopentenyladenine (IP; Fig. 4.29D) and trans-zeatin (TZ; Fig. 4.29E) levels were significantly reduced by AB-G treatment. The detected concentrations of GAs and CKs were relatively low, ranging from 1.5 to 0.25 ng gDW^{-1}.

In contrast, the concentration of ABA and IAA reached 200 ng gDW^{-1} and 20 ng gDW^{-1}, respectively. However, the concentration of ABA in the phloem was not changed by the AB-G treatment at 74 DAT (Fig. 4.29C). For IAA, the concentration in the phloem of AB-G shoots significantly decreased compared to ON shoots at 8 DAT but not at 74 DAT (Fig. 4.29F, G). These results suggest that auxin transport in the phloem remained active after treatment.

In the leaves, the two phenotypes capable of flowering the following spring, OFF and AB-G, showed an increased accumulation of tryptophan (the precursor amino acid of IAA) throughout the flowering induction period, reaching a threefold increase in relative concentration at 74 DAT compared to ON shoots (Fig. 4.30).

In the buds, the concentration of active GAs (GA_1 and GA_4) showed no significant differences between ON and AB-G shoots at 4 DAT (Fig. 4.31C and D). Cytokinins showed a similar pattern to that observed in the phloem, with the concentration of IP being significantly lower in both OFF and AB-G shoots than in ON

shoots (Fig. 4.31B). Finally, the concentration of IAA was significantly lower in the buds of AB-G shoots at 4 DAT (Fig. 4.31A).

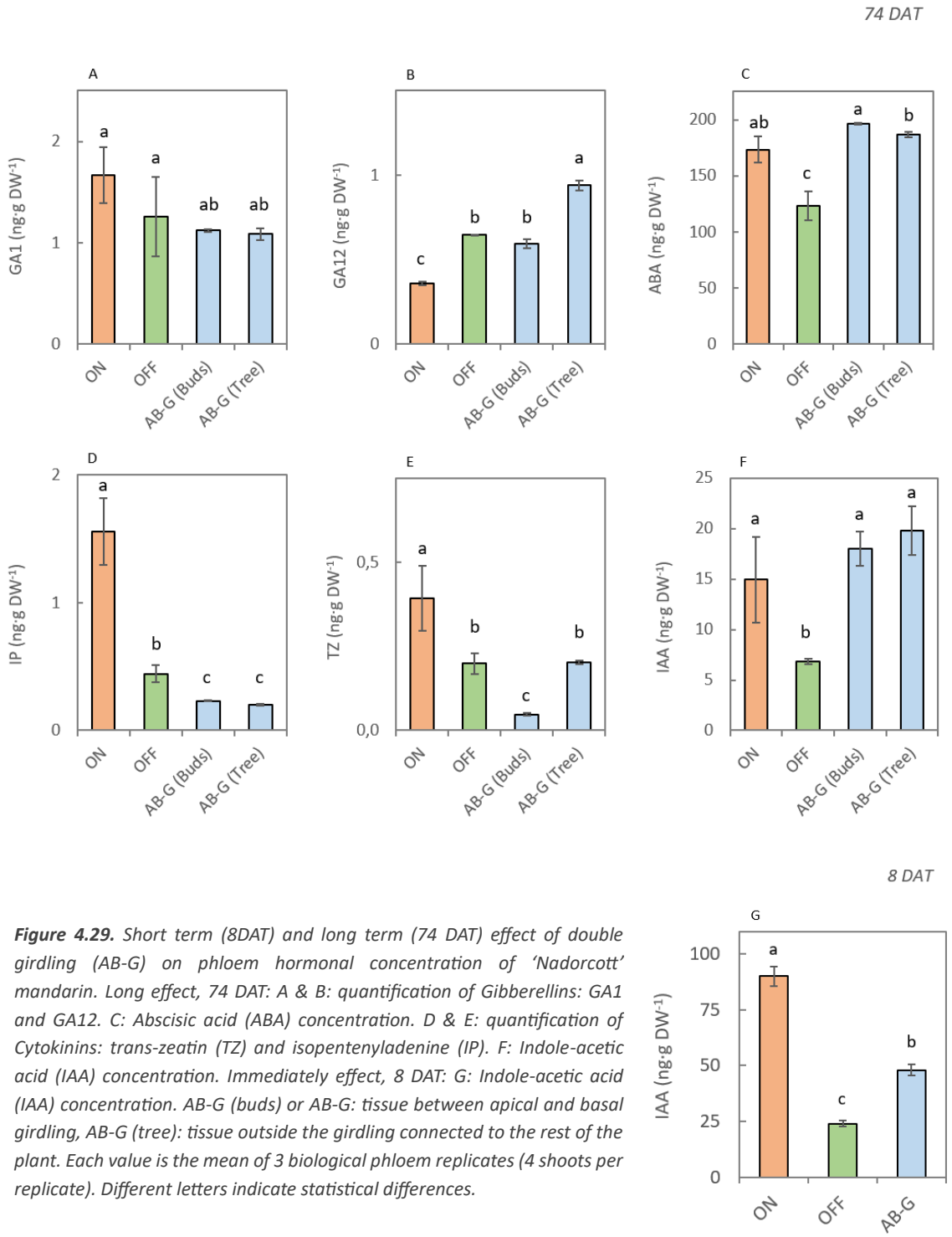


Figure 4.29. *Short term (8DAT) and long term (74 DAT) effect of double girdling (AB-G) on phloem hormonal concentration of 'Nadorcott' mandarin. Long effect, 74 DAT: A & B: quantification of Gibberellins: GA1 and GA12. C: Abscisic acid (ABA) concentration. D & E: quantification of Cytokinins: trans-zeatin (TZ) and isopentenyladenine (IP). F: Indole-acetic acid (IAA) concentration. Immediately effect, 8 DAT: G: Indole-acetic acid (IAA) concentration. AB-G (buds) or AB-G: tissue between apical and basal girdling, AB-G (tree): tissue outside the girdling connected to the rest of the plant. Each value is the mean of 3 biological phloem replicates (4 shoots per replicate). Different letters indicate statistical differences.*

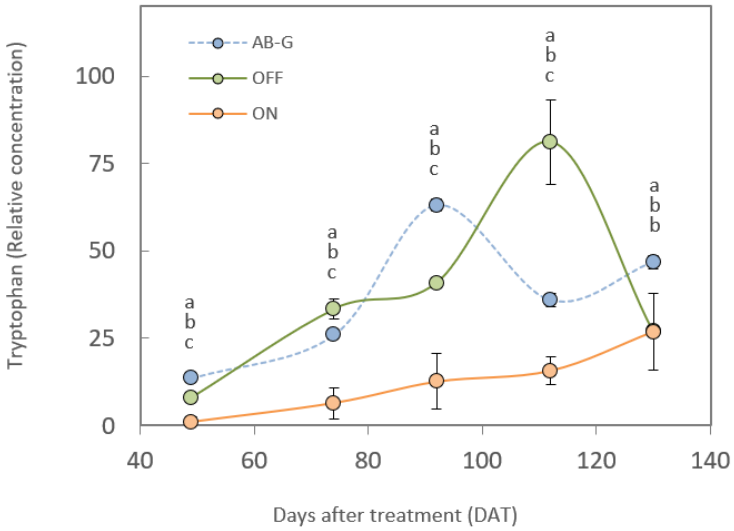


Figure 4.30. *Effect of double girdling (AB-G) on tryptophan relative concentration in the leaves during flowering inductive period. Each value is the mean of 3 mature leaves. Treatment 20th of September. Different letters indicate significative differences.*

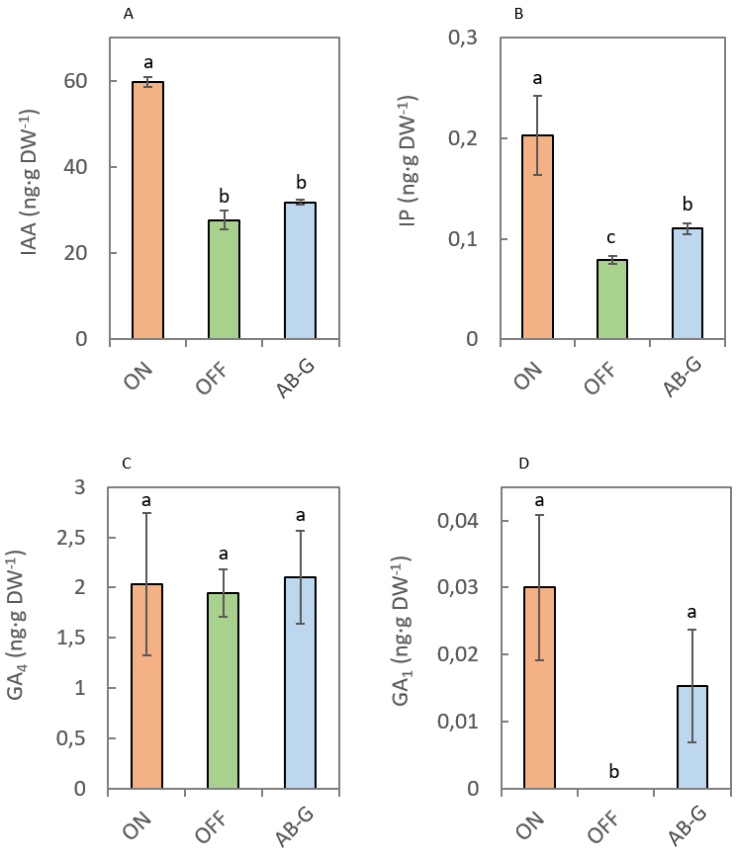


Figure 4.31*. Short term effect of double girdling (AB-G) in the bud hormonal concentration (A: auxin, B: Cytokinins, C & D: Gibberellins) at the beginning of the differentiation period in 'Nadorcott' mandarin. Treatment 29th of November and sampling 4 DAT. Each value is the mean of 3 biological bud replicates (20 buds per replicate). Different letters indicate significative differences.*

These results suggest that the maintenance of the IAA gradient in the phloem during the induction period (Fig. 4.29F) occurs by a different pathway than in ON shoots. Leaves accumulate more tryptophan in flowering phenotypes (Fig. 4.30) and have a lower IAA gradient in the buds (Fig. 4.31A). So, what is the mechanism that activates the shoot to restore this IAA flux once it has been removed from the influence of the fruit?

When we evaluated the natural behaviour between ON and OFF shoots, we found the same results as in Chapter III. At the beginning of autumn, before flowering induction, the axillary buds of ON and OFF shoots do not differ in the expression of *PIN3* (Fig. 4.32A). However, during the period of flower induction, the meristems of OFF shoots initiate polar auxin transport, doubling the relative expression of *PIN3* compared to ON buds (Fig. 4.32B), which can be maintained during the period of flower differentiation (Fig. 4.32C).

On the other hand, double girdling (AB-G), which activates the expression of *CiFT3* in the short term (4-8 DAT), regardless of the time of treatment, does the same with the expression of *PIN3* before (Fig. 4.32A) or during (Fig. 4.32C) the temperature drop. In addition, the buds of AB-G shoots maintain polar auxin transport activity in the long term, at 74 DAT (Fig. 4.32B) or at 21 and 50 DAT (Fig. 4.32C).

