UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Genetic and Molecular Basis of Flowering Time in Almond

Bases Genéticas y Moleculares de la Época de Floración en Almendro

Dª Ángela Sánchez Prudencio

2019

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

TESIS DOCTORAL

Bases genéticas y moleculares de la época de floración en almendro

Genetic and molecular basis of flowering time in almond

Doctoranda

Ángela Sánchez Prudencio

Directores

Dr. Pedro Martínez-Gómez

Dr. Federico Dicenta López-Higuera

Murcia, 2019

Agradecimientos

El Ministerio de Economía y Competitividad hizo posible el desarrollo de la presente Tesis Doctoral mediante la concesión de la beca de Formación de Personal Investigador (FPI) BES-2014-070554, asociada al proyecto "Mejora genética del almendro" (AGL2013-48577-C2-1-R). Así mismo, la estancia breve EEBB-I-18-12749, financiada por la misma entidad, ha contribuido a la formación investigadora de la doctoranda, permitiendo el desarrollo de su actividad en la Universidad de La Coruña (España).

A título personal, me gustaría agradecer a todas las personas a las que voy a hacer mención a continuación su contribución a este trabajo:

En primer lugar, a mis directores, Federico Dicenta y Pedro Martínez-Gómez, por depositar en mí su confianza y respetar mi ritmo de trabajo, así como por la paciencia que han tenido conmigo a la hora de organizar información y de comunicar resultados. Gracias por darme la libertad para aportar ideas y apoyo para desarrollarlas.

Gracias a Encarna, por su amabilidad y generosidad, y por su colaboración en la propuesta de nuevos experimentos.

Agradezco sobre todo a la familia del Grupo de Mejora Genética de Frutales del CEBAS su cariño, su compañía y su ayuda cuando lo he necesitado. Gracias Manolo por implicarte en las actividades de tus compañeros y aportar calidad a su trabajo. Gracias David y Pepe por vuestro asesoramiento en los temas de acumulación de frío y salida del letargo. Tere y Alberto, gracias por ser mi guía en las salidas al campo y por los mil favores que me habéis hecho, y sobre todo por vuestra comprensión y consejos.

Gracias Jesús, nuestro *variety manager*, por su alegría y su energía y por echarme un cable cuando me hizo falta.

A Pedro J. le agradezco haber aportado con su aptitud y herramientas mucho respecto al análisis de la salida del letargo. A Raquel, por haber llegado como un soplo de aire fresco, por ser inyección de motivación y haberme dado recursos para investigar.

¿Qué decir de las dos incondicionales que siempre han estado conmigo? Eva y Eli, habéis sido mis madres, mis hermanas y hasta mi pareja llegado al caso. La amistad con vosotras ha sido fundamental en el trabajo y fuera de él.

Jorge, gracias por todo lo que me has enseñado, por tu confianza y sobre todo por tu sentido del humor. Gracias a ti la experiencia en el CEBAS y en Murcia ha sido especial.

Bea, gracias por tener siempre una palabra de ayuda cuando he acudido a ti. Ha sido una suerte contar con la experiencia de una persona tan profesional.

Agradezco a esas chicas del CEBAS, con las que compartí buenos momentos en las comidas y los cafés del día a día, que me arroparan cuando era "la nueva": Ana, Irene, Sara, Mariajo... Chu, gracias por todo lo que compartimos mientras estuviste en Murcia. Elena y Gema, habéis sido mucho más que compañeras y amigas fuera del trabajo, y gracias a vosotras me he sentido muy querida.

Gracias a mucha más gente que me ha acompañado en este camino:

A Mery, por su apoyo constante y su franqueza, sobre todo en los momentos difíciles. Gracias.

A Margarida, por aprender tan rápido, por su trabajo y por su actitud vital en el laboratorio.

A Jesús, Juan y Víctor, por ser unos excelentes compañeros y mejores personas.

A mis compañeros de departamento Pepe y Pedro, por su colaboración en este trabajo y su incondicional simpatía. A Lorenzo, Nuria, Lydia y Lola, por su ayuda y sus consejos.

A Olaf y Rosa, por su disposición e implicación siempre que hemos trabajado juntos.

A la profesora Mª Esperanza Cerdán Villanueva, a Mónica Lamas Maceiras y a todos los compañeros de laboratorio que me recibieron con los brazos abiertos en el grupo EXPRELA de la Universidad de La Coruña: Juanjo, María, Ana, Aida, Agustín… con vosotros no dejé de aprender conocimientos y valores.

A Frank Hoeberichts y Peter Balk, por su compromiso con nuestro trabajo y su ayuda en el análisis transcriptómico.

A todos los que me habéis demostrado que no puedo hacerlo sola.

A mis padres por ser principio y ejemplo. Gracias por apoyarme, entenderme y cuidarme. Gracias Dani, por tu valor y sacrificio. Gracias Ale, por tu coraje y seguridad. Gracias Marcos, por haber venido. Gracias Manuel, por lo que has sido. Gracias a todos aquellos que siguen siendo mis amigos.

A Marcos:

Por darme razones

GENERAL INDEX **Page**

FIGURE INDEX **Page**

TABLE INDEX

ABBREVIATIONS

5mC	5´ Methylated Cytosine
ABA	Abscisic acid
ABC	ATP BINDING CASSETTE
AFP	ABI5-BINDING PROTEIN
AGL	AGAMOUS-LIKE
AIP2	ABI3 INTERACTING PROTEIN 2
ARF	AUXIN RESPONSE FACTOR
ARP4	AUXIN RELATED PROTEIN 4
ATHB12	ARABIDOPSIS THALIANA HOMEOBOX-LEUCINE ZIPPER PROTEIN 12
AWPM19	ABA-INDUCED WHEAT PLASMA MEMBRANE 19
bHLH	Basic helix-loop-helix
Bp	Base pair
BR	Brassinosteroid
CBF	C-REPEAT BINDING FACTOR
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CCAA	Cooperativas Agro-Alimentarias
4CL	4-COUMARATE:CoA LIGASE
CO	CONSTANS
CP	Chill Portion
CR	Chilling requirement
CV	Coefficient of Variation
CytP450	Cytochrome P-450
DAM	DORMANCY ASSOCIATED MADS-BOX
DEG	Differentially Expressed Gene
DMF	Differentially Methylated Fragment
DMG	Differentially Methylated Gene
DREB2c	DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2c
ELF3	EARLY FLOWERING 3
EMF2	EMBRYONIC FLOWER 2
epiGBS	epi-Genotyping By Sequencing
EXO7	EXORDIUM 7
FAR1	FAR-RED IMPAIRED RESPONSE 1
FLC	FLOWERING LOCUS C
FT	FLOWERING LOCUS T
GAs	Gibberellins
GA2ox	GIBBERELLIN 2-OXIDASE
GA20ox	GIBBERELLIN 20-OXIDASE
GDH	Growing Degree Hour
GDSL	Gly, Asp, Ser, and Leu consensus aminoacid motif sequence
GH	Glucosyl Hydrolase
GMQE	Global Model Quality Estimate

GO	Gene Ontology
GPI	Glycerophosphatidylinositol
H3K27me3	Trymethylation of Histone 3 on Lis (K) residue 27
HR	Heat requirement
HSP	HYDROPHOBIC SEED PROTEIN
IPCC	Intergovernmental Panel on Climate Change
Lb	*Late blooming*
LBs	Lipid Bodies
LEA	LATE EMBRYOGENESIS ABUNDANT
LG	LINKAGE GROUP
LHY	LATE ELONGATED HYPOCOTYL
LOX3.1	LINOLEATE 13S-LIPOXYGENASE 3-1
LRR-TIR	LEUCINE RICH REPEAT-TOLL INTERLEUKIN 1 RECEPTOR
MAP	MITOGEN-ACTIVATED PROTEIN
MIZ1	MIZU-KUSSEI 1
NF-Y	HISTONE LIKE TRANSCRIPTION FACTOR NF-Y
NGS	Next Generation Sequencing
PBL15	PROBABLE SERINE/THREONINE-PROTEIN KINASE 15
PIE1	PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1
PcG	Polycomb Group
POX	Peroxidase
PRCII	POLYCOMB REPRESSIVE COMPLEX II
PTM	PostTranslational histone Modifications
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
QMEAN	Qualitative Model Energy ANalysis
QTL	Quantitative Trait Loci
RGA1	RESISTANCE GENE ANALOGUE 1
RPKM	Reads Per Kilobase per Million reads
ROS	Reactive Oxygen Species
RS5	RAFFINOSE SYNTHASE 5
SA	Salicylic Acid
SAM	S-Adenosyl Methionine
SEA	Singular Enrichment Analysis
SNP	Single Nucleotide Polymorphism
SVP	SHORT VEGETATIVE PHASE
SSH	Supressive Substractive Hybridization
SSR	Single Sequence Repeat
SOC1	SUPPRESSOR OF CONSTANS 1
SUVH5	SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG PROTEIN 5
SWR1	SWI2/SNF2-RELATED 1
TE	TRANSPOSABLE ELEMENT
Tm	Melting temperature
TSS	TRANSCRIPTIONAL START SITE
UTR	UNTRANSLATED REGION
WSD1	WAX ESTER SYNTHASE/DIACYLGLYCEROL ACYLTRANSFERASE 1

1. INTRODUCTION

1. INTRODUCTION

1.1. Almond culture

Almond, a temperate tree crop cultivated around the world, is a very important crop in Spain, closely linked to the Mediterranean landscape and culture. It has been traditionally cultivated under dry conditions, implanted in marginal lands and with poor care by farmers.

However, due to the recent increase of the prices of almond at international level, almond culture is having an amazing expansion, not only in Spain but also in all the producer countries. The efficient horticultural practices in irrigated orchards and the introduction of new cultivars better adapted to the climatic conditions of each area are allowing growers to obtain a high productivity, low costs of production and so profitable benefits.

In this new context, research, experimentation and transference activities are required for the release of new almond cultivars adapted to each area, that position almond cultivation as a profitable option.

1.1.1. Taxonomy, botany and origin of the almond species

The almond (*Prunus dulcis* (Mill.) D. A. Webb) is a deciduous stone fruit species belonging to the *Rosaceae* family. Its taxonomic classification is as follows:

Kingdom: *Plantae*
Division: *Spermatophyta*
Subdivision: *Angiospermae*
Class: *Magnoliopsida*
Order: *Rosales*
Family: *Rosaceae*
Subfamily: *Prunoidea*
Genus: *Prunus*
Subgenus: *Amygdalus* (L.) Focke
Species: *Prunus dulcis* (Mill.) D.A. Webb

The term *Prunus dulcis* has been used by several botanists from Spach (1843) to Graselly (1976) for almond designation. In 1964, the General Committee for botanical nomenclature decided that the official name would be *P. dulcis* (Mill.) D.A. Webb. In addition, *P. amygdalus* (Batsch) and *P. communis* (L.) Archang. are accepted as synonyms (Punt, 1964).