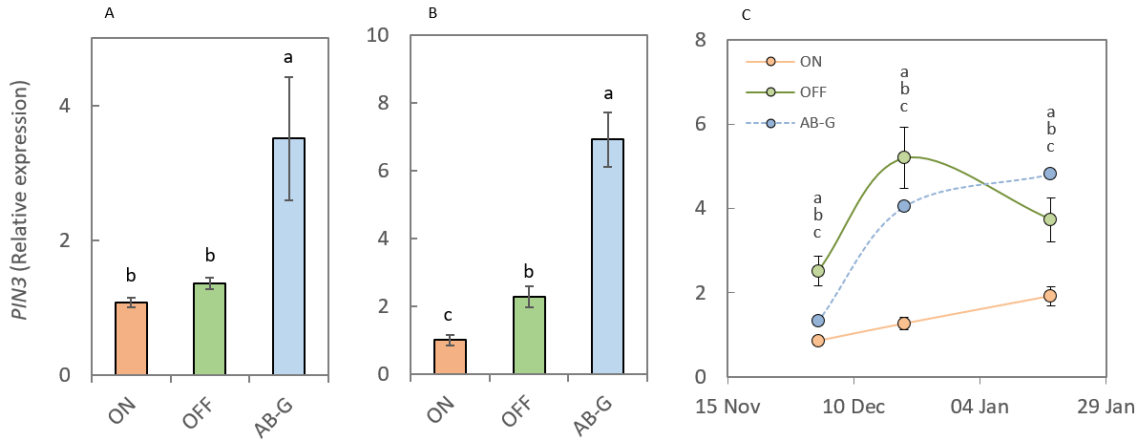


Figure 4.32. *Effect of double girdling (AB-G) on auxin transport in 'Nadorcott' mandarin buds measured by the relative expression of the gene PIN3, an auxin efflux carrier. A: Short term effect of AB-G, 8 DAT, treatment 1st of October. B: Long term effect of AB-G, 74 DAT, treatment 20th of September. C: Effect of AB-G at the end of flowering inductive period, treatment 29th of November, 4 DAT, 21DAT and 50 DAT. Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Different letters indicate significative differences.*

Finally, in the case of gibberellins, the relative expression of the regulatory enzymes for synthesis (*GA20ox2* and *GA3ox2*) and catabolism (*GA2ox2*) in the buds during flowering induction shows lower synthesis and higher catabolism in the two phenotypes that flower (OFF and AB-G) compared to the ON shoots (Fig. 4.33). These results are in line with those developed in Chapter II, where greater GA synthesis is observed in the buds that will remain vegetative, specifically those of ON shoots, and correlates with the expression of the *CEN* gene.

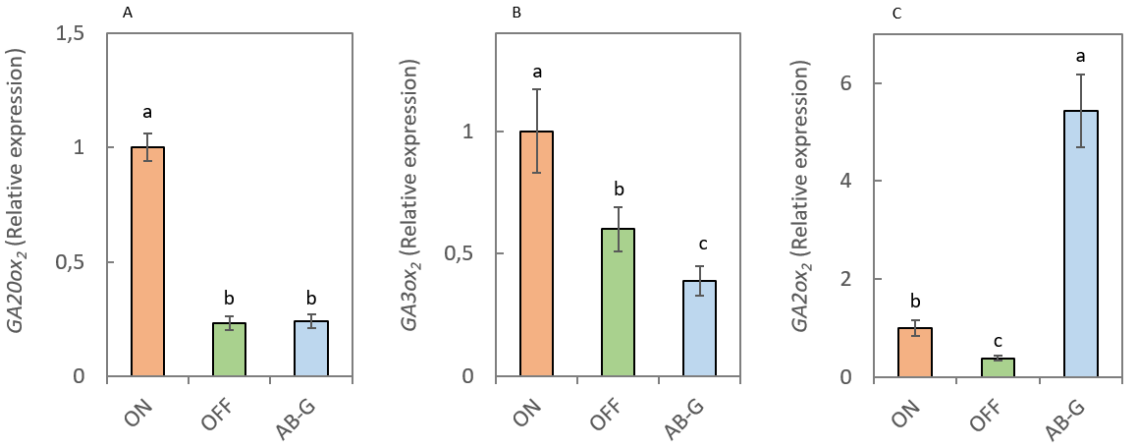


Figure 4.33. *Long term effect of double girdling (AB-G) on gibberellin metabolism in 'Nadorcott' mandarin buds. A: Relative expression of GA20ox₂. B: Relative expression of GA3ox₂ C: Relative expression of GA2ox₂. Each value is the mean of 3 biological bud replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Different letters indicate significative differences. Measurements were performed 74 days after the girdling treatment, made the 1st of October.*

Taken together, the results suggest that the buds isolated by the double girdling treatment become the new main sinks, as the treatment isolates them from the influence of the rest of the tree fruit, freeing the main auxin pathway. This allows the buds to reactivate polar IAA transport. Consequently, in citrus trees, auxins would play a dual role in the buds: (i) in fruit-bearing shoots (ON), where the high exported concentration suppresses the reactivation of the sink capacity of axillary meristems, preventing their development, and (ii) in fruitless shoots (OFF), where the absence of a primary sink (when the shoot has stopped growing) allows axillary meristems to synthesise and export auxins, allowing floral differentiation. However, the extent to

which auxin export from the fruit is the primary explanation for the inhibition of floral induction remains unknown.

So, if auxins modulate the sink capacity of buds, how do carbohydrates act in this process? Can AB-G treatment alter the nutrient balance? All this will be analysed in the last section of this chapter.

4.5. Effect of double girdling (AB-G) on carbohydrates metabolism

The relative concentration of carbohydrates in the leaves was significantly altered by girdling at 74 DAT. The levels of sucrose, fructose and glucose in the leaves of AB-G shoots were significantly lower than in ON shoots, showing a relative reduction of 20%, 70% and 80%, respectively (Fig. 4.34A, B, C). In addition, the concentration of trehalose 6-phosphate (a stress signalling sugar) (Fig. 4.34D) also showed significant differences, decreasing by up to 50% compared to ON shoots.

At 92 DAT the behaviour was similar. The relative concentration of sucrose, fructose, glucose and trehalose 6-phosphate in leaves of AB-G shoots remained significantly lower than in ON shoots (Fig. 4.34F, G, H, I), with an identical pattern of behaviour to OFF shoots. However, where we found temporal differences was in the relative concentration of maltose. At 74 DAT, only the OFF shoots contained maltose (a product of starch hydrolysis) (Fig. 4.34E). In contrast, at 92 DAT, the concentration of maltose decreased in OFF shoots and started to accumulate in AB-G shoots (Fig. 4.34J). This metabolite was not detected in ON shoots.

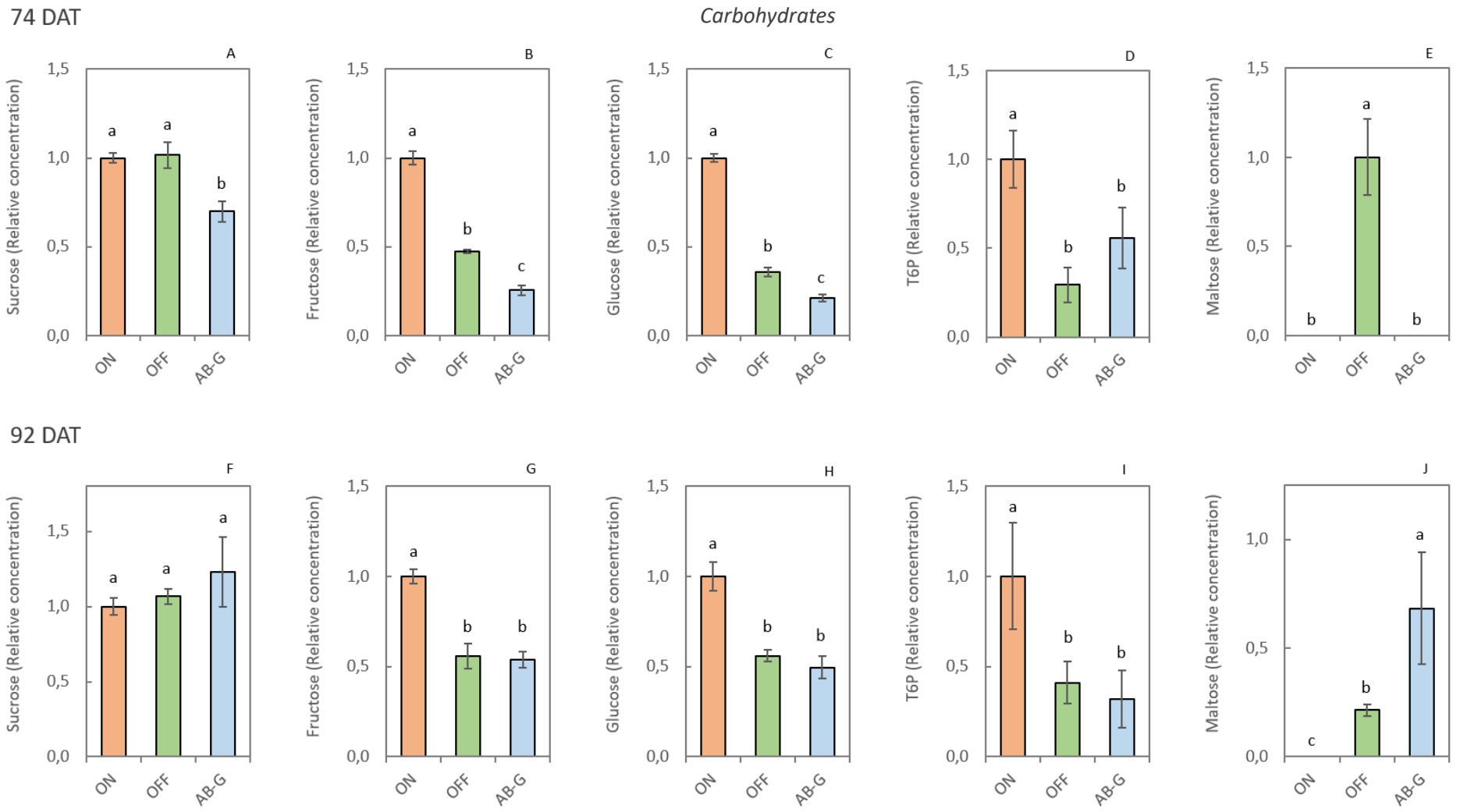


***Figure 4.34**. Effect of double girdling (AB-G) on the relative concentration of carbohydrates in the leaves, during the floral induction period (74 DAT) and prior to bud differentiation (92 DAT). Treatment 20th of September. Each value is the mean of 3 mature leaves. Different letters indicate statistical differences.*

On the other hand, among the amino acids analysed in the leaves of the shoots, methionine (Fig. 4.35A) showed no significant differences between treatments. Methionine is a key amino acid in the ethylene synthesis pathway. However, putrescine, the most abundant polyamine in citrus, showed significant differences. Its relative concentration was 90-80% higher in ON and OFF shoots than in AB-G shoots at 74 and 92 DAT (Fig. 4.35B, E). Its relationship with the floral induction stimulus induced by double girdling does not seem relevant, as there were no significant differences between fruit-bearing (ON) and fruitless (OFF) controls. The relationship between putrescine content and cold-induced flowering has been studied in sweet orange, although no consistent conclusion has been reached (Ali and Lovatt, 1995). Nevertheless, the effect