Morphologically, almond is a tree with a different size depending on the cultivar, soil and horticultural practices. The leaves are alternate and lanceolate and flowers are grouped or isolated. The almond flower is hermaphrodite, white or rose depending on the genotype. After successful pollination, almond fruit starts growing during the spring and maturation takes place in summer (Figure 1.1).

The almond fruit is oval-shaped with a tip, and it is classified as a drupe with a pubescent skin (exocarp), a fleshy but thin hull (mesocarp), and a hardened shell (endocarp) that contains the seed (kernel), made up of the embryo and the testa. The fruit grows during development and the hull opens and dries at maturity. The mature endocarp hardness ranges from soft to hard depending on the genotype. Horticulturally, almonds are classified as a "nut" in which the edible seed (the kernel) is the commercial product (Figure 1.1).

Figure 1.1. The almond production cycle. A. Flowering and pollination. B. Fruit development. C. Fruit maturation (opened mesocarp). D. Edible seeds (almonds).

Early researchers proposed that cultivated almond resulted from selection within a species listed originally as *Amygdalus communis* L., based on studies of two natural populations containing large numbers of sweet seeded individuals, rather than the bitter ones typically found in the wild almond species (Watkins, 1979).

One population is located in the Kobet Dag mountain range in central Asia (between Iran and Turkmenistan) and the other in the lower slopes of the Tian Shan Mountains (between Kyrgyzstan and western China) (Vavilov, 1931; Grasselly, 1976). The natural range of distribution would extend and overlapped with known sites of early almond cultivation, and distinction between cultivated and wild forms gradually disappeared (Denisov, 1988; Kester et al., 1991).

However, because the variable kernel phenotype of almond, the emerging consensus is that cultivated forms possibly derived from *Prunus fenzliana* Fritsch through natural hybridizations with a range of wild species including *Prunus bucharica* (Korsh.) Hand.-Mazz., *Prunus kuramica* (Korsh.) Kitam. and *Prunus triloba* Lindl (Ladizinsky, 1999; Zeinalabedini et al., 2010). According to this view, the Kobet Dag and Tian Shian populations would come from later domesticated orchards (Kester et al., 1991; Graselly and Crossa Raynaud, 1980; Socias i Company, 2002). In addition, Felipe (2000) indicated that *P. fenzliana* and *P. bucharica* are middle-late flowering species.

The subsequent diffusion of cultivated almond occurred in three main stages (Figure 1.2):

1. Asiatic. The Asiatic stage included the initial domestication and expansion along major prehistoric trade routes (Grasselly, 1976). The range centers on present-day Iran extending east to western China, northwest India, northern Pakistan, northwest through Turkey and southwest into the uplands and deserts of central Israel and Syria. Almond culture continues to the present time in these areas, mainly under dryland and subsistence agricultural practices similar to those used 2,000 BCE (Gradziel, 2009).

2. Mediterranean. Cultivated almonds appear to have been brought into Greece prior to 300 BCE and subsequently they were introduced in other regions bordering the climate-moderating Mediterranean Sea (Bacarella et al., 1991). Subsequent introductions occurred in 500-600 CE with the conquest of North Africa by Arabs who also brought almonds into southern Spain and Portugal (Gradziel, 2009). In these regions, orchards were established in specific locations where well-defined ecotypes have evolved (Muncharaz, 2004).

3. New World. Along the 18th and 19th century, almonds were distributed to North America, South America and Australia (Kester et al., 1990; Gradziel and Kester, 1996). Hard-shelled germplasm was brought from Spain and later France introduced soft-shelled material. In California, almond production was moved from marginal coasts to Central Valley. High-input orchard practices, together with favourable soil and climatic conditions and water abundance made California the top almond producer worldwide (Gradziel, 2009). Nowadays, 'Nonpareil' is the most abundant cultivar, often used in breeding programs as genitor.

Almond culture was later introduced in Australia and South America (Chile and Argentina overall), and it is cultivated in Mediterranean zones under the Californian production system (Gradziel, 2011). The vegetative multiplication of local cultivars in the 20th century allowed the stabilization of plant material, that constitutes the starting material of breeding programs.

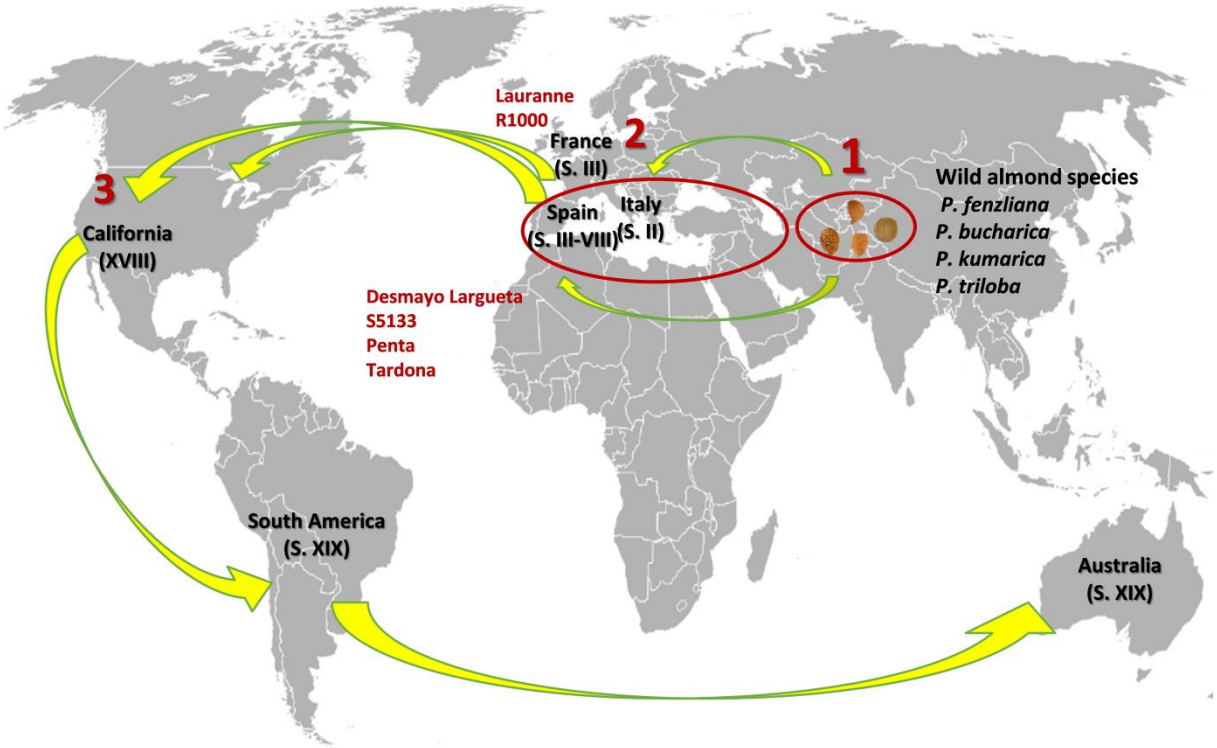


Figure 1.2. Origin of the cultivated almond (*P. dulcis*) and diffusion phases (1, 2, 3). Wild almond species and the century of introduction (between parenthesis) are indicated. Almond cultivars involved in this Thesis are also indicated in red. (Adapted from Martínez-Gómez et al., 2017).

1.1.2. Distribution and economical importance of almond

Due to its great variability almond is nowadays cultivated in a wide variety of ecological niches. Almonds are cultivated in more than 50 countries, with a 95% of production located in California, Australia and the Mediterranean basin (http://faostat.fao.org).

Regarding the surface, according to the Food and Agriculture Organization of the United Nations (FAO), in 2017 there was 1,925,887 ha dedicated to almond cultivation in the world. Spain is the first country with 633,562 ha, representing 33% of the global total, followed by USA (21%), Tunisia (9.4%) and Morocco (9%). However, despite our large surface cultivated, Spain is still the third world producer due to the lower productivity of the Spanish orchards (100 kg/ha on average) compared to that of the Californian and Australian (2,500 kg/ha), due to cultivation techniques and irrigation.

Production of almond in Spain was of 62,027 t of kernel in 2018, according to Cooperativas Agro-Alimentarias (CCAA) (http://www.agro-alimentarias.coop). Aragón, Castilla La Mancha and Andalucía were the top regional producers, followed by Comunidad Valenciana and Región de Murcia (Figure 1.3). The establishment of new large irrigated orchards in Spain will rise our production to 100,000 t in the following years.

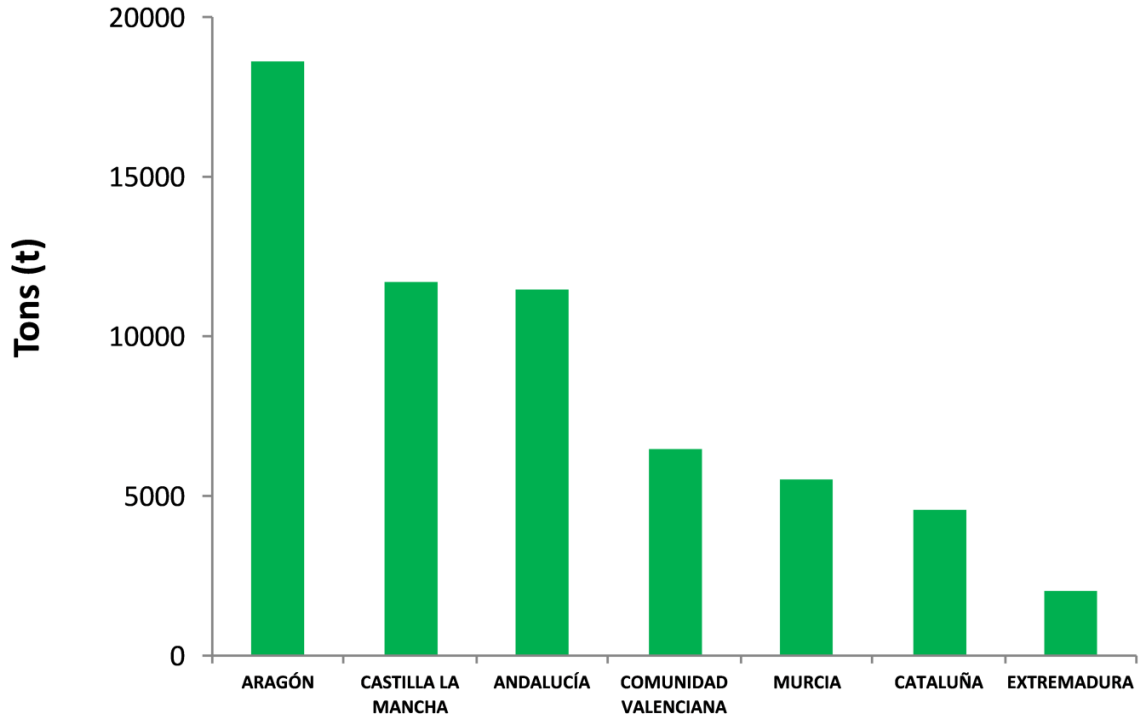


Figure 1.3. Spanish almond production (t) in 2018 by regions.

1.1.3. Food uses and applications of almonds

The almond kernel is consumed either in the natural state or processed, and it has many food uses. Kernels may be roasted or fried in oil. The processed kernel can be used blanched, and it is combined with chocolate in confectionery, also sliced in pastry and ground into paste for bakery products or marzipan (Gradziel, 2009). Additionally, almond kernel oil can be used for cosmetics.

Although almond consumption is largely based on sweet cultivars, bitter and slightly bitter almonds are used for marzipan and amaretto liquors (Dicenta and García, 1993). Bitterness results from hydrolysis of amygdalin, which produces benzaldehyde (conferring the "amaretto" flavour) and the toxic compound cyanide (Sánchez-Pérez et al., 2008). Bitter kernels are usual in wild almond species to protect the seed against herbivores. The domestication of wild bitter to cultivated sweet almonds was elucidated by the sequencing of the almond genome, finding that a single mutation in a basic helix-loop-helix (bHLH) transcription factor switched off the accumulation of amygdalin in sweet almonds (Sánchez-Pérez et al., 2019).

Historically, almonds were consumed as fresh and processed food, and it is nowadays considered as a functional food with both nutritional and health properties. Almonds are among the most dietetic nuts. They are a good source of essential fatty acids, (between 50-60% of kernel weight, the oleic acid representing 65-80%), vitamins (mainly vitamin E and folic acid), minerals and fibre (Saura-Calixto et al., 1981; Schirra, 1997; Sabate and Haddad, 2001). Almond consumption is associated to the prevention of coronary disease, cancer and cataracts (Fulgoni et al., 2002; Gradziel and Martínez-Gómez, 2013) with anti-inflammatory and hypocholesterolaemic properties (Poonam et al., 2011; Musa-Veloso et al., 2016). On the other hand, almonds are not highly allergenic to consumers (Sathe et al., 2001). In addition, the uses of sweet or bitter almond ointment included the treatment of asthma and the use in soothing salves for burns (Gradziel, 2009).

1.1.4. Factors affecting almond production

Productivity of the almond tree is mainly determined by the plant material, culture practices and environmental conditions (García, 1986). Selection of the plant material includes both the cultivar and the rootstock. Both determine production, fruit quality and cultivation costs. The rootstock must be compatible with the cultivar and adapted to the type of soil (permeability, minerals and pathogens presence) and irrigation system.

Suitable culture practices are equally important. Orchard designing, pruning, fertilization, weeds control, pesticides treatments and irrigation. In Mediterranean countries almond has been traditionally considered a rainfed species. However, the production is directly related to water availability. In non-irrigated orchards, low rainfall drastically reduces yield and almond quality (Egea et al., 2010). Moderate to high rainfall, together with introduction of early ripening cultivars, which ripen before the driest summer season, can improve production and quality.

Environmental conditions also affect almond production. The main limiting factor in most of areas in Spain (and other countries) is the late frost. During the winter, dormant buds are very resistant to low temperatures, but open flowers and mainly young fruits are highly susceptible to frosts and can be seriously damaged. During flowering, bad weather (wind, low temperatures, fog and rain) difficults the pollination of flowers by insects, delays anthers dehiscence and reduces successful fertilization of flowers for fruit set. The use of late flowering cultivars self-compatible is recommended for minimizing both problems in cold areas (Martínez-García et al., 2011 and Dicenta et al., 2016).

1.1.5. Almond breeding

For the above mentioned factors that limit almond production in cold areas, late flowering and self-compatibility together with a high production and fruit quality (size, shape, absence of double kernels, etc.) are desirable traits for almond breeding programs in Spain. Other important traits for breeders are pest and disease resistance and drought tolerance. Obviously, sweet kernel is a compulsory characteristic.

Classical almond breeding is a long and tedious process, similar to that of other fruit species, affected by its woody nature and the long juvenile period. For this reason, breeders must have a rigorous and extensive knowledge of the species, the culture, and the genetic control of the most important traits, to design the new cultivars. The new cultivars released will have an impact in the sector (nurseries and growers) many years later since they were created by breeders. The minimum time to release a new almond cultivar could be 10 years.

Once fixed the objectives of breeding, the first key decision of the breeder is the selection of the genotypes that will be used as genitors. This election will determine the success or failure of the breeding program. Breeder usually crosses two cultivars with complementary characteristics to obtain a new cultivar with the most interesting traits of both genitors. Transgressive seedlings (with lower or higher

values than genitors) for the main traits are usually selected to advance more and more in the improvement of these traits.

The first desirable trait is a high productivity. For this, a high floral density and a good fruit set rate have to be assured. In addition, floral and fruit production must be stable year by year, which depends on the cultivar, but also on culture practices.

In cold areas, late flowering is very important to escape the late frost since the risk of frost decreases along the season. Self-compatibility allows that flowers can be fertilized by its own pollen. This trait allows an easier orchard management, based on mono-varietal plantations (Dicenta et al., 2000; Ortega and Dicenta, 2004). Early ripening is also important in these areas, to avoid the harvest under cold and wet conditions of October. Ripening time and flowering time are quite independent traits, so extra-late flowering and early ripening like 'Penta' and 'Makako' cultivars can be released.

The first almond breeding programs were developed in the USA (1925) and Russia (1932). Later on, other programs were carried out in the Mediterranean basin, with important results in France, Italy and Spain (Kester and Gradziel, 1996). Finally, Australia joined to the list of almond breeders at the end of the XX century (Wirthensohn and Sedgley, 2001). Nowadays, the most important breeding programs are located in the USA (California), Spain (Zaragoza, Reus and Murcia) and Australia (Adelaide).

Almond breeding was initiated at the University of California Davis (UC Davis) and a high number of new cultivars were released. Some of them are selections of 'Nonpareil', 'Texas' ('Mission'), 'Ne Plus Ultra' and 'Peerless' (Kester et al., 1984), whereas others came from breeding programs, like 'Butte', 'Ruby' and 'Sweet Heart' (Gradziel et al., 2001; Gradziel and Martínez-Gómez, 2013). Other cultivars from private companies are 'Shasta' or 'Independence', being the first commercial self-compatible American almond cultivars. The main objective of the almond breeding program at UC Davis is currently the release of cultivars compatible with 'Nonpareil' (like 'Sonora' and 'Winters'), as well as self-compatible cultivars, pest and disease resistant (Gradziel and Kester, 1994; 1998; 1999; Gradziel et al., 2007).

In Spain, there are three different breeding programs carried out in Aragón, Región de Murcia and Cataluña. Self-compatibility and late flowering are the primary common objectives.

The research on almond was initiated in Zaragoza in 1966 in the current Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). The breeding program was started in 1974 (Felipe and Socias i Company, 1985). The first releases

were the cultivars 'Guara', 'Aylés' and 'Moncayo' (Felipe and Socias I Company, 1987), later 'Blanquerna', 'Cambra' and 'Felisia' (Socias i Company and Felipe, 1999) and more recently 'Soleta', 'Belona', 'Mardía' and 'Vialfas' (Socias i Company and Felipe, 2007; Socias i Company et al., 2008; Segura et al., 2016). Recently, the analysis by Single Sequence Repeat (SSR) markers has shown that 'Guara' is actually the Italian cultivar 'Tuono' (Dicenta et al., 2015), as it was shown with 'Supernova' (Marchese et al., 2008).

The almond breeding program developed at Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), in Murcia was started in 1971 (Egea et al., 1985; Egea and García, 1988). First crossings were made from an 81 varieties collection, with the objective of maintaining the characteristics of the autochthonous cultivars (productivity, quality kernel and hard shell) and introducing self-compatibility and late-flowering. The first cultivars released were 'Antoñeta' and 'Marta' (Egea et al., 2000). Later, 'Penta' and 'Tardona' the first extra-late flowering cultivars, were released (Dicenta et al., 2009). 'Tardona' is the latest flowering almond cultivar released so far. More recently, the extra-late cultivar 'Makako' has been registered (Dicenta et al., 2017a).

In Catalonia, the almond breeding program started in 1975 at the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), in Reus. The first cultivars obtained were 'Masbovera', 'Glorieta' and 'Francolí' (Vargas et al., 1984; Vargas and Romero, 1993) and later were released 'Constantí', 'Vairo', 'Marinada' and 'Tarraco' (Vargas et al., 2006).

Finally, the most recent almond breeding program began in 1995 at the University of Adelaide. The main objectives are self-compatibility and good kernel quality (Bertozzi et al., 1998; Sedgley and Collins, 2002). Spanish parents as 'Marta' and 'Antoñeta' from CEBAS-CSIC (Murcia) have been used for these breeding purposes.

1.1.6. Perspectives of almond culture in Spain

The almond culture has undergone a vertiginous change in our country in the last years. The rise in almond prices, consequence of the high international demand, has caused a never-known interest in this crop.

Traditional growers have begun to invest in their plantations to improve the production and large companies from the almond sector, or from other sectors, are investing in almond culture, establishing large modern farms with irrigation and

yields similar to those of the Californians. Traditional rainfed almond farming will have to look for the benefit of organic farming, for example, in order to compete with the low production costs of large farms.

All this has been possible thanks to the release of the new self-compatible cultivars with late and extra-late flowering time, which can currently be grown in any Spanish region, with a low risk of frost. The development of cultivation techniques adapted to our highly variable edaphoclimatic conditions has also contributed to this new situation.