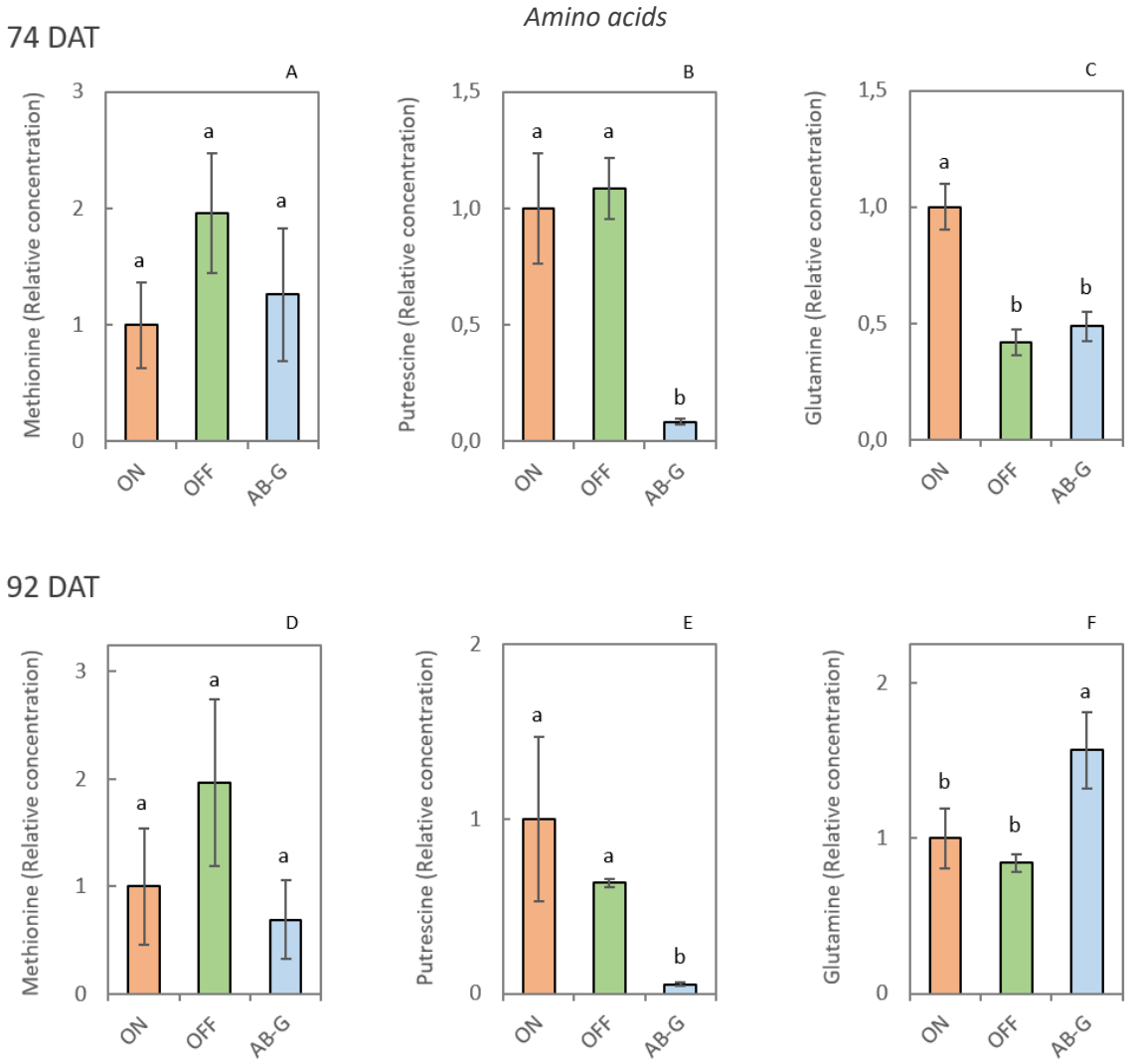


***Figure 4.35**. Effect of double girdling (AB-G) on the relative concentration of aminoacids in the leaves, during the floral induction period (74 DAT) and prior to bud differentiation (92 DAT). Treatment 20th of September. Each value is the mean of 3 mature leaves. Different letters indicate statistical differences.*

of AB-G treatment consistently reduces the relative concentration of this amino acid. Finally, glutamine in girdled shoots varies throughout the period, showing a significantly lower concentration at 74 DAT (Fig. 4.35C) and a higher concentration at 92 DAT (Fig. 4.35F) compared to control shoots with fruit.

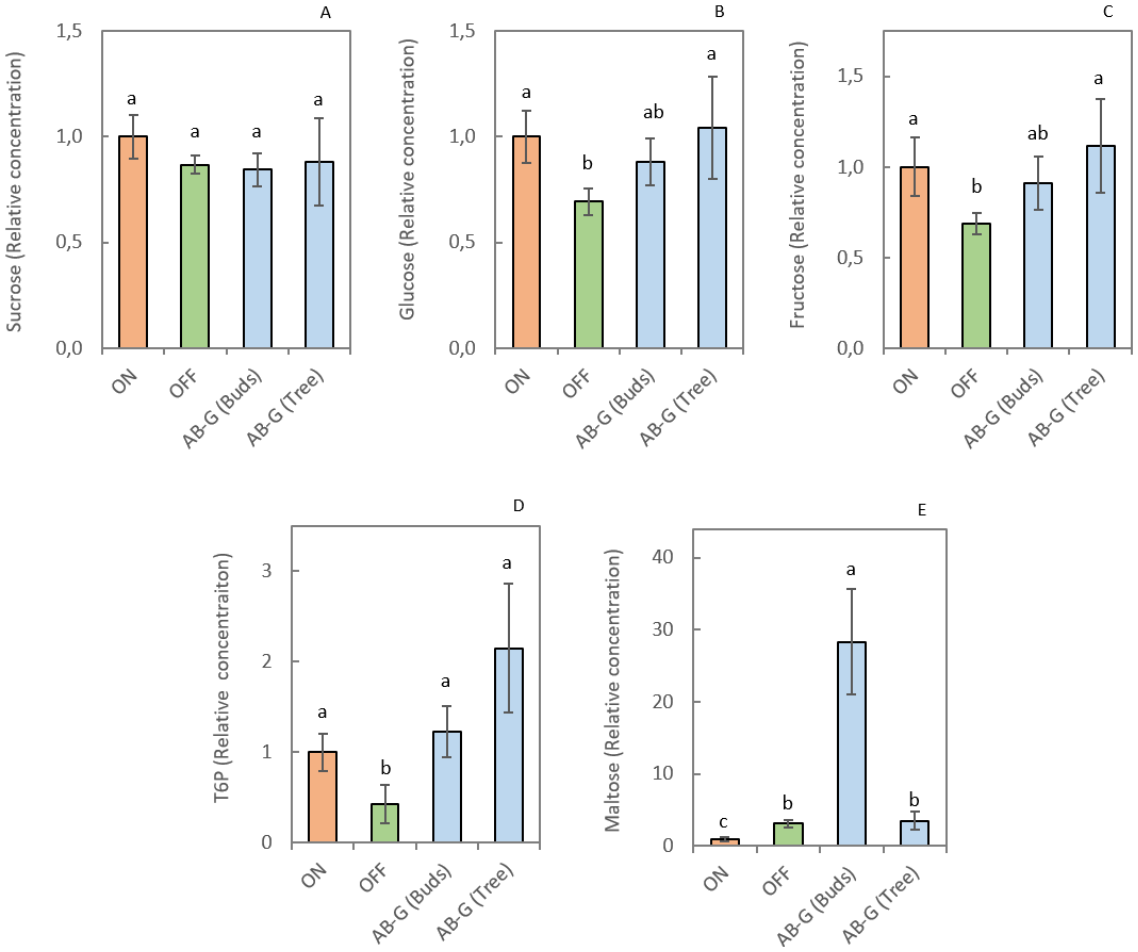
In the phloem, the relative concentration of carbohydrates at 74 DAT was similar between AB-G and ON shoots. The concentration of sucrose, glucose, fructose and trehalose 6-phosphate showed no significant differences between the tissue isolated by the AB-G treatment and the phloem tissue of ON shoots (Fig. 4.36A, B, C, D). However, the relative content of maltose in OFF shoots was twice that of fruit-bearing shoots and, more importantly, the relative concentration in AB-G shoots increased by a factor of 29 in the tissue isolated by the treatment (AB-G buds; Fig. 4.36E). The phloem tissue that remained connected to the rest of the plant (AB-G Tree) maintained the same relative maltose content as the OFF shoots. This is in agreement with the results obtained in the leaves, where maltose is also a differential metabolite.

Amino acids in the phloem showed a different behaviour than in the leaves. Glutamine showed no significant differences between treatments (Fig. 4.36G). In contrast, the concentration of putrescine at 74 DAT was significantly higher in the phenotypes that would flower the following spring (Fig. 4.36F). Finally, in the case of methionine, AB-G shoots reduced the relative concentration compared to the control treatments (Fig. 4.36H).

Consequently, the results suggest that the double girdling treatment (AB-G) induces an alteration in sugar metabolism, reducing the relative concentration of metabolites such as fructose, glucose or trehalose 6-phosphate to values similar to those of the non-grown control (OFF). It also increases the accumulation of maltose in leaves and phloem. However, the concentration of other metabolites such as sucrose remains constant in all tissues.

Is this similar concentration of sucrose between the three treatments due to a stable content, or is it due to ongoing synthesis and utilisation?

Carbohydrates



Amino acids

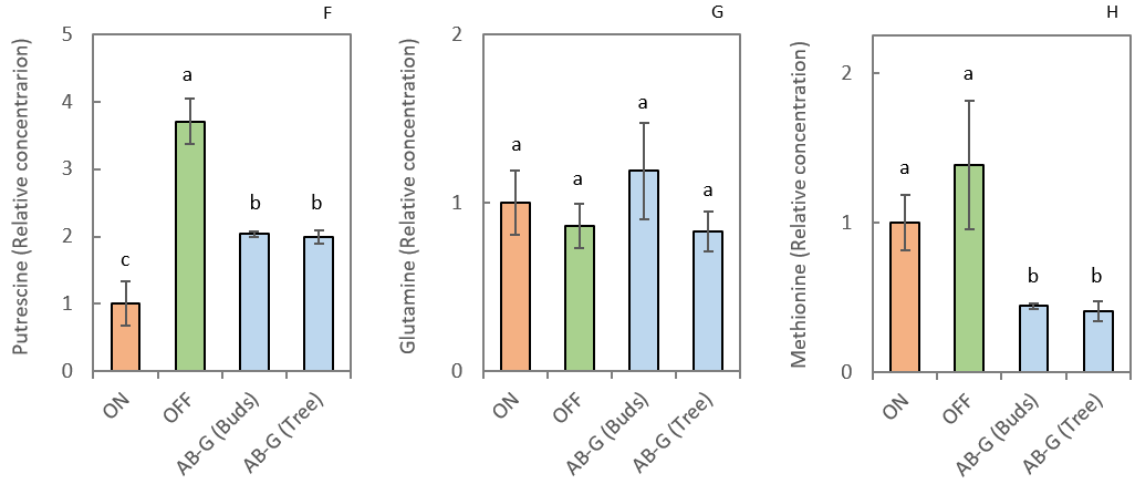


Figure 4.36. *Effect of double girdling on the relative concentration of arbohydrates (A, B, C, D & E) and aminoacids (F, G & H) in the phloem, at the floral induction period (74 DAT). Treatment 20th of September. Each value is the mean of 3 biological phloem replicates (4 shoots per replicate). Different letters indicate statistical differences.*

To address this, the expression of genes related to sucrose hydrolysis and transport was analysed, taking into account the overexpressed and repressed sequences from the RNA-seq analysis (Section II, Chapter IV).

The kinase *SnRK1* works in conjunction with another kinase (*TOR*) to sense nutrient levels and antagonistically promote or inhibit growth. Low sugar levels promote *SnRK1 expression*, while high levels regulate *TOR* activity (Deprost et al., 2007). The SnRK1 protein complex consists of three subunits (α, β, γ). In the buds of AB-G shoots, the relative expression of all three subunits, *α-SnRK1*, *β-SnRK1* and *γ-SnRK1*, is significantly twice as high as in the buds of fruit-bearing control shoots at 74 DAT (Fig. 4.37A, B, C). This result suggests that the buds are under nutrient deficiency. Consequently, metabolic pathways are activated to supply sugars to the demanding meristems. On the one hand, the expression of the enzyme *α-amylase* (*α-AMY*) doubles its relative expression compared to ON shoots (Fig. 4.37D). This reactivates starch hydrolysis in the meristems, releasing maltose (Figure 4.36E). In addition, analysis of enzymes involved in sucrose metabolism, such as *cell wall* (*CWINV*), *cytoplasmic* (*CIN* and *SUS1*) and *vacuolar* (*VIN*) *invertases*, which are responsible for breaking down sucrose molecules into glucose and fructose, showed that double girdling (AB-G) altered their expression patterns. *CWINV* and *VIN* invertases were significantly downregulated compared to control treatments (Figure 4.37E, H). This suggests that neither the sucrose in the cell environment nor the sucrose stored in the vacuoles has initiated hydrolysis. However, the expression of *CIN* remains high and, more importantly, the relative expression of *SUS1* increased fourfold compared to ON and OFF controls (Fig. 4.37G), indicating that sucrose hydrolysis is occurring directly in the cell cytoplasm in AB-G shoots.

The results suggest that double girdling (AB-G) activates the primary metabolism of axillary meristems during the floral induction period. The interruption of transport in the shoot leads to a nutrient deficit in the buds (activation of the SnRK1 complex), which triggers starch hydrolysis and sucrose transport to the cytoplasm, where sucrose hydrolysis is initiated to promote cell development.

But is this effect immediate or does it occur in the long term?