Due to the expansion of the crop in our country and the increase of productivity of the orchards, it is predictable that Spanish production will soon exceed 100,000 t, and that it will continue to grow exponentially in the coming years. Given the imminent climate change, the emergence of new pests and diseases and the increasing consumer demand, breeders have a great job to do releasing new varieties adapted to these new challenges mainly in aspects related to bud dormancy and flowering.

1.2. Dormancy in temperate fruit trees

Phenology is defined as the sequential developmental stages of the annual growth cycle and their timing. Thus, the key developmental stages of the annual growth are phenological events as growth cessation, dormancy, dormancy release and bud burst (sprouting and flowering) (Singh et al., 2017). Phenological states from endodormancy to flowering of flower buds were described by Felipe (1977) (see Figure 2.6 in Chapter 2).

Both flower and vegetative almond buds begin their development as vegetative buds. By mid-summer the potential floral buds undergo a transition to the reproductive state, a process marked by the enlargement of the shoot apical meristem. The floral whorls are initiated sequentially during late summer (Lamp et al., 2001; Imani et al., 2012). Almond flower buds can be presented on axial nodes or in spurs, and can be differentiated from vegetative buds by shape. Vegetative buds are conical, whereas flower buds are egg-shaped (Figure 1.4). Differentiation of flower buds is not possible until late summer, and may require bud dissection and microscopy (Polito et al., 2002). The formation of terminal apical buds, prior to termination of cell proliferation, is a major indicator of the dormancy beginning of trees (Hamilton et al., 2016).

Figure 1.4. Almond bud formations. Vegetative (V) buds are indicated by arrows.

1.2.1. Dormancy phases

Dormancy has been defined as a temporary suspension of visible growth of any plant structure containing a meristem (Campoy, 2009). Many types of plant dormancy have been described, regarding seed and bud dormancy (Dennis, 1994). Dormancy period is a critical developmental phase of some plants of temperate climates, in which the plant must be protected from the cold injuries of the winter (Vitasse et al., 2014; Ding and Nilsson, 2016).

Almond harvest usually occurs between August and September. Dormancy period is established during summer and remains during the winter. Once dormancy is overcome, flowering takes place. Depending on cultivar and region, almond flowering in Spain ranges from January to April (Figure 1.5).

According to Lang (1987), dormancy can be divided in three sequential phases: Paradormancy, endodormancy and ecodormancy (Figure 1.5). In paradormancy, the bud growth is inhibited by other buds within the tree. During endodormancy, growth inhibition is controlled by the inhibited bud meristem itself. Finally, during ecodormancy maintenance depends on environmental cues, and the bud will be able to restart its active growth under favourable conditions.

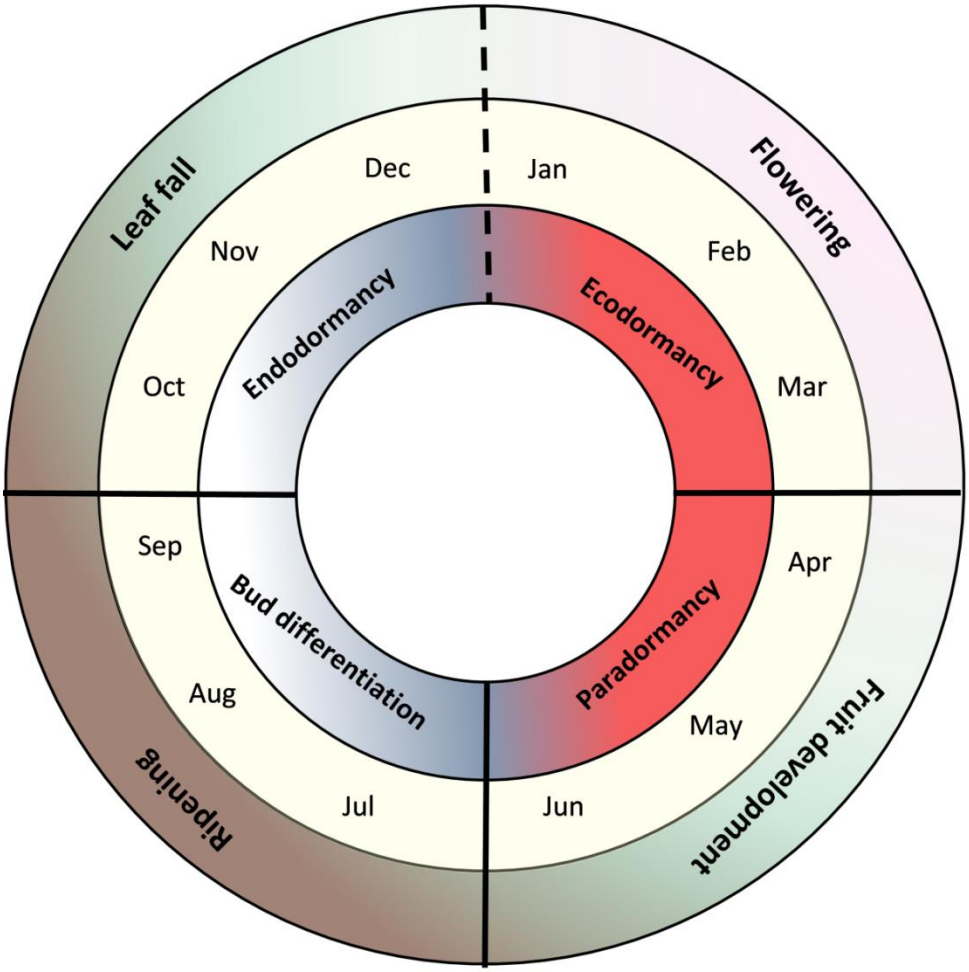


Figure 1.5. Almond annual life cycle. From outer to inner: Phenological events, months, and physiological processes. Schedule of phenology is approximated and can vary depending on the cultivar and area. Dashed line indicates endodormancy to ecodormancy transition.

Paradormancy

According to Champagnat (1983), dormancy starts with paradormancy, what consist on the inhibition of axillary buds growth by the apical dominance of terminal bud (Hillman, 1984). Paradormancy gradually becomes endodormancy (Figure 1.5) what is also known as dormancy induction phase.

Apical dominance is based on the acropetal circulation of nutrients, water, minerals and growth promoters as cytokinins because of the auxin synthesis by the leaves at shoot apex. This phenomenon implies a gradient in dormancy depth that decreases basipetally (Arias and Crabbé, 1975). Thus, dormancy depth is variable

within the three and dormancy state can be assessed in three different levels: whole tree, shoots or single node cuttings.

Endodormancy

Endodormancy is established in the late-summer and the deepest point is reached in autumn (beginning of November in Spain) although this characteristic depends on climatic factors, the species and even the cultivar studied (Figure 1.5).

Factors affecting induction and depth of endodormancy

The induction and depth of endodormancy depends on environmental and endogenous factors.

- Environmental factors:

The most important environmental factors affecting tree dormancy induction and depth are photoperiod, temperature and horticultural practices.

Photoperiod is necessary for endodormancy establishment in a wide range of tree species (Arora et al., 2003; Ruttink et al., 2007). However, response to photoperiod is variable within members from the *Rosaceae* family, and it depends on the interaction with temperature (Heide and Prestud, 2005; Heide, 2008; Cook, 2005).

Summer high temperatures promote and intensify endodormancy (Jonkers, 1979; Heide, 2003), as well as falling temperatures of the autumn (Hatch and Walker, 1969; Ben Ismail, 1989; Cook et al., 2005).

On the other hand, horticultural practices can also affect bud endodormancy, depending on the moment of application. If the case of pruning, if the shoot apex is removed too late, the effect on endodormancy release of axillary buds will be low. Regarding defoliation, if it is performed before endodormancy establishment, the removal of growth inhibitors synthetized in leaves can alleviate dormancy state (Mielke and Dennis, 1978; Erez, 1982).

- Endogenous factors

Hormone content and tree characteristics are the main endogenous factors affecting endodormancy establishment and depth.

Abscisic Acid (ABA) is generally considered a growth inhibitor (Addicot, 1983). ABA synthesis is promoted upon endodormancy induction, probably by ethylene hormone (Rodrigo et al., 2006). Then, ABA content decreases concomitantly with endodormancy progression (Tamura et al., 1992; Horvath et al., 2008). Moreover, exogenous ABA application prevented endodormancy release of sour cherry (*Prunus cerasus* L.) and peach (*Prunus persica* (L.) Batsch) (Mielke and Dennis, 1978). The effect of ABA in endodormancy maintenance is probable mediated by other molecules, as C-REPEAT/DRE BINDING FACTORS (CBFs) and DEHYDRATION-RESPONSIVE-ELEMENT BINDING (DREB) transcription factors (Lata and Prasad, 2011; Rubio et al., 2018) that regulate expression of genes responsive to cold and other abiotic stresses (Wisniewski et al., 2006) (see Chapters 1.2.4 and 5).

On the other hand, gibberellins are growth promoter molecules that have been traditionally used to stimulate buds to growth and to measure dormancy depth. High levels of gibberellins (GAs) are required for endodormancy release (Ramsay and Martin, 1970; Tamura et al., 1992).

Finally, auxin levels decrease during endodormancy while rise in ecodormancy (Bennet and Skoog, 1938; Rodríguez and Sánchez-Tamés, 1986; Aloni and Peterson, 1997).

Regarding tree characteristics, vigour and age increase endodormancy depth. Moreover, Tromp (1976) showed that vegetative vigour, induced by high temperatures, can inhibit floral initiation. The branch position and orientation is also important (Crabbé, 1984) due to hormone balance. For grafted trees, if the rootstock used is low-compatible, endodormancy release can be advanced (Erez, 2000).

Endodormancy release

Low temperatures are needed for endodormancy overcoming. The concept of chilling requirement was introduced to define the environmental conditions controlling endodormancy release. Chilling requirement consist on the amount of chill accumulated necessary for endodormancy release, and this trait is dependent on the species and cultivar, even on the type of bud (floral or vegetative).

The first to observe the relationship between dormancy and low temperatures was Coville (1920). Different models for chill accumulation have been proposed (see Chapter 1.2.2). Although these models can predict when chilling requirements will be satisfied, there is little information on the physiological basis of this process.

In this regard, endodormancy has been conceptualized by two different schools:

- The classical school proposed a model based on the balance of growth promoters and inhibitors (hormones) (Amen, 1968; Seeley, 1990) that has remained aside.
- The French school focused on a "morphogenetic factor" dependent on different correlative influences and beginning from paradormancy (Champagnat, 1983).

Regardless of the conceptualization of endodormancy, this is considered a quantitative state that is released progressively. For this reason, endodormancy release is established according to an arbitrary criterion to estimate chilling requirement and to compare results between species and cultivars.