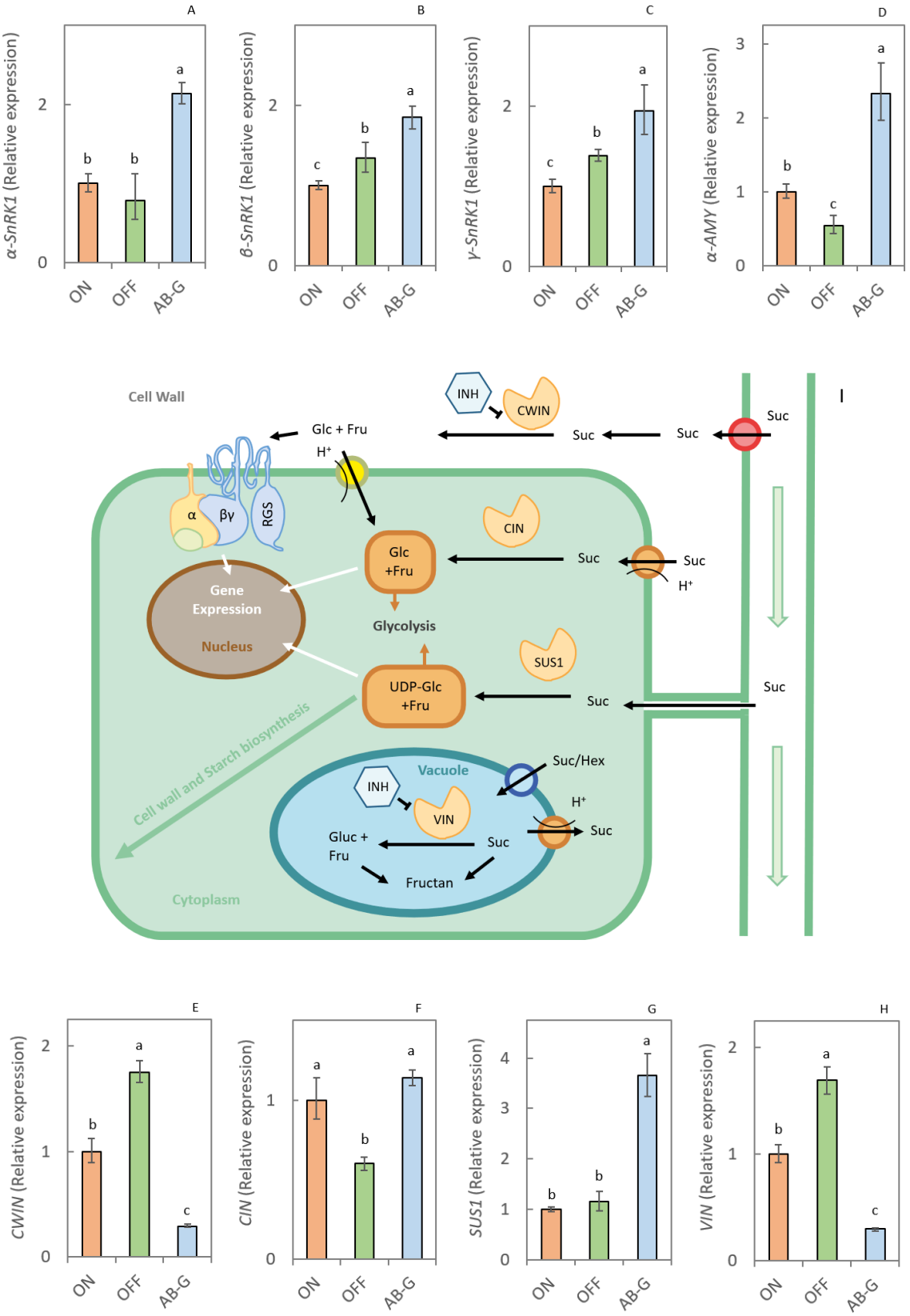


Figure 4.37. *Molecular markers related with carbohydrate metabolism and nutritional stress in 'Nadorcott' mandarin buds, 74 DAT (20th September). A, B, C: relative expression of three subunits of the protein SnRK1. D: relative expression of α-Amylase enzyme. E, F, G, H: expression of sucrose invertases and synthase located in: cell wall (CWIN), cytoplasm (CIN, SUS1) and vacuole (VIN). I: Diagram of sucrose unloading, transport, and metabolism in sink tissues. Each value is the mean of 3 biological replicates (20 buds per replicate). The PCR reference gene was Lycopene (LYC). Different letters indicate statistical differences. Modified scheme adapted from Ruan et al., 2014.*

The double girdling treatments (AB-G), carried out in October before the flower induction period, were sampled 8 days after treatment (8 DAT). Both leaves and buds overexpressed the *α-AMY* gene, increasing its expression by fivefold (leaves; Fig. 4.38A) and sixfold (buds; Fig. 4.38B) compared to the fruit-bearing (ON) and fruitless (OFF) control treatments. In addition, when the AB-G treatment was applied at a completely different time, after the induction period, in January, double girdling immediately reactivated the shoot photosynthetic rate (An) and remained high even 24 hours after treatment (Fig. 4.37C).

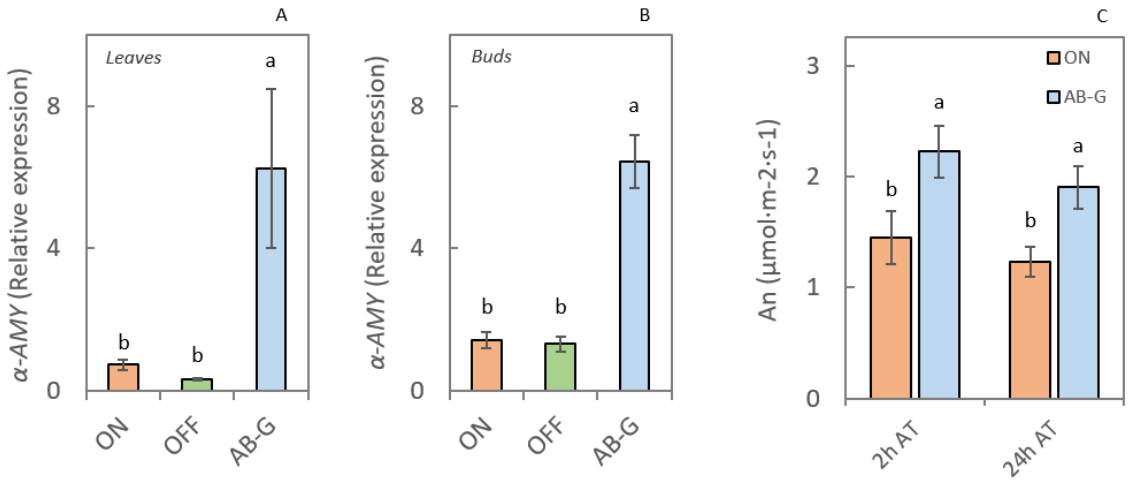


***Figure 4.38**. Immediate effect of AB-G on primary metabolism. A, B: relative expression of α-Amylase enzyme in 'Lane late' (Citrus sinensis) leaves and Buds, 8 DAT (Treatment 1st October). Each value is the mean of 3 mature leaves or 3 biological buds replicates (20 buds per replicate). The PCR reference gene for buds was Lycopene (LYC) and Actine (ACT) in leaves. C: net photosynthesis (An) 2h and 24h after AB-G (treatment 15th January). Each value is the mean of 5 shoots (3 measurements per shoot). Equipment: Portable Photosynthesis System (LI-COR). Different letters indicate statistical differences.*

In conclusion, double girdling (AB-G) immediately reactivates the primary metabolism of the shoot, regardless of the time of treatment. Furthermore, this effect can be maintained over time.

Discussion

1. *Which endogenous signals are the most likely candidates to regulate Flower Induction?*

Plant hormones are the signals that regulate growth (quantitative changes) and differentiation (qualitative changes) in all physiological processes of the tree, such as vegetative growth, apical dominance, assimilate partitioning, fruit set and growth, stress alleviation, etc. In each cycle, the development of new vegetative buds in citrus coincides with fruit growth during summer and autumn, and will only flower the following spring if they are in other branches away from *fruit development signals* (FDS). According to the composition of the fruit exudate (Fig. 2.5), the most likely FDS candidates to interfere citrus FI, in order from highest to lowest concentration, are JA, SA and AIA ($\cong 10$ ng g^{-1}), ABA ($\cong 10^{-1}$ ng g^{-1}) and GA ($\cong 10^{-2}$ ng g^{-1}). Similar results have been recently reported in apple (Milyaev et al., 2022).

Among these hormones, GA and IAA have been considered the most likely FDS candidates to interfere with FI. However, regarding GA, Bangerth (2009) believes it controversial to consider endogenous GA as 'targeted' FDS to inhibit FI in trees, even though GA_3 treatments inhibit flowering in most fruit tree species. In particular, Bangerth stated that "it would be difficult to assume GA transport from a fruit, a strong sink, to the apical or axillary bud, a weak sink". However, other experiments in pea and Arabidopsis have shown that GA_{20}, GA_1, and particularly GA_{12} moves from root to shoot in the xylem and from shoot to root in the phloem to regulate plant growth (Regnault et al., 2012). On the other hand, IAA is strongly transported basipetally from the sink to weak organs (apical and ‘king fruit’ dominance), and it has therefore also been suggested that polar IAA transport could be an alternative to GA as a secondary messenger (Bangerth 2006).

This type of transport is usually referred to as fruit-to-bud transport. However, FI takes place in the leaf and it is not known whether these fruit-derived signals reach the leaf. A recent study showed that sink/source identities and phloem translocation can be reversed (from taproots to shoots in sugar beet), induced by vernalization, to promote

flowering, suggesting that mature leaves can receive signals from other sinks (Rodrigues et al., 2020).

To date, it is not clear whether endogenous GA play a direct signalling role in citrus FI, and the extent to which auxin export from the fruit is the primary explanation for flowering regulation remains open (Sadka et al., 2023). This PhD thesis studies the role of GA and auxins in citrus FI and flower bud differentiation.

2. Neither GA nor auxin activate CcMADS19 in the leaf

In the OFF-tree leaf, the results suggest an inverse relationship between endogenous GA and *CiFT3* expression (Figs. 1.4B & 1.5). Summer-fall vegetative shoots develop in the months before FI. During leaf development, GA, IAA and CK are synthesized to promote cell division and expansion (Nelissen et al., 2012). Although it is necessary to establish a cause-and-effect relationship, as cold might affect both GA synthesis (Yamaguchi et al., 2004) and *CiFT3* upregulation (Nishikawa et al., 2007), the results suggest the hypothesis that it is necessary for GA levels to decrease prior to *CiFT3* upregulation, and that any increase in GA levels (exogenous treatments) reduces *CiFT3* expression, as previously reported (Muñoz-Fambuena et al., 2012).

The expression of *CcMADS19* slightly decreased during cold, coinciding with GA depletion (Fig. 1.4C). However, GA_3 treatments before, during, or continuously throughout the FI period in the OFF trees inhibited flowering without activating *CcMADS19*, in contrast to the fruit mechanism based on the upregulation of this floral repressor (Agustí et al., 2020). Finally, neither *CcMADS19* expression (data not shown) nor flowering (Martínez-Fuentes et al., 2013) was altered by PBZ treatment of the ON tree.

Fruit thinning reversed the inhibitory effect on *CiFT3* expression, as previously shown (Shalom et al. 2014; Agustí et al., 2020). The results of this thesis show that this effect is immediate, regardless of the time of thinning, since thinning in July and November doubles the expression of *CiFT3* after 48 hours and, in the case of November,

even equals the expression of the OFF control. Shalom et al. (2014) showed similar results, but thinned in September and measured *CiFT3* expression in buds instead of leaves after 7 days. On the other hand, the presence of the fruit does not stimulate the expression of the *GA3ox1* and *GA3ox2* genes in the leaves (Figs. 1.6C, D), whereas thinning does, at least during July, up to the OFF-tree level. These results are not in agreement with previous experiments on mango, where the expression of GA synthesis genes is higher in ON trees (Nakagawa et al., 2012). However, there is a significant difference between citrus and mango: in the former, the fruit is present during the FI period, whereas in the latter, the fruit is not present during the FI period due to harvesting 4 months earlier (in August). Therefore, the results of Nakagawa et al. may reflect increased vegetative development rather than fruit-mediated inhibition of FI.

Accordingly, the hypothesis that the inhibitory effect of fruit on flowering is mediated by its effect on GA levels during FI (Monselise and Goldschmidt, 1982) is not supported by our observations: 1) in the OFF tree, GA_3 treatments downregulate *CiFT3* without increasing *CcMADS19* expression, and GA_1 depletion in the leaf correlates with activation of *CiFT3* expression independent of *CcMADS19*, and 2) in the ON tree, the fruit activates the flowering repressor through a signal unrelated to GA synthesis and content in the leaf. Thus, the results suggest that GA and fruit do not use the same mechanism to repress *CiFT3* up-regulation, i.e., *Citrus* FI.

All these findings raise important questions: 1) How does GA regulate flowering time in *Citrus*? In our experiments, GA_3 treatments significantly reduced the relative expression of *NF-YA1* (Fig. 1.10), a transcription factor that activates *CiFT3* expression by binding to the promoter (Zhou et al., 2022), suggesting a direct effect on *CiFT3* transcription probably mediated by DELLA proteins (Davière, and Achard, 2016). 2) If it is not GA, what is the signal from the fruit that activates *CcMADS19* in the leaf? Is it auxin, as Bangerth suggested?

The effect of 2,4-D treatment on *CiFT3* expression (Fig. 3.4) contrasts with the results obtained with GA_3 treatment (Figs. 1.9A, C & E), which show a high consistency in the reduction of *CiFT3* expression. All species treated with GA_3 (sweet orange, lemon, grapefruit, and clementine mandarin) significantly reduced *CiFT3* expression

immediately after application and maintained the inhibitory effect throughout the FI period. The auxin 2,4-D, however, showed an erratic response in controlling the expression of this gene and thus FI. Similar results were previously obtained by García-Luís et al. (1986) on the inhibition of flowering by 2,4-D treatments. Neither GA_3, nor 2,4-D treatment consistently promoted the expression of the *CcMADS19* gene in the non-bearing trees (Figs. 1.9 & 3.4).

The endogenous concentration of IAA in the leaf did not fully correlate with FI. Thus, early fruit thinning (July) temporarily reduced IAA concentration in leaves and internodes before the FI period and restored 100% of flowering capacity, whereas late thinning (September) reduced IAA concentration but did not fully restore flowering, although it increased it with respect to fruiting branches (Martínez-Fuentes et al., 2010; Haim et al., 2021). However, in both treatments the leaf IAA concentration was not reduced compared to the ON tree during the FI period (Fig. 3.10A), a result that contrasts with the IAA concentration of the OFF tree (higher than that of the ON tree). Previous studies found no significant differences in leaf IAA concentration between ON and OFF trees (Koshita et al 1999) and between ON and fruit-thinned trees (Okuda 2000).

In our fruit-bearing shoot model, the leaf is an 8-month-old mature leaf during the FI period, source of carbohydrates, and it is unclear if it can be a sink for fruit-derived hormonal signals. According to previous studies in the annual plant *Phaseolus vulgaris* (Tamas et al., 1981), IAA is involved in the correlative control of axillary bud development and leaf senescence by fruits. IAA released from the fruit may play the role of FDS, which translocate to the target organ where it stimulates the synthesis or accumulation of ABA. ABA, in turn, may be responsible for inhibiting axillary bud development and promoting leaf senescence. However, this type of correlative leaf control has been rejected in Arabidopsis (Noodén and Peney, 2001). To our knowledge, correlative control of leaf development by fruits has not been investigated in evergreen trees such as citrus.

In conclusion, as with GA, the results obtained do not establish a strong cause-effect relationship to explain the fruit inhibition effect on FI in terms of auxin being the primary inhibitory signal. Further studies are needed to determine which signals, hormonal or otherwise, are the most likely candidates to regulate FI in trees.

3. Both GA and auxin regulate flower bud differentiation

On the other hand, the correlative control of axillary bud development is very well documented in the 'king fruit' and apical dominance studies, and a role for auxin and GA has been proposed (Bangerth, 2000; Beveridge et al., 2023). In citrus, however, flower bud differentiation occurs simultaneously with bud release, making it difficult to separate the role of hormones in regulating flower identity.

In ON trees, a significant peak of GA (high concentration of GA_4 and upregulation of its precursor *GA3ox*) was detected in the buds, coinciding with the period of floral differentiation (Figs. 2.2B, H & 2.3). On the contrary, the opposite was observed in OFF and defruited trees, where GA concentration and synthesis were practically zero and stable throughout the period studied, with no significant differences between them (Fig. 2.2). To our knowledge, there are no published studies analysing GA concentration and synthesis (*GA20ox* and *GA3ox* expression) in citrus buds in the same experiment. In contrast to our results, previous studies found no differences in *GA-like substances* in the bud of ON and OFF trees during the dormant period (Jones et al., 1977). However, according to Hedden (2020), the quantification of GA and their precursors and catabolites is becoming more realistic with the increasing sensitivity of methods such as UPLC-MS (the one used in our analyses) compared to previous methods. Furthermore, considering that the GA concentration correlates with *GA20ox* and *GA3ox* expression, it is concluded that endogenous GA play a significant role in inhibiting flower bud differentiation.

The increase in GA concentration in ON-tree buds could be related to (i) the arrival of endogenous GA from the ripe fruit, as suggested by Gambetta et al. (2012), and (ii) that the buds initiated the biosynthesis of GA *in situ* in response to an endogenous stimulus. The GA_1 and GA_4 content of the fruit exudate in November (colour change/FI) and December (flower differentiation) was less than 1 ng g^{-1} or zero for GA_1 and GA_4, respectively (Fig. 2.5). Furthermore, the phloem concentration of the bioactive GA precursors GA_{12} and GA_{20} in ON, OFF and fruit-thinned trees did not correlate with the GA_4 concentration in the buds (Fig. 2.4), and similar results were reported for apple

(Milyaev et al., 2022). Therefore, the results suggest the hypothesis that the increase in GA concentration in the buds of ON trees is not due to transport from the fruit, but rather to the fact that the presence of the fruit prevents the expression of *CiFT3* in the leaf (Agustí et al., 2020) and the upregulation of *LFY* (Collani et al., 2019; Zhu et al., 2020). In Arabidopsis, this fact activates GA synthesis to inhibit the floral meristem (Yamaguchi et al., 2014). More specifically, to overcome the inhibitory effect of GA on flower formation, the LFY transcription factor activates the expression of *ELA1*, an enzyme involved in the catabolism of these hormones. Therefore, an increase in *LFY* expression (Fig. 2.6A) leads to a reduction in the concentration of GA (Fig. 2.2), as we observed in the buds of the OFF trees. Consequently, the low level of GA in the meristem allows the accumulation of DELLA proteins, which are involved in the upregulation of the *AP1* gene (Yamaguchi et al., 2014). Our results confirm that, at least at the meristem level, GA inhibit flower formation in woody species as they do in Arabidopsis. It remains paradoxical that GA have a positive effect on the switch to reproductive development (flowering time) in most annual plants, but a negative effect in woody species.

Finally, our results link the role of GA, both endogenous and exogenous, to the upregulation of *CsCEN*, whose expression is localized in the vegetative leaf primordia (Fig. 2.11). The expression of *CsCEN* in indeterminate axillary meristems not only maintains stem cell activity but also represses axillary bud outgrowth to regulate tree shoot architecture (Zhang et al., 2021).

Given the link between auxin and GA in other physiological processes, such as fruit set (Bermejo et al., 2015), it was hypothesised whether auxin and GA have a coordinated effect in inhibiting floral differentiation. First, we tested whether 2,4-D applied to the OFF tree had a direct effect on inhibiting differentiation or whether it did so by stimulating GA synthesis. Unexpectedly, 2,4-D inhibited GA biosynthesis in both the *GA20ox* and *GA3ox* rate-limiting steps (Fig. 3.6), which differs from previous results (Frigerio et al., 2006; Dorcey et al., 2009). However, it needs to be confirmed whether this is a direct or indirect effect, as it was only recorded at one sampling date one month after treatment.

On the other hand, both 2,4-D and GA_3 significantly reduced auxin synthesis (*YUCCA4*, *TRN2*) and transport (*PIN3*, *LAX3*) in the buds (Fig. 3.7). The effect of GA has already been shown in other plant systems, for example in rice root tips, where GA regulates local auxin biosynthesis and polar auxin transport by modulating the expression of *OsYUCCA6* and *PIN* (Li et al., 2020). The effect of 2,4-D can be similar to the apical dominance mechanism (Beveridge et al., 2023). Thus, the high concentration of 2,4-D in the stem might suppress auxin synthesis and export capacity in axillary buds, reducing cell division (*CYCB2*; Fig. 3.7E) and the expression of *LFY* and *AP1* (Figs. 3.5A, B). Although in the apical dominance model endogenous GA acts downstream of auxin efflux to regulate bud release (Beveridge et al., 2023) through the control of cell division (Nelissen et al., 2012; Mesejo et al., 2016), in our experiment GA_3 treatment acted as a primary signal inhibiting *PIN3* in the bud.

Overall, the results may partially explain the auxin-GA regulation of bud differentiation in citrus. Thus, the primary signal would be the high auxin level in the stem (caused by exogenous applications of 2,4-D or by the fruit), which limits the synthesis and efflux transport of IAA in the axillary buds, suggesting that the high auxin gradient generated by the fruit (dominant organ) limits the capacity for synthesis and transport of IAA from the axillary buds (dominated organ), reducing their capacity for floral differentiation (Bangerth 2000; Haim et al., 2021). Given that both 2,4-D treatments and fruit reduce the expression of *LFY* (floral fate) and *CYCB2* (cell division), and that in citrus bud differentiation coincides with bud release, do auxins regulate (negatively) floral fate, bud release or both? Or instead, is the absence of the FT protein the primary regulatory signal? In either case, *LFY* inhibition leads to a downstream increase in GA synthesis (secondary signal) and indeterminate vegetative growth development.

The fact that auxins and *LFY* initiate flower meristem development in Arabidopsis (Yamaguchi et al., 2013; Denay et al., 2017), together with the critical examination of our data, suggests that it must be the same mechanism of flower meristem regulation in citrus, but on a different time scale compared to Arabidopsis (Fig. D1). Our model supports the view that the absence of FT protein in the bud, rather than fruit-derived auxin, is the reason for maintaining the vegetative state of the meristem.

During the vegetative bud phase, *LFY* is inhibited by *TFL1* and *FD* (probably in both ON and OFF buds), and the fruit inhibits bud development by high stem auxin concentrations (Fig. D1, and Haim et al., 2021). At the time of FI (early November) in OFF trees, *CiFT3* expression is upregulated and the FT protein recruits FD and allows *LFY* to be up-regulated and determine flower fate (Yamaguchi, 2021). However, at this stage, the IAA concentration in the stem does not differ between ON and OFF trees, although a higher IAA concentration is found in the buds of ON trees (Fig. D1). Our experiments cannot confirm that this difference in IAA levels is the cause of the *LFY* block, but the increase in IAA synthesis, efflux transport, *LFY* expression, IAA bud concentration and flowering in the buds of OFF trees along the flower determination and primordium formation stages suggests the opposite (Fig. D1), as occurs in Arabidopsis (Yamaguchi et al., 2013 Denay et al., 2017). Indeed, there is no evidence to suggest that meristematic control of flower development differs between species, quite the opposite. Further evidence for this is the *LFY* - DELLA correlation in flowering OFF buds (Fig. 2.7), as occurs in Arabidopsis (Yamaguchi et al., 2014).