Several methods for study the endodormancy release have been proposed (Dennis, 2003; Fadón and Rodrigo, 2018). One of them is to expose plant material (single-node cuttings, branches) to forcing conditions (Seeley, 1996). Such conditions of temperature (23-25°C) and relative humidity (40-60%) allow the phenological evolution of buds. The criterion selected for defining endodormancy release is diverse (Felker and Robitaille, 1985). Endodormancy release can be considered when 50% of buds shows a green tip (Tabuenca, 1964) or when buds gain 30% of weight or reach the Baggioglini stage (Baggioglini, 1952) (see Chapter 2.2).

During flower bud endodormancy, cell division, enlargement, and differentiation are taking place (Reinoso et al., 2002; Fadón and Rodrigo 2018). Some biological processes as tetrad formation during microsporogenesis in apricot (Julian et al., 2011), xylem vessels differentiation in apricot and peach (Bartolini et al., 2006; Andreini et al., 2012; Viti et al., 2013) and carbohydrates hydrolysis and uptake by floral primordia in peach (Bonhomme, 2005) were related to endodormancy release.

Ecodormancy

Once endodormancy is overcome, sprouting or flowering of tree buds depends on high temperatures of spring. This period is called ecodormancy (Lang, 1987) and the amount of high temperatures needed for each cultivar is defined as heat requirements.

While the influence of chilling requirements on dormancy release is a well-studied phenomenon (Couvillon and Erez, 1985a; Erez and Couvillon 1987), the effect of heat requirements on flowering time is less clear in *Prunus* species (Citadin et al., 2001; Couvillon and Erez, 1985b).

Heat units accumulation expressed as Growing Degree Hours (GDH), proposed by Richardson et al. (1975), is one of the most suitable models to estimate the heat requirements (Darbyshire et al., 2014). However, the model showed inter-annual and inter-location variability in a given cultivar (El Yaacoubi et al., 2016) (see Chapter 1.2.2).

During ecodormancy, starch accumulated during endodormancy starts vanishing as heat accumulation increase (Fadón et al., 2018). Following starch decrease, the ovary cells become active and processes of cell division, chromatin decondesation and vacuolization are observed (Horvath et al., 2003).

1.2.2. Models for dormancy monitoring

Weinberger (1950) initially established that effective temperatures for chill accumulation should be below 7°C. Later, Richardson et al. (1974) established a range of temperatures that corresponded to an amount of Chill Units (CU). The Richardson model has been the most and frequently used to estimate chilling requirements of cultivars, but it was developed for the cold winters of Utah (USA). This resulted in the inaccuracy of the model in warm-winter areas (see Chapter 3).

Whereas Richardson model considers temperatures above 16° have negative effects on chill accumulation, Erez and Couvillon (1987) reported that moderate temperatures ranging 13-15ºC actually performs a synergistic effect with lower temperatures. Moreover, high temperatures in brief daily periods reinforce the positive effect of low temperatures for chill accumulation

The dynamic model (Fishman, 1987a; 1987b), developed in Israel, took into account the considerations mentioned above and the irreversibility of chill accumulated. This model has been successfully applied in Mediterranean climates as in Spain (see Chapter 3).

In *Prunus* species, chilling requirement for endodormancy release is considered the major factor determining flowering time (Tabuenca et al., 1972; Couvillon and Erez, 1985a; Citadin et al., 2001; Egea et al., 2003; Albuquerque et al., 2008). While Harrington et al. (2010) suggested that chilling and heat requirements are not independent processes, but they overlapped in time, Okie and Blackburn (2011)

considered that heat requirements are subordinate to chilling requirements in peach. The contribution of heat requirements to dormancy release was estimated in 30% in apricot with a 70% of contribution of chilling requirements (Ruiz et al., 2007). Thus, the chilling requirement of each cultivar determines its suitable adaptation to each area.

For proper calculation of chilling requirements it is very important to establish the beginning time of chilling accumulation. According to different authors this time could be when a persistent amount of chilling is being accumulated (Richardson et al., 1974; Ruiz et al., 2007).

On the other hand, temperature data must be accurately recorded. Thus, is worthy to take into account the solar radiation and the wind, what could modify the temperature of the bud, usually not recorded by dataloggers (Weinberger, 1950; Erez, 2000; Naor, 2003).

1.2.3. Dormancy and fruit production

Successful dormancy completion is necessary for a good production in terms of fruit quantity and quality. Climatic conditions (mainly temperatures) affect the progression of endodormancy, ecodormancy, and the processes of flowering, fruit set and fruit development (Cooke et al., 2012; Hamilton et al., 2016).

Effects of temperatures during endodormancy

Dormancy has to be overcome by satisfying the chilling requirement of the tree, which is critical to successfully complete the reproductive phase and provide a high production of quality (Byrne and Bacon, 1992; Erez, 2000).

The term "residual effect of dormancy" is used when crops show symptoms of insufficient chilling. These symptoms are usually a deficient and irregular flowering, the uneven foliation and a low fruit set (Felker and Robitaille, 1985; Couvillon and Erez, 1985a).

Insufficient chill accumulation has been related to an excessively long ecodormancy period (El Yaacoubi et al., 2016). The lack of chilling must be considered since last years are among the warmest since 1850, with a significant trend of temperature to increase in spring and winter in the northern locations (Schawrtz, 2006; Cordero et al., 2011; Marengo and Camargo, 2008). Moreover, temperature will continue increasing according to data provided by the Intergovernmental Panel on Climate Change (IPCC) report (2018). Such warming has also been reported in Spain

(Martínez et al., 2010; Rodríguez et al., 2019). Others parameters under consideration when looking at climate change are the variability of rainfall and the more frequent drought periods. The effect of high temperatures during endodormancy is discussed in Chapter 3.

Effects of temperatures during ecodormancy

During ecodormancy period, tree tissues lose cold hardiness (dehardening) and are ready to resume growth (Kalberer et al., 2006). For this reason, from ecodormancy to flowering, low temperatures are dangerous because the tree hardening potential is low (Hänninen, 2016; Lenz et al., 2016).

Dehardening potential of *Prunus* species (*Prunus avium* (L.) L.) is higher than that the exhibited by other tree species, as heat requirements are lower (Vitra et al., 2017). Moreover, frost resistance of peach flower buds decreased rapidly in response to heat accumulation, during ecodormancy (Szálay et al., 2010). Frost resistance declined even more rapidly after a mild winter. Thus, high temperatures during the endodormancy period can affect the hardening potential during ecodormancy.

Furthermore, negative effects of high temperatures during ecodormancy and flowering are explained in Chapter 4.

1.2.4. Genetic and Molecular control of dormancy and flowering time

Dormancy phases are regulated by several molecular pathways interconnected (Beauvieux et al., 2018). In recent decades, many studies based on metabolic, physiological, subcellular, genetic, and genomic analyses have unravelled mechanisms underlying bud dormancy progression. However, the molecular switch leading to endodormancy release has not been uncovered to date.

Genetic control

Flowering time is a heritable quantitative trait in fruit tree species. Due to its importance for tree adaptation and success in breeding programs, many studies on the genetic control of this trait have been carried out.

The transmission of flowering time has been studied by different authors in almond (Kester, 1965; Grasselly, 1972; Vargas et al., 1984; Sánchez-Pérez et al., 2007; 2012) and high values (0.8-1) of heritability has been calculated by regression (Kester and Asay, 1973; Kester et al., 1977; Dicenta et al., 1993). Dicenta and García (1993)

showed that flowering time is controlled by nuclear DNA, and no maternal effects were observed.

On the other hand, different genomic regions involved in the control of flowering time have been identified in almond and other *Prunus* species by Quantitative Trait Loci (QTLs) studies (Sánchez-Pérez et al., 2014). Using Random Amplified Polymorphic DNAs (RAPDs), a major gene in the variance control of flowering time was found. This and other two markers were found in linkage group (LG) 4 (Ballester et al., 2001). The localization of the *Lb* gene in LG4 was confirmed using Simple Sequence Repeat markers (SSRs) in the almond population R1000 x Desmayo Largueta and other QTLs were identified in an almond (Sánchez-Pérez et al., 2007; 2012) and almond x peach progeny (Silva et al., 2005) and located in LG1, LG2, LG3, LG5, LG6 and LG7. Moreover, some of these QTLs were linked to chilling requirements and in LG2 and LG7 were found QTLs linked to heat requirements (see Chapter 5).

Based on the model derived from the long-day herbaceous plant Arabidopsis, *LEAFY* (*LFY*) and *FLOWERING TIME* (*FT*) genes, among others, were identified as determinants of flowering time. In some *Prunus* species, homologs to that flowering time regulators have been isolated and characterized (An et al., 2012; Srinivasan et al., 2012; Yarur et al., 2016). In almond, *LFY* but no *FT* homolog has been reported to date (Silva et al., 2005). However, homologous genes to *FT* and *LFY* and others involved in flowering time control in the model plant Arabidopsis did not colocalize with the *Lb* gene. More recently, candidate genes underlying *Lb* were investigated in sweet cherry and key genes were identified for chilling requirements and flowering time (Castède et al. 2015) (see Chapter 5).

The peach *evergrowing* (*evg*) mutant carries a deletion in *EVG* locus affecting up to four genes that prevents terminal buds from endodormancy (Bielenberg et al., 2004). The map-based cloning analyses of *EVG* locus revealed that it included six tandemly arrayed genes, called *Dormancy Associated MADS-box* (*DAM*) genes (Bielenberg et al., 2004; 2008) (see Chapter 5).

Other QTLs were also described in different LGs in peach (Fan et al., 2010; Romeu et al., 2014; Bielenberg et al., 2015), and apricot (*Prunus armeniaca* L.) (Olukolu et al., 2009; Salazar et al., 2016).

Transcriptional regulation

Seasonal cues in terms of photoperiod and temperature regulate dormancy state through gene expression control. Gene expression regulation can operate at different levels. Several strategies for decipheration of transcriptional networks and epigenetic marks involved in the control of dormancy and flowering time in fruit tree species have been performed. One of them was to apply the knowledge acquired from herbaceous and tree model species (Arabidopsis and poplar respectively) to non-model tree species like almond.

Furthermore, in this Thesis, transcription regulation (Chapter 6) and DNA methylation (Chapter 7) were targeted for analysis during endodormancy progression.

- Circadian clock genes

Plants and animals perceive seasonal changes, as photoperiod and temperature, by the circadian clock. Such environmental cues occur during the induction and release of endodormancy.