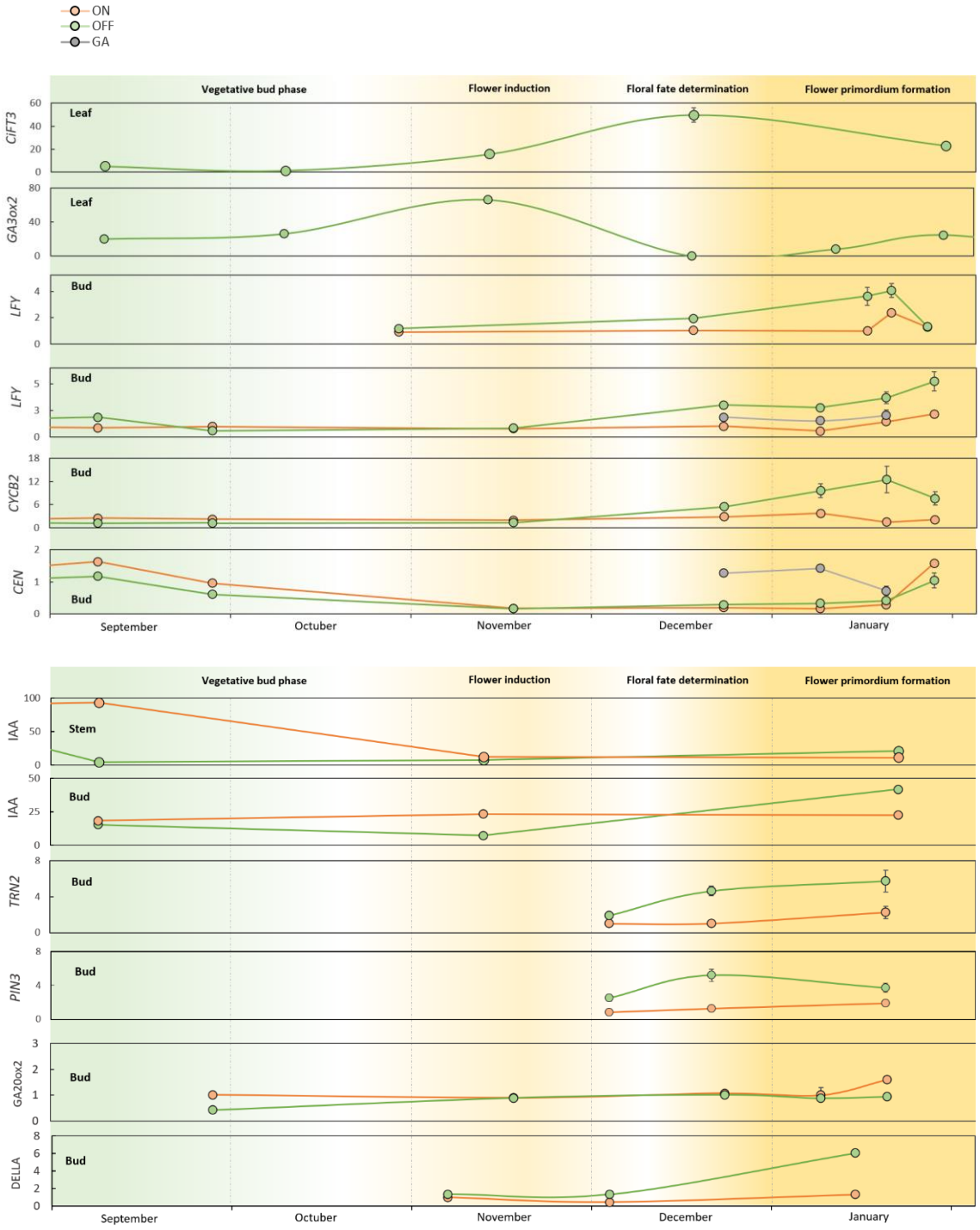


***Figure Discussion 1**. A model for the role of auxin and gibberellin in the regulation of citrus flowering in ON and OFF trees. The figures are selected results from all the experiments developed in this PhD thesis, most of them using Citrus sinensis as plant material. The vegetative buds phase was considered to be the period of time without CiFT3 and CsLFY expression; the flower induction phase was consider to start at the onset of CiFT3 expression; the floral fate determination phase was consider to start at the onset of CsLFY expression; the flower primordium formation phase was considered to be the period between maximum CsLFY expression and bud release.*

4. Double girdling but no single girdling promotes FI

In the absence of a consistent relationship between the fruit, the action of GA and auxins in leaves and the stimulation of the transcription factor *CcMADS19*, disrupting phloem transport between fruit and leaves/buds by girdling was proposed to 1) disrupt the fruit signal and promote flowering, and 2) analyze the gene response to find primarily repressed inhibitory signals.

Surprisingly, single girdling in summer, either apical or basal, only stimulated vegetative bud sprouting, whereas double girdling (apical and basal) stimulated flowering, suggesting different mechanisms of action. In fact, the summer apical girdling allowed us to explain the epigenetic resetting of *CcMADS19* in the OFF tree. Fruit promotes H3K4 trimethylation of *CcMADS19* in nearby leaves to inhibit the response to floral inductive signals in winter, but repressive H3K27me3 is maintained in buds (Agustí et al., 2020). In spring, the new leaves formed during bud release maintain the repressed state of *CcMADS19* thanks to increased *CcVIN3* activity, allowing them to respond to subsequent floral inductive cold temperatures. Summer apical-girdling advances bud release to early autumn instead of the following spring, and the new leaves are ready to respond to cold temperatures in the immediate winter, causing the induction of *CiFT3*. These results are consistent with the mode of flowering regulation proposed for *Arabidopsis* (monocarpic) and *Arabis alpina* (polycarpic), in which *FLC* and *PEP1* (*FLC* homolog) silencing is promoted by vernalisation and mediated by H3K27me3 (De Lucía et al., 2008; Wang et al., 2009). A striking finding was that whereas in *Arabidopsis VIN3* induction is triggered by exposure to low temperature (Hepworth et al., 2020) and modulated by the circadian clock (Hepworth et al., 2018), in *Citrus*, *CcVIN3* expression would be associated with a developmental phase transition, such as the resumption of vegetative growth from dormant buds.

Double-girdled shoots flowered like the OFF tree, as did buds from shoots with small (undeveloped) fruits. RNA-seq analysis at the FI time point reveals different behaviour of these 3 flowering phenotypes in leaves and buds. Whereas in leaves there is a greater similarity between the two phenotypes bearing a fruit (AB-G and SF), in buds

there is a greater similarity between the two most flowering phenotypes (OFF and AB-G). Moreover, the behaviour between leaves and buds is also quantitatively different, since in buds the 3 phenotypes share 39% of the DEGs, whereas in leaves they share only 4.1% (Figs. 4.15B & 4.18B).

All this suggests that the 3 phenotypes have different mechanisms for regulating flowering, especially for the process of FI in the leaves. Indeed, in this case, the three phenotypes shared only one transcription factor related to ABA and stress, and none related to flowering time, according to the TFs prediction analysis. In particular, this result is consistent with previous omics studies showing oxidoreductase activity and stress responses in ON vs. OFF 'Moncada' mandarin leaves (Muñoz-Fambuena et al. 2013).

In the buds, however, the three phenotypes shared important TFs of the flowering process, such as *AP1* and *SOC1*, as well as other TFs related to the hormonal response. In addition, the study in the buds of the 2429 DEGs shared by the three comparisons revealed interesting results indicating how the three flowering phenotypes (OFF, AB-G, SF) differ from the non-flowering phenotype (ON). The differences observed in cell division, some hormonal pathways and primary metabolism of the buds stand out. All three processes showed a similar pattern of regulation between OFF, AB-G and SF with respect to ON shoots. In the case of hormones, the main pathway affected was the auxin-related pathway (Fig. 4.22), which showed changes both in homeostasis and, more importantly, in polar transport. The signalling pathways of ABA and JA, and to a lesser extent GA and CK, were also altered. The pathways of carbohydrate production and transport were also differentially expressed at several regulatory sites.

The RT-qPCR and metabolomic analyses show on the one hand that double girdling is able to induce *CiFT3* expression in the leaf, independent of low temperatures. The effect of the treatment is immediate, both in the leaf and in the bud, and alters carbohydrate metabolism. It was found that: 1) it increased the net photosynthetic rate (Fig. 4.38C); and 2) it increased starch hydrolysis in the leaf (upregulation of the α-AMY enzyme; Fig. 4.38A). and bud, with the upregulation of α-AMY being extended over time (74 DAT; Fig. 4.37D); 3) it increased sucrose hydrolysis in the bud (upregulation of

cytoplasmic enzymes like *CIN* and *SUS1*; Ruan et al., 2014); and 4) all these responses were likely triggered in response to the carbohydrate deficit state, given the upregulation of the SnRK1 protein complex, which signals a carbohydrate deficit (Robaglia et al., 2012; Fig. 4.37A, B, C). Expression was also upregulated in genes involved in polar auxin transport (*PIN3*; Fig. 4.32), cell division (*CYCB2*; Fig. 4.27H), floral differentiation (*LFY* and *AP1*; Fig. 4.27F, G), and GA catabolism (*GA2ox*; Fig. 4.33).

Overall, although further studies are needed, double girdling appears to work through nutritional stress and independently of cold temperature. The method confirms in buds the major role of auxins and sugar metabolism during the period of bud differentiation. Not flowering buds in ON trees, show reduced efflux auxin transport resulting in a reduction in cell division compared to flowering buds in OFF trees. As a result, ON buds remain in a state of basal metabolism, with reduced energy-producing processes such as glycolysis and starch hydrolysis, and reduced sucrose transport. The results support the view previously proposed in the model (Fig. D1) and most of the hypotheses proposed by other authors (Bangerth 2000, 2009; Goldschmidt and Sadka, 2021; Haim et al., 2021).

Conclusions

1. Treatment with GA_3 during the flowering induction period does not increase the expression of the flowering repressor *CcMADS19* but reduces the expression of the transcription factor *NF-YA1* in the leaf. In the bud, GA_3 increases *CEN* expression regardless of the fruit and the time of application. The response is species independent.

2. Fruit does not activate GA synthesis in the leaf, but in the bud, together with *CEN* expression. *CEN* expressed at leaf primordia in vegetative bud.

3. High levels of auxin in the stem (from fruit or 2,4-D treatments) inhibit auxin synthesis and efflux transport in the bud, which is inversely correlated with flowering.

4. In the buds of the ON trees, the repressive H3K27me3 marks of the *CcMADS19* locus are transmitted to the newly emerging leaves, which can induce flowering in the next OFF season.

5. Double girdling activates *CiFT3* expression in the leaf. In the bud, it increases cell division, auxin synthesis and transport, carbohydrate consumption and *CsLFY* expression, whereas it decreases gibberellin synthesis and *CsCEN* expression. All isolated buds from fruit influence between the apical and basal girdling can flower the following spring.

References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., ... & Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science*, *309*(5737), 1052-1056.

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

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

Agustí, M., García-Marí, F., & Guardiola, J. L. (1982). The influence of flowering intensity on the shedding of reproductive structures in sweet orange. *Scientia Horticulturae*, *17*(4), 343-352.

Agustí, M., Mesejo, C., Muñoz-Fambuena, N., Vera-Sirera, F., de Lucas, M., Martínez-Fuentes, A., ... & Blázquez, M. A. (2020). Fruit-dependent epigenetic regulation of flowering in *Citrus*. *New Phytologist*, *225*(1), 376-384.

Agustí, M., Reig, C., Martínez-Fuentes, A., & Mesejo, C. (2022). Advances in citrus flowering: A review. *Frontiers in Plant Science*, *13*, 868831.

Alquézar, B., Zacarías, L., & 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.

Atay, A. N., & Koyuncu, F. (2016). Manipulating regular bearing in '*Golden Delicious'*/M9 apple trees using GA4+ 7 and ethephon. *International Journal of Fruit Science*, *16*(1), 10-22.

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

Bangerth, K. F. (2006). Nature and significance of correlative hormonal signals in growth and development of annual and perennial plants. In *XXVII International Horticultural Congress-IHC2006: International Symposium on Endogenous and Exogenous Plant Bioregulators 774* (pp. 379-390).

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.

Bermejo, A., Granero, B., Mesejo, C., Reig, C., Tejedo, V., Agustí, M., ... & Iglesias, D. J. (2018). Auxin and gibberellin interact in citrus fruit set. *Journal of plant growth regulation*, *37*, 491-501.

Beveridge, C. A., Rameau, C., & Wijerathna-Yapa, A. (2023). Lessons from a century of apical dominance research. *Journal of Experimental Botany*, *74*(14), 3903-3922.

Blázquez, M. A., Soowal, L. N., Lee, I., & Weigel, D. (1997). *LEAFY* expression and flower initiation in Arabidopsis. *Development*, *124*(19), 3835-3844.

Beveridge, C. A., Rameau, C., & Wijerathna-Yapa, A. (2023). Lessons from a century of apical dominance research. *Journal of Experimental Botany*, *74*(14), 3903-3922.

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

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., & Coen, E. (1997). Inflorescence commitment and architecture in *Arabidopsis*. *Science*, *275*(5296), 80-83.

Chan, B. G., & Cain, J. C. (1966). A rapid quantitative method for routine determination of monosaccharides and oligosaccharides from plants by paper chromatography. *Journal of Chromatography A*, *22*, 95-101.

Chen, C. N., Chu, C. C., Zentella, R., Pan, S. M., & David Ho, T. H. (2002). *AtHVA22* gene family in *Arabidopsis*: phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. *Plant molecular biology*, *49*, 631-642.

Chica, E. J., & 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.

Cho, L. H., Yoon, J., & An, G. (2017). The control of flowering time by environmental factors. *The Plant Journal*, *90*(4), 708-719.

Collani, S., Neumann, M., Yant, L., & Schmid, M. (2019). FT modulates genome-wide DNA-binding of the bZIP transcription factor FD. *Plant Physiology*, *180*(1), 367-380.

Corbesier, L., Lejeune, P., & Bernier, G. (1998). The role of carbohydrates in the induction of flowering in *Arabidopsis thaliana*: comparison between the wild type and a starchless mutant. *Planta*, *206*, 131-137.