In several tree species as poplar, photoperiod plays a major role in endodormancy induction. *LHY* and *TOC1* were the first clock genes described in chestnut (*Castanea sativa* (Mill.)) and poplar (*Populus tremula* (L).) (Ramos et al., 2005). These genes regulate the module *CONSTANS/FLOWERING LOCUS T* (*CO/FT*) that control flowering, as occurs in Arabidopsis (Böhlenius et al., 2006). Additionally, induction of *FT* by cold towards dormancy release has been reported in poplar (Rinne et al., 2011) and in sweet cherry flower buds (Gericke, 2015). Moreover, in Arabidopsis, *FT* expression is regulated by *GIGANTEA* (*GI*) (Sawa and Kay, 2011). In almond, an increase of *FT* transcript abundance was observed upon anthesis and *PdGIGANTEA* expression increased during endodormancy of flower buds (Barros et al., 2017).

Another gene related to photoperiod is *ELF3* (*EARLY FLOWERING 3*), identified as a light signal transductor for flowering in Arabidopsis. In trees, *ELF3,* was identified as up-regulated in endodormant buds of leafy spurge (Doğramacı et al. 2010).

- Cold tolerance

During endodormancy meristematic and surrounding tissues inside the bud acquired cold hardiness, promoted in autumn by the fall of temperatures.

This process enables the tree to survive to lower and freezing temperatures during the winter (Wisniewski et al., 2018).

Cold hardiness is based on the synthesis of molecules that prevents frost injury by the stabilization of cell membranes. This mechanism is triggered in Arabidopsis by CBFs and DREB transcription factors (Wisniewski et al., 2011), whose expression depends on photoperiod (Artlip et al., 2013) and low temperatures. Moreover, *CBFs* are regulated by *LHY* (Ibáñez et al., 2010).

In almond, gene expression analysis of cold-responsive *PdCBF1* and *PdCBF2* in floral buds suggested a downregulation of these genes during ecodormancy (Barros et al., 2012).

- MADS-box genes

MADS box genes are an extent gene family of transcription factors that regulate floral development. In addition *DAM* (*Dormancy Associated MADS-box*) genes are responsible for endodormancy maintenance and its expression decrease concomitantly with endodormancy release (Yamane et al., 2011; Xu et al., 2014; Zhu et al., 2015; Rothkegel et al., 2017). Actually, CBF transcription factors are known to target *DAM* genes for transcriptional regulation in Japanese apricot (*Prunus mume* (Siebold) Siebold & Zucc.) (Zhao et al., 2018).

Other MADS-box genes controlling flowering in *Arabidopsis* are those belonging to *AGAMOUS-LIKE* (*AGL*) family, together with *SUPRESSOR OF CONSTANS* (*SOC1*) and *SHORT VEGETATIVE PHASE* (*SVP*). *SVP* and *SOC1* expression decreased during dormancy release in poplar vegetative buds (Howe et al., 2015) and Japanese apricot flower buds (Kitamura et al., 2016). On the other hand, SVP accumulation is influenced by ELF3 (Yoshida et al., 2009), which relates expression pattern with photoperiod.

According to the regulatory model proposed by Wu et al. (2017), it seems that SVP and DAM are in fact floral repressors whose expression becomes reduced during chill accumulation and dormancy release (Kumar et al., 2016a; Singh et al., 2018). Nevertheless, a role associated to dormancy rather than to flowering transition has been proposed for *SOC1* in apricot and kiwifruit (Trainin et al., 2013; Voogd et al., 2015). Recently, the interaction of SOC1 and DAM6 has been reported in Japanese apricot (Kitamura et al., 2016).

In almond, MADS box genes *PdMADS1-PdMADS3*, gradually increase expression levels during ecodormancy of flower buds (Barros et al., 2012).

Gibberellins pathway

Based on the expression pattern of the *GIBBERELLIN 20-OXIDASE* (*GA20ox*) and *GIBBERELLIN 2-OXIDASE* (*GA2ox*) genes, involved in GAs biosynthesis of bioactive and inactive GAs, respectively, a change in GA metabolism was proposed to take place upon endodormancy release (Silva et al., 2005; Barros et al., 2012). Actually, GAs have been proposed as signal molecules for promotion of growth reactivation, together with FT (Singh et al., 2017). Interestingly, gibberellins are involved in the regulation of cell elongation in stamen filament as well as in cellular development in anthers, in Arabidopsis (Cheng et al., 2004). In sweet cherry, *GA2ox* was a highlighted candidate gene related to flowering time from QTL analysis (Castède et al., 2015) (see Chapter 5).

Epigenetic regulation

Finally, the expression regulation at epigenetic level cannot be discarded during endodormancy (Yaish et al., 2011). Genome-wide patterns of DNA and histones modifications highlighted the importance of this process in endodormancy release (Santamaría et al., 2009; De la Fuente et al., 2015). Furthermore, the epigenetic regulation of a *DAM* gene was reported in peach (Leida et al., 2012a).

In peach, Zhebentyayeva et al. (2014) described PcG (Polycomb Group) genes, which are involved in the regulation of flowering at the epigenetic level in the model species Arabidopsis. The *POLYCOMB REPRESSIVE COMPLEX II* (*PRCII*) components *EMBRYONIC FLOWER 2* (*EMF2*) and *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* (*PIE1*) colocalize with a flowering time QTL in sweet cherry (Castède et al., 2015) (see Chapter 5).

Epigenetic marks regarding DNA methylation in almond will be discussed in detail in Chapter 7.

1.3. Objectives

The main objective of this PhD Thesis is the analysis of the genetic and molecular basis of endodormancy release and flowering time in almond to identify molecular markers linked to these important agronomic traits. For this general purpose, several specific objectives were included:

1. Assessment of chill accumulation for flower bud dormancy release using different models and evaluation of heat accumulation for flowering in almond cultivars with different flowering time.
2. Evaluation of the effect of different controlled temperature conditions on flowering time, fruit set, fruit characteristics and maturity time in almond cultivars with different flowering times.
3. Expression analysis of candidate genes linked to endodormancy release and flowering time in almond cultivars with different flowering times and evaluation of their use as potential expression markers for monitoring endodormancy release and flowering time.
4. Identification of new expression markers by using whole transcriptome sequencing (RNA-Seq) of endodormant and ecodormant flower buds from almond cultivars with different chilling requirements and flowering time.
5. Identification of epigenetic marks based on DNA methylation in endodormant and ecodormant flower buds from almond cultivar with different flowering time.

These specific objectives correspond to the different experimental chapters of this Thesis.

2. PLANT MATERIAL

2. PLANT MATERIAL

2.1. Almond cultivars assayed

In terms of flowering time, five different groups of cultivars can be considered in almond (Dicenta et al., 2017b) (Figure 2.1):

- **Extra-Early flowering cultivars:** Traditional cultivars of unknown genetic origin resulting from selection carried out by farmers. These cultivars flower in the Mediterranean area in January.
- **Early flowering cultivars:** Traditional cultivars of unknown genetic origin resulting from selection carried out by farmers flowering in the Mediterranean area in early February.
- **Late flowering cultivars:** Cultivars obtained from crosses between local cultivars with other late flowering cultivars, usually coming from breeding programs. These cultivars flower 20-30 days after early cultivars.
- **Extra-late flowering cultivars:** Cultivars obtained from crosses between previously obtained late cultivars. These cultivars flower 50-60 days after early cultivars.
- **Ultra-late flowering cultivars:** Recently, an almond cultivar which flowers in April (more than 60 days later than early cultivars) has been obtained: 'Tardona'.

The plant material included in this Thesis consisted of a traditional almond cultivar of extra-early flowering ('Desmayo Largueta') and cultivars provided by the Almond Breeding Program of "Centro de Edafología y Biología Aplicada del Segura" (CEBAS-CSIC): the extra-late cultivar 'Penta' and the ultra-late cultivar 'Tardona'.

These almond cultivars were choosen due to its different flowering time and endodormancy release including the three main genetic pools used to delay the flowering time in almond: North of Italy, Californian mutation 'Tardy Nonpareil' and Ukraine (Figure 2.1).

The trees are located in the experimental station "Tres Caminos" of CEBAS-CSIC located in Santomera (Murcia, South-East Spain, 38º 6' 34.28'' N; 1º 2' 16.71'' O). With an altitude of 130 meters on average, this farm is characterised by very hot summers and cool winters, with minimum temperatures usually higher than 0 ºC. The trees are ten-years old, grafted onto 'Garrigues' seedling rootstock and drip irrigated (Figure 2.2).