Crevillén, P., Yang, H., Cui, X., Greeff, C., Trick, M., Qiu, Q., ... & Dean, C. (2014). Epigenetic reprogramming that prevents transgenerational inheritance of the vernalized state. *Nature*, *515*(7528), 587-590.

Dag, A., Bustan, A., Avni, A., Tzipori, I., Lavee, S., & 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.

Das, A., Geetha, G. A., Ravishankar, K. V., Shivashankara, K. S., Roy, T. K., & Dinesh, M. R. (2019). Interrelations of growth regulators, carbohydrates and expression of flowering genes (*FT, LFY, AP1*) in leaf and shoot apex of regular and alternate bearing mango (*Mangifera indica* L.) cultivars during flowering. *Scientia Horticulturae*, *253*, 263-269.

Dastkar, E., Soleimani, A., Jafary, H., de Dios Alche, J., Bahari, A., Zeinalabedini, M., & Salami, S. A. (2020). Differential expression of genes in olive leaves and buds of ON-versus OFF-crop trees. *Scientific Reports*, *10*(1), 15762.

Davis, L. D. (1957). Flowering and alternate bearing. In *Proc. Amer. Soc. Hort. Sci* (Vol. 70, No. 545, p. 56).

De Lucia, F., Crevillen, P., Jones, A. M., Greb, T., & Dean, C. (2008). A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of *FLC* during vernalization. *Proceedings of the National Academy of Sciences*, *105*(44), 16831-16836.

Delgado, R., Casamayor, R., Rodriguez, J. L., Cruz, P., & Fajardo, R. (1985, September). Paclobutrazol effects on oranges under tropical conditions. In *V International Symposium on Growth Regulators in Fruit Production 179* (pp. 537-544).

Denay, G., Chahtane, H., Tichtinsky, G., & Parcy, F. (2017). A flower is born: an update on *Arabidopsis* floral meristem formation. *Current opinion in plant biology*, *35*, 15-22.

Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Nicolaï, M., ... & Meyer, C. (2007). The *Arabidopsis TOR* kinase links plant growth, yield, stress resistance and mRNA translation. *EMBO reports*, *8*(9), 864-870.

Dolgikh, V. A., Pukhovaya, E. M., & Zemlyanskaya, E. V. (2019). Shaping ethylene response: the role of *EIN3/EIL1* transcription factors. *Frontiers in Plant Science*, *10*, 1030.

Dorcey, E., Urbez, C., Blázquez, M. A., Carbonell, J., & Perez-Amador, M. A. (2009). Fertilization-dependent auxin response in ovules triggers fruit development through the modulation of gibberellin metabolism in *Arabidopsis*. *The Plant Journal*, *58*(2), 318-332.

Durand, J. B., Allard, A., Guitton, B., Van de Weg, E., Bink, M. C., & Costes, E. (2017). Predicting flowering behavior and exploring its genetic determinism in an apple multi-family population based on statistical indices and simplified phenotyping. *Frontiers in Plant Science*, *8*, 858.

El-Otmani, M., Coggins Jr, C. W., Agustí, M., & Lovatt, C. J. (2000). Plant growth regulators in citriculture: World current uses. *Critical reviews in plant sciences*, *19*(5), 395-447.

Elsysy, M. A., & Hirst, P. M. (2017). The role of spur leaves, bourse leaves, and fruit on local flower formation in apple: An approach to understanding biennial bearing. *HortScience*, *52*(9), 1229-1232.

Endo, T., Shimada, T., Fujii, H., & Omura, M. (2006). Cloning and characterization of 5 MADS-box cDNAs isolated from citrus fruit tissue. *Scientia horticulturae*, *109*(4), 315-321.

Fang, Q., Wang, Q., Mao, H., Xu, J., Wang, Y., Hu, H., ... & Luo, K. (2018). AtDIV2, an RR-type *MYB* transcription factor of *Arabidopsis*, negatively regulates salt stress by modulating ABA signaling. *Plant Cell Reports*, *37*, 1499-1511.

Ferrándiz C (2001). Non-Radioactive *In Situ* Hybridization In *Arabidopsis*-A Laboratory Manual. (Weigel, D., and Glazebrook, J. eds), pp 195-203. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor*. Sessions A.

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

Fonseca, S., Chico, J. M., & Solano, R. (2009). The jasmonate pathway: the ligand, the receptor and the core signalling module. *Current opinion in plant biology*, *12*(5), 539-547.

Frigerio, M., Alabadí, D., Pérez-Gómez, J., García-Cárcel, L., Phillips, A. L., Hedden, P., & Blázquez, M. A. (2006). Transcriptional regulation of gibberellin metabolism genes by auxin signaling in Arabidopsis. *Plant physiology*, *142*(2), 553-563.

Gambetta, G., Martínez-Fuentes, A., Bentancur, O., Mesejo, C., Reig, C., Gravina, A., & 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*, 273-282.

Garcia-Luis, A., Fornes, F., & Guardiola, J. L. (1986). Effects of gibberellin A3 and cytokinins on natural and post-harvest, ethylene-induced pigmentation of Satsuma mandarin peel. *Physiologia Plantarum*, *68*(2), 271-274.

Garcia-Luis, A., Fornes, F., & Guardiola, J. L. (1995). Leaf carbohydrates and flower formation in *Citrus*. *Journal of the American Society for Horticultural Science*, *120*(2), 222-227.

García-Luís, A., Kanduser, M., Santamarina, P., & Guardiola, J. L. (1992). Low temperature influence on flowering in *Citrus*. The separation of inductive and bud dormancy releasing effects. *Physiologia Plantarum*, *86*(4), 648-652.

Goldberg-Moeller, R., Shalom, L., Shlizerman, L., Samuels, S., Zur, N., Ophir, R., ... & 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., Aschkenazi, N., Herzano, Y., Schaffer, A. A., & Monselise, S. P. (1985). A role for carbohydrate levels in the control of flowering in citrus. *Scientia Horticulturae*, *26*(2), 159-166.

Goldschmidt, E. E., & Golomb, A. (1982). The Carbohydrate Balance of Alternate-bearing *Citrus* Trees and the Significance of Reserves for Flowering and Fruiting1. *Journal of the American Society for Horticultural Science*, *107*(2), 206-208.

Goldschmidt, E. E., & Sadka, A. (2021). Yield alternation: horticulture, physiology, molecular biology, and evolution. *Horticultural reviews*, *48*, 363-418.

Goldschmidt, E. E., Tamim, M., & Goren, R. (1997). Gibberellins and flowering in citrus and other fruit trees: A critical analysis. In *VIII International Symposium on Plant Bioregulation in Fruit Production 463* (pp. 201-208).

Gottschalk, C., Zhang, S., Schwallier, P., Rogers, S., Bukovac, M. J., & van Nocker, S. (2021). Genetic mechanisms associated with floral initiation and the repressive effect of fruit on flowering in apple (*Malus x domestica Borkh*). *Plos one*, *16*(2), e0245487.

Gravina, A., Arbiza, H., Ferenczi, A., Orlando, L., Severino, V., Gambetta, G., ... & Agustí, M. (2000). Estudio del comportamiento alternante de la naranja "*Salustiana*" [*Citrus sinensis* (L.) osb.] en diferentes condiciones productivas. *Agrociencia-Sitio en Reparación*, *4*(1), 17-22.

Guardiola, J. L., Agustí, M. & García-Marí, F. 1977. Gibberellic acid and flower bud development in sweet orange. *Proc. Int. Soc. Citriculture*, 696–699.

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

Guitton, B., Kelner, J. J., Celton, J. M., Sabau, X., Renou, J. P., Chagné, D., & Costes, E. (2016). Analysis of transcripts differentially expressed between fruited and deflowered '*Gala*' adult trees: a contribution to biennial bearing understanding in apple. *BMC Plant Biology*, *16*(1), 1-22.

Haberman, A., Ackerman, M., Crane, O., Kelner, J. J., Costes, E., & Samach, A. (2016). Different flowering response to various fruit loads in apple cultivars correlates with degree of transcript reaccumulation of a *TFL 1*-encoding gene. *The Plant Journal*, *87*(2), 161-173.

Haberman, A., Bakhshian, O., Cerezo-Medina, S., Paltiel, J., Adler, C., Ben-Ari, G., ... & Samach, A. (2017). A possible role for flowering locus T-encoding genes in interpreting environmental and internal cues affecting olive (*Olea europaea* L.) flower induction. *Plant, Cell & Environment*, *40*(8), 1263-1280.

Haim, D., Shalom, L., Simhon, Y., Shlizerman, L., Kamara, I., Morozov, M., ... & Sadka, A. (2021). Alternate bearing in fruit trees: fruit presence induces polar auxin transport in citrus and olive stem and represses IAA release from the bud. *Journal of Experimental Botany*, *72*(7), 2450-2462.

Halevy, A. H., Monselise, S. P., & Plaut, Z. (1964). Effects of gibberellin on translocation and on dry matter and water content in several plant species. *Physiologia plantarum*, *17*(1), 49-62.

Hepworth, J., Antoniou-Kourounioti, R. L., Berggren, K., Selga, C., Tudor, E. H., Yates, B., ... & Dean, C. (2020). Natural variation in autumn expression is the major adaptive determinant distinguishing Arabidopsis *FLC* haplotypes. *Elife*, *9*, e57671.

Hepworth, J., Antoniou-Kourounioti, R. L., Bloomer, R. H., Selga, C., Berggren, K., Cox, D., ... & Dean, C. (2018). Absence of warmth permits epigenetic memory of winter in *Arabidopsis*. *Nature communications*, *9*(1), 639.

Hoijemberg, M., & Cerdán, P. D. (2020). Epigenetic accounting of a previous harvest. *New Phytologist*, *225*(1), 10-12.

Hussain, S. B., Guo, L. X., Shi, C. Y., Khan, M. A., Bai, Y. X., Du, W., & Liu, Y. Z. (2020). Assessment of sugar and sugar accumulation-related gene expression profiles reveal new insight into the formation of low sugar accumulation trait in a sweet orange (*Citrus sinensis*) bud mutant. *Molecular biology reports,* 47, 2781-2791.

Jones, W. W., Coggins Jr, C. W., & Embleton, T. W. (1977). Growth regulators and alternate bearing. In *Proc. Int. Soc. Citricult* (Vol. 2, pp. 657-660).

Koshita, Y., Takahara, T., Ogata, T., & 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*(3-4), 185-194.

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

Lee, E. J., Matsumura, Y., Soga, K., Hoson, T., & Koizumi, N. (2007). Glycosyl hydrolases of cell wall are induced by sugar starvation in *Arabidopsis*. *Plant and cell physiology*, *48*(3), 405-413.

Li, Z., Li, B., Liu, J., Guo, Z., Liu, Y., Li, Y., ... & Dong, A. (2016). Transcription factors *AS1* and *AS2* interact with *LHP1* to repress *KNOX* genes in *Arabidopsis*. *Journal of Integrative Plant Biology*, *58*(12), 959-970.

Li, C. Y., Weiss, D., & Goldschmidt, E. E. (2003). Girdling affects carbohydrate-related gene expression in leaves, bark and roots of alternate-bearing citrus trees. *Annals of Botany*, *92*(1), 137-143.

Li, J., Yang, Y., Chai, M., Ren, M., Yuan, J., Yang, W., ... & Fan, H. (2020). Gibberellins modulate local auxin biosynthesis and polar auxin transport by negatively affecting flavonoid biosynthesis in the root tips of rice. *Plant Science*, *298*, 110545.

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

Lord, E. M., & Eckard, K. J. (1985). Shoot development in *Citrus sinensis* L. (Washington navel orange). I. Floral and inflorescence ontogeny. *Botanical Gazette*, *146*(3), 320-326.

Mahmoud, L. M., Vincent, C. I., Grosser, J. W., & Dutt, M. (2021). The response of salt-stressed Valencia sweet orange (*Citrus sinensis*) to salicylic acid and methyl jasmonate treatments. *Plant Physiology Reports*, *26*, 137-151.

Martínez-Alcántara, B., Iglesias, D. J., Reig, C., Mesejo, C., Agustí, M., & 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., & 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., Muñoz-Fambuena, N., Reig, C., González-Mas, M. C., Iglesias, D. J., ... & Agustí, M. (2013). Fruit load restricts the flowering promotion effect of paclobutrazol in alternate bearing *Citrus* spp. *Scientia Horticulturae*, *151*, 122-127.

Martínez-Fuentes, A., Mesejo, C., Reig, C., & 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.

Mason, M. G., Ross, J. J., Babst, B. A., Wienclaw, B. N., & Beveridge, C. A. (2014). Sugar demand, not auxin, is the initial regulator of apical dominance. *Proceedings of the National Academy of Sciences*, *111*(16), 6092-6097.

Matsoukas, I. G., Massiah, A. J., & Thomas, B. (2012). Florigenic and antflorigenic signaling in plants. *Plant and Cell Physiology*, *53*(11), 1827-1842.

Mandel, MA., Gustafson-Brown, C., Savidge, B., & Yanofsky, M. F. (1992). Molecular characterization of the Arabidopsis floral homeotic gene *APETALA1*. *Nature, 360*(6401), 273-277.

Mesejo, C., Martínez-Fuentes, A., Reig, C., & Agustí, M. (2022). Ringing branches reduces fruitlet abscission by promoting *PIN1* expression in '*Orri*' mandarin. *Scientia Horticulturae*, *306*, 111451.

Mesejo, C., Marzal, A., Martínez-Fuentes, A., Reig, C., & Agustí, M. (2021). On how auxin, ethylene and IDA-peptide relate during mature *Citrus* fruit abscission. *Scientia Horticulturae*, *278*, 109855.

Mesejo, C., Martínez-Fuentes, A., Reig, C., & Agustí, M. (2022). Ringing branches reduces fruitlet abscission by promoting PIN1 expression in 'Orri'mandarin. *Scientia Horticulturae, 306, 111451.*

Mesejo, C., Marzal, A., Martínez-Fuentes, A., Reig, C., de Lucas, M., Iglesias, D. J., ... & Agustí, M. (2022). Reversion of fruit-dependent inhibition of flowering in Citrus requires sprouting of buds with epigenetically silenced *CcMADS19*. *New Phytologist*, *233*(1), 526-533.

Mesejo, C., Yuste, R., Reig, C., Martínez-Fuentes, A., Iglesias, D. J., Muñoz-Fambuena, N., ... & Agustí, M. (2016). Gibberellin reactivates and maintains ovary-wall cell division causing fruit set in parthenocarpic *Citrus* species. *Plant Science*, *247*, 13-24.

Milyaev, A., Kofler, J., Moya, Y. A. T., Lempe, J., Stefanelli, D., Hanke, M. V., ... & Wünsche, J. N. (2022). Profiling of phytohormones in apple fruit and buds regarding their role as potential regulators of flower bud formation. *Tree Physiology*, *42*(11), 2319-2335.

Monselise, S. P. (1979). The use of growth regulators in citriculture; a review. *Scientia Horticulturae*, *11*(2), 151-162.

Monselise, S. P., & Goldschmidt, E. E. (1982). Alternate bearing in fruit trees. *Horticultural reviews*, *4*(1), 128-173.

Muñoz-Fambuena, N., Mesejo, C., Agustí, M., Tárraga, S., Iglesias, D. J., Primo-Millo, E., & González-Mas, M. C. (2013). Proteomic analysis of "*Moncada*" mandarin leaves with contrasting fruit load. *Plant Physiology and Biochemistry*, *62*, 95-106.

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

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

Muñoz-Fambuena, N., Nicolas-Almansa, M., Martínez-Fuentes, A., Reig, C., Iglesias, D. J., Primo-Millo, E., ... & Agustí, M. (2019). Genetic inhibition of flowering differs between juvenile and adult *Citrus* trees. *Annals of botany*, *123*(3), 483-490.

Moss, G. I. (1971). Effect of fruit on flowering in relation to biennial bearing in sweet orange (*Citrus sinensis*). *Journal of Horticultural Science*, *46*(2), 177-184.

Na, X., Jian, B., Yao, W., Wu, C., Hou, W., Jiang, B., ... & Han, T. (2013). Cloning and functional analysis of the flowering gene *GmSOC1-like*, a putative *SUPPRESSOR OF OVEREXPRESSION CO1/AGAMOUS-LIKE 20 (SOC1/AGL20)* ortholog in soybean. *Plant cell reports*, *32*, 1219-1229.

Nakagawa, M., Honsho, C., Kanzaki, S., Shimizu, K., & 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.

Nelissen, H., Rymen, B., Jikumaru, Y., Demuynck, K., Van Lijsebettens, M., Kamiya, Y., ... & 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.

Nishikawa, F., Endo, T., Shimada, T., Fujii, H., Shimizu, T., Omura, M., & 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.

Nunes, C., O'Hara, L. E., Primavesi, L. F., Delatte, T. L., Schluepmann, H., Somsen, G. W., ... & Paul, M. J. (2013). The trehalose 6-phosphate/SnRK1 signaling pathway primes growth recovery following relief of sink limitation. *Plant physiology*, *162*(3), 1720-1732.

Ollas, C. D., Arbona, V., Zandalinas, S. I., & Gómez-Cadenas, A. (2012). Jasmonic acid signals citrus response to drought. *Levante Agricola*, *51*(410), 105-109.

Pelaz, S., Ditta, G. S., Baumann, E., Wisman, E., & Yanofsky, M. F. (2000). B and C floral organ identity functions require *SEPALLATA MADS-box* genes. *Nature*, *405*(6783), 200-203.

Pillitteri, L. J., Lovatt, C. J., & Walling, L. L. (2004). Isolation and Characterization of *LEAFY* and *APETALA1* Homologues from *Citrus sinensis* L. Osbeck Washington'. *Journal of the American Society for Horticultural Science*, *129*(6), 846-856.

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

Regnault, T., Davière, J. M., Wild, M., Sakvarelidze-Achard, L., Heintz, D., Carrera Bergua, E., ... & Achard, P. (2015). The gibberellin precursor GA_{12} acts as a long-distance growth signal in *Arabidopsis*. *Nature Plants*, *1*(6), 1-6.

Robaglia, C., Thomas, M., & Meyer, C. (2012). Sensing nutrient and energy status by SnRK1 and TOR kinases. *Current opinion in plant biology*, *15*(3), 301-307.

Rodrigues, C. M., Müdsam, C., Keller, I., Zierer, W., Czarnecki, O., Corral, J. M., ... & Pommerrenig, B. (2020). Vernalization alters sink and source identities and reverses phloem translocation from taproots to shoots in sugar beet. *Plant Cell*, *32*(10), 3206-3223.

Roessner, U., Wagner, C., Kopka, J., Trethewey, R. N., & Willmitzer, L. (2000). Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry. *the plant journal*, *23*(1), 131-142.

Roxrud, I., Lid, S. E., Fletcher, J. C., Schmidt, E. D., & Opsahl-Sorteberg, H. G. (2007). GASA4, one of the 14-member *Arabidopsis* GASA family of small polypeptides, regulates flowering and seed development. *Plant and Cell Physiology*, *48*(3), 471-483.

Ruan, Y. L. (2014). Sucrose metabolism: gateway to diverse carbon use and sugar signaling. *Annual review of plant biology*, *65*, 33-67.

Ruan, J., Zhou, Y., Zhou, M., Yan, J., Khurshid, M., Weng, W., ... & Zhang, K. (2019). Jasmonic acid signaling pathway in plants. *International journal of molecular sciences*, *20*(10), 2479.

Sadka, A., Walker, C. H., Haim, D., & Bennett, T. (2023). Just enough fruit: understanding feedback mechanisms during sexual reproductive development. *Journal of Experimental Botany*, *74*(8), 2448-2461.

Sanchez-Capuchino, J. A., & Casanova, R. (1973). Induccion floral en mandarinos Clementina sin hueso y Satsuma. In *First International Congress Citriculture* (Vol. 2, pp. 223-225).

Seifert, R., & Wenzel-Seifert, K. (2002). Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn-Schmiedeberg's archives of pharmacology*, *366*, 381-416.

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.

Schneider, E. F. (1968). The rest period of Rhododendron flower buds I. Effect of the bud scales on the onset and duration of rest. *Journal of Experimental Botany*, *19*(4), 817-824.

Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Doron-Faigenboim, A., Blumwald, E., & 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.

Sheldon, C. C., Rouse, D. T., Finnegan, E. J., Peacock, W. J., & Dennis, E. S. (2000). The molecular basis of vernalization: the central role of *FLOWERING LOCUS C (FLC). Proceedings of the National Academy of Sciences*, *97*(7), 3753-3758.

Soppe, W. J., Vinegra de la Torre, N., & Albani, M. C. (2021). The diverse roles of *FLOWERING LOCUS C* in annual and perennial Brassicaceae species. *Frontiers in Plant Science*, *12*, 627258.

Southwick, S. M., Weis, K. G., Yeager, J. T., & Zhou, H. (1995). Controlling cropping in peach using gibberellin: effects on flower density, fruit distribution, fruit firmness, fruit thinning, and yield. *Journal of the American Society for Horticultural Science*, *120*(6), 1087-1095.

Sung, S. K., Yu, G. H., Nam, J., Jeong, D. H., & An, G. (2000). Developmentally regulated expression of two MADS-box genes, *MdMADS3* and *MdMADS4*, in the morphogenesis of flower buds and fruits in apple. *Planta*, *210*, 519-528.

Tamas, I. A., Engels, C. J., Kaplan, S. L., Ozbun, J. L., & Wallace, D. H. (1981). Role of indoleacetic acid and abscisic acid in the correlative control by fruits of axillary bud development and leaf senescence. *Plant Physiology*, *68*(2), 476-481.

Tang, L., & Lovatt, C. J. (2019). Effects of low temperature and gibberellic acid on floral gene expression and floral determinacy in '*Washington*' navel orange (*Citrus sinensis* L. Osbeck). *Scientia horticulturae*, *243*, 92-100.

Valiente, J. I., Albrigo, L. G., & Beck, H. W. (2004). Testing a Flowering Expert System for the 'Decision Information System for Citrus'. In *VII International Symposium on Modelling in Fruit Research and Orchard Management 707* (pp. 17-24).

Wahl, V., Ponnu, J., Schlereth, A., Arrivault, S., Langenecker, T., Franke, A., ... & Schmid, M. (2013). Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis* thaliana. *Science*, *339*(6120), 704-707.

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

Waters, M. T., Scaffidi, A., Sun, Y. K., Flematti, G. R., & Smith, S. M. (2014). The karrikin response system of *Arabidopsis*. *The Plant Journal*, *79*(4), 623-631.

Whittaker, C., & Dean, C. (2017). The *FLC* locus: a platform for discoveries in epigenetics and adaptation. *Annual Review of Cell and Developmental Biology*, *33*, 555-575.

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

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

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

Yamaguchi, N., Wu, M. F., Winter, C. M., Berns, M. C., Nole-Wilson, S., Yamaguchi, A., ... & Wagner, D. (2013). A molecular framework for auxin-mediated initiation of flower primordia. *Developmental cell*, *24*(3), 271-282.

Zhang, F., Wang, Y., & Irish, V. F. (2021). CENTRORADIALIS maintains shoot meristem indeterminacy by antagonizing THORN IDENTITY1 in Citrus. *Current Biology*, *31*(10), 2237-2242.

Zhao, D., Yu, Q., Chen, M., & Ma, H. (2001). The ASK1 gene regulates B function gene expression in cooperation with UFO and LEAFY in Arabidopsis.

Zhong, C., Xu, H., Ye, S., Wang, S., Li, L., Zhang, S., & Wang, X. (2015). Gibberellic acid-stimulated Arabidopsis6 serves as an integrator of gibberellin, abscisic acid, and glucose signaling during seed germination in Arabidopsis. *Plant Physiology*, *169*(3), 2288-2303.

Zhou, H., Duan, H., Liu, Y., Sun, X., Zhao, J., & Lin, H. (2019). Patellin protein family functions in plant development and stress response. *Journal of plant physiology*, *234*, 94-97.

Zhou, H., Zeng, R. F., Liu, T. J., Ai, X. Y., Ren, M. K., Zhou, J. J., ... & Zhang, J. Z. (2022). Drought and low temperature-induced NF-YA1 activates FT expression to promote citrus flowering. *Plant, Cell & Environment*, *45*(12), 3505-3522.

Zhu, Y., Klasfeld, S., Jeong, C. W., Jin, R., Goto, K., Yamaguchi, N., & Wagner, D. (2020). TERMINAL FLOWER 1-FD complex target genes and competition with FLOWERING LOCUS T. *Nature communications*, *11*(1), 5118.

Ziv, D., Zviran, T., Zezak, O., Samach, A., & 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. *PloS one*, *9*(10), e110613.