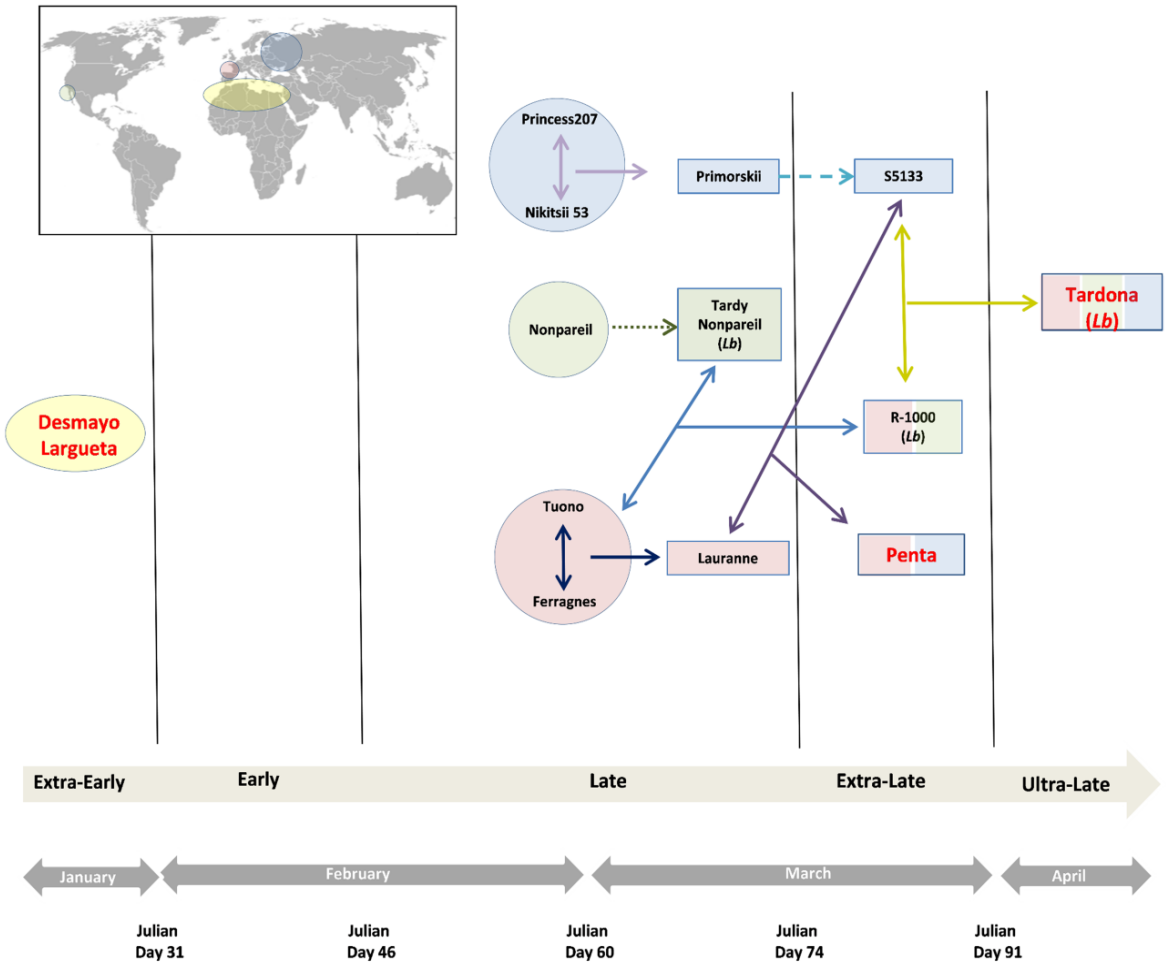


Figure 2.1. Pedigree representation of the almond cultivars used as plant material 'Desmayo Largueta', 'Penta' and 'Tardona' (colored in red). New releases (squares) were obtained by crosses of traditional cultivars (circles), indicated by arrows. The origin of 'Tardy Nonpareil' is a natural mutation (*Lb*, *Late blooming*) of 'Nonpareil', and the selection 'S5133' is an open pollination of 'Primorskii' cultivar. The early-flowering genetic pool is in yellow, the medium-late Mediterranean pool is in red, the medium-late Californian pool is in green, and the late Ukrainian pool is in blue. Adapted from Dicenta et al. (2017) and Martínez-Gómez et al. (2017).

Desmayo Largueta: A traditional Spanish cultivar of unknown origin, dating from the 19th century in Tarragona and spread throughout the Mediterranean area. It is self-incompatible, with extra-early flowering and late ripening times, and fruit hard-shelled and elongated. Its high productivity and good quality makes its price high in the market (Figure 2.3).

Figure 2.2. Trees from cultivars used in this work. From left to right: 'Penta', 'Tardona' and 'Desmayo Largueta' grafted into 'Tardona' in the experimental orchard of CEBAS-CSIC.

Figure 2.3. 'Desmayo Largueta' tree and shoot with developing fruits (March). The black line indicate 1cm.

Penta: A Spanish cultivar obtained at CEBAS-CSIC from the cross between the late flowering Spanish selection S5133 (open pollination of the Ukrainian cultivar 'Primorskii') and Lauranne ('Ferragnès' x 'Tuono'). It is self-compatible, with extra-late flowering and early ripening times. The fruit is elongated and hard-shelled. Penta combines sources of late-flowering from Italy and Ukraine (Figure 2.4).

Figure 2.4. Penta mother tree and shoot with pollinated flowers (March) and fruits set. The black line indicates 1cm.

Figure 2.5. Tardona mother tree and shoot with flowers in different phenological stages (C-G) (March). The black line indicates 1cm.

Tardona: A Spanish cultivar obtained at CEBAS-CSIC from the cross between S5133 and the late flowering French cultivar 'R1000' ('Tardy Nonpareil' × 'Tuono'). 'Tardona' combines sources of late flowering from Italy and Ukraine, as well as the spontaneous *Lb* mutation from the Californian cultivar 'Tardy Nonpareil'. 'Tardona' can flower up to 60 days later than 'Desmayo Largueta', and it is the latest flowering almond cultivar released in the world to date (Figure 2.5).

2.2. Plant material for endodormancy release evaluation

In all the developed assays in this Thesis three branches (mixed twigs, 1-year shoots, 40 cm in long and 5 mm in diameter) of each almond cultivar were picked weekly from the same tree in the field for dormancy release evaluation described in Chapter 3. Phenological evolution of buds was assessed based on classification of Felipe (1977) (Figure 2.6).

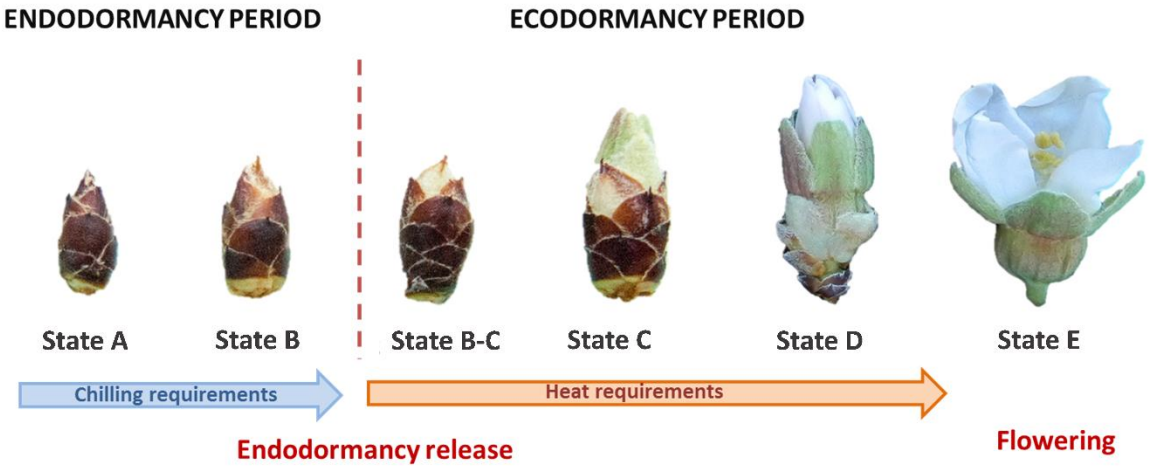


Figure 2.6. Almond flower buds phenological states as described by Felipe (1977).

2.3. Plant material for molecular analysis

The plant material used for molecular analysis included endodormant (A state) to ecodormant (B state) flower buds (Figures 2.6 and 2.7) used for DNA extraction and RNA extraction protocols referred in next Chapters 5, 6 and 7. Flower buds were picked randomly from each cultivar tree in the field and kept on ice. Samples were collected weekly from November 10th until the dormancy release date, during two seasons: 2015-2016 and 2016-2017. 'Tardona' samples from December 1st, 9th, 29th of 2016 and January 28th of 2017 could not be used for molecular analysis.

Once at laboratory, buds were peeled and frozen at -80ºC. A group of flower buds was not frozen, but kept for observations with stereoscopic microscope Olympus SZ61 (Olympus, Hamburg, Germany), and pictures were taken with camera Olympus SC30 (Olympus, Hamburg, Germany) (Figure 2.7 and Figure 2.8).

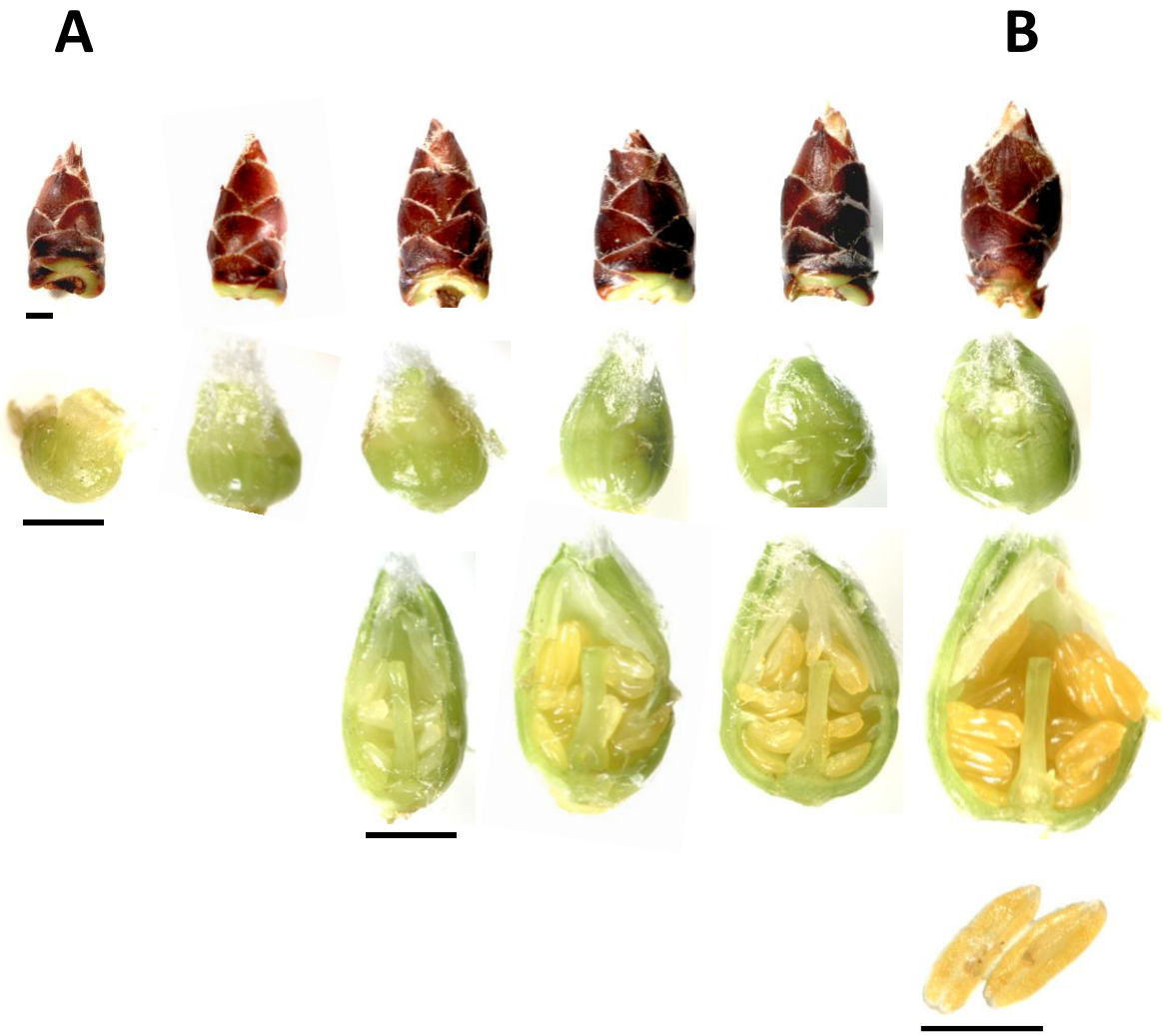


Figure 2.7. Endodormant (A state) to ecodormant (B state) flowering buds sampled and processed for molecular analysis. Line indicates 1mm. From upper to lower: Intact flower buds with brown scales, peeled flower buds with green scales and chopped flower buds (developing pistil and anthers). From endodormancy to ecocodormancy flower bud is size increasing.

Figure 2.8 shows a detailed comparison of flower buds in B state (ecodormancy) of the cultivars used as plant material: 'Desmayo Largueta', 'Penta' and 'Tardona'. It is necessary to note that different flowering time is reflected in a different development of the anther in the analysed almond flower buds.

Figure 2.8. Comparison of flower buds in B state (ecodormancy) of the cultivars used as plant material: 'Desmayo Largueta', 'Penta' and 'Tardona'. Line indicates 1 mm.

3. NATURAL PROGRESSION OF DORMANCY AND FLOWERING IN ALMOND

3. NATURAL PROGRESSION OF DORMANCY AND FLOWERING IN ALMOND

3.1. Introduction

As we indicated in the Introduction of this Thesis, one of the main objectives of almond breeding is the development of new extra-late flowering cultivars to avoid damage from spring frosts. The expansion of almond cultivation from the Mediterranean Basin to colder areas in Northern Europe and America has been made possible by delaying flowering time through classical breeding methods (Gradziel and Martínez-Gómez, 2013; Dicenta et al., 2016; Martínez-Gómez et al., 2017). This delay in flowering time has its limits, however, and success is conditioned by the extent to which the climatic requirements are met for the suitable development of vegetative and flower buds and the processes of pollination, fruit set and fruit development (Alonso Segura et al., 2017; Dicenta et al., 2017).

The chilling and heat requirements (CR and HR, respectively) determine flower bud dormancy release and the subsequent flowering in temperate fruit species (Campoy et al., 2011). Although chill and heat accumulation are inter-dependent processes (Erez and Couvillon, 1987), chilling requirement is considered to be the major factor that determines flowering time in almond and the rest of the *Prunus* species, rather than heat requirement (Egea et al., 2003; Ruiz et al., 2007; Albuquerque et al., 2008; Sánchez-Pérez et al., 2012; Atkinson et al., 2013). Chilling requirement is therefore a very important adaptive trait specific to each cultivar (Egea et al., 2003; Ruiz et al., 2007; Campoy et al., 2011), and it determines the length of the dormancy period. The dormant state is cumulative and quantitative with endogenous and environmental signals continually altering its depth in woody plants (Cooke et al., 2012). For an adequate flowering the chilling and heat requirements of each cultivar have to be fulfilled. Otherwise this essential process could be negatively affected also affecting final production (Luedeling, 2012) and even fruit shape (Yong et al., 2016).

According to Lang et al. (1987), there are three distinct types of dormancy: endodormancy, due to control factors inside the bud; paradormancy, controlled by agents outside the dormant bud; and ecodormancy, depending on the temperature once the chilling requirement has been supplied (see Chapter 1.2) (Figure 3.1). Chilling requirement therefore mainly affects the endodormancy period, with high chilling requirement determining long endodormancy periods and vice versa.

Plant production can be affected by dormancy in two ways: through increasing the chances of survival during inclement climatic conditions, and through affecting flower and fruit development during the growing season (Campoy et al., 2011; Martínez-Gómez et al., 2017).

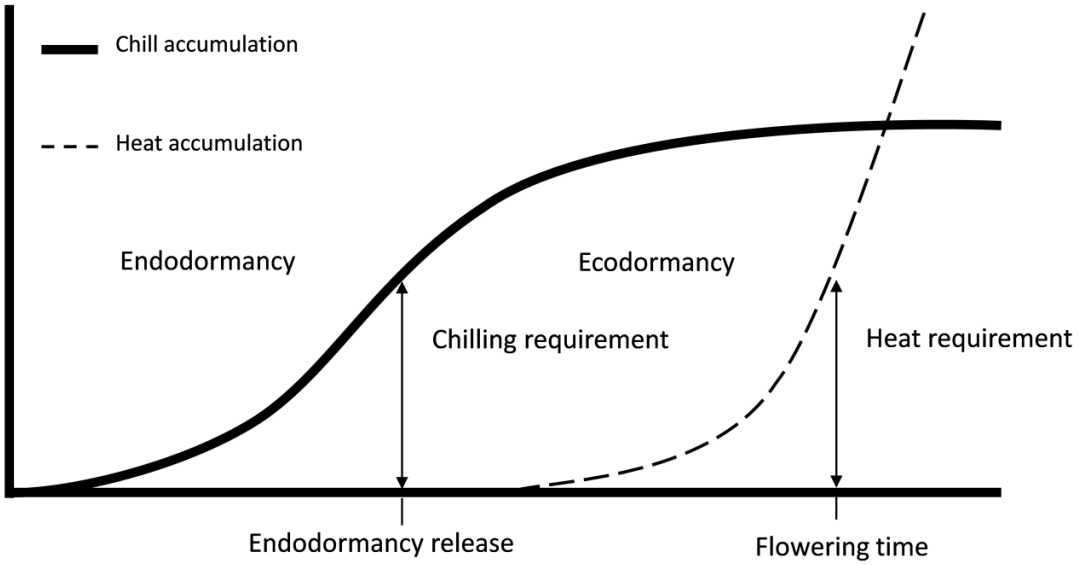


Figure 3.1. Chill and heat accumulation as conceptualized by Lang et al. (1987) and phenologic events related to them. Adapted from Luedeling et al. (2009).

To explain dormancy release, different models have been developed, including the Richardson model (Richardson et al., 1974) for cold areas and the Dynamic model (Fishman et al., 1987a; 1987b; Erez et al., 1990) for mild-winter areas (see Chapter 1.2.2). Studies comparing the Richardson and Dynamic models have been performed using historical phenology records in peach (Maulión et al., 2014), almond (Benmoussa et al., 2017) and sweet cherry (Gannouni et al., 2017). These studies have shown clear differences in the estimation of chill requirements using both methods. Maulión et al. (2014) recommended Richardson model for the evolution of chilling requirements in peach in Argentina conditions whereas Benmoussa et al. (2017) and Gannouni et al. (2017) recommended the Dynamic models in almond and sweet cherry in the North of Africa conditions.

From the perspective of global climate change, dormancy regulation has recently drawn the attention of more researchers (Luedeling et al., 2009; Luedeling, 2012; Campoy et al., 2011; Viti et al., 2013). Such studies are of great interest in mild-winter areas like South-East of Spain where warmer winters are anticipated in the future due to the climate change (Ponti et al., 2014; IPCC, 2018). In the Mediterranean region, a delay has been observed in dormancy release and flowering time in peach

(Ghrab et al., 2014) and cherry (Oukabli and Mahhou, 2007; Gannouni et al., 2017) in the context of this climate change. This situation has also been observed in Western Europe for apple (*Malus domestica* Borkh.) (Legave et al., 2013).

In relation to the genetic basis of endodormancy release and flowering time in almond, the objective of this first experimental chapter of the Thesis was the assessment of chill and heat accumulation for flower bud dormancy release and flowering in almond, using Richardson and Dynamic models, in three almond cultivars with different flowering time.

3.2 Material and methods

3.2.1 Plant material

The plant material assayed included the traditional Spanish extra-early flowering almond cultivar 'Desmayo Largueta' in addition to the extra-late 'Penta' and the ultra-late 'Tardona', both releases of the CEBAS-CSIC breeding programme (Table 1). This study has been performed onto ten-years old trees grafted onto 'Garrigues' seedling rootstocks (see Chapter 2). Trees, four replications per cultivar, were growth in standard cultivation conditions in a drip-irrigated orchard.

3.2.2 Evaluation of chilling and heat requirements

Experiments for the evaluation of chilling and heat requirements were conducted over a period of three seasons in the experimental field of CEBAS-CSIC, in Murcia (south-eastern Spain) at an altitude of 150 m.s.l. Between 2014 and 2017, temperatures were recorded hourly with a data-logger (HOBO® UX100-003) from November 1st to April 7th. Mixed twig branches were weekly collected during the period from November 1st to April 1st. The full flowering time of each cultivar (when 50% of flowers were open, F50) was recorded in the field. The calculation of chilling accumulation was performed in Chill Units (CUs) according to the model described by Richardson et al. (1974) and in Chill Portions (CPs) according to the Dynamic model (Fishman et al., 1987a; 1987b).

Using the Richardson model, the initial date for chilling accumulation was determined when consistent chilling accumulation occurred and temperatures producing a negative effect (chilling negation) were rare (Erez et al., 1979).

Finally, the period of ecodormancy (between dormancy release and flowering), when heat is theoretically accumulated, was considered as the number of days between dormancy release and the full flowering date (Figure 3.1). Heat requirements (HR) were estimated during this period according to Richardson et al. (1975) as Growing Degree Hours (GDHs), defined as the hourly temperatures minus 4.5°C.

3.2.3 Endodormancy release evaluation

Three branches (mixed twigs, 1-year shoots 40 cm in long and 5 mm in diameter) (Figure 3.2) of each cultivar were picked weekly from the same tree in the field and placed in a growth chamber in controlled conditions (23±1ºC, RH of 40% during the 16h of photoperiod and 20±1ºC, RH of 50% during the dark period). A fresh cut was made at the base of the almond branches, which were placed in the growth chamber in 5 % sucrose 0.1 % aluminium sulphate solution. The date of endodormancy release was established when 50% of the flower buds were in the B-C state (Felipe, 1977) (Figure 2.6) after 10 days in the growth chamber.

3.2.4. Flowering density and productivity evaluation

Flowering density and productivity were evaluated in the four ten-season-old trees of each cultivar on a scale of 1 to 5 (minimum to maximum) during the three seasons of the study (2015, 2016 and 2017): 0. - no flowering or production; 1.- very irregular flowering or production; 2.- low flowering or production; 3.- medium flowering or production; 4.- high flowering or production 5.- very high (complete) flowering or production.

3.3. Results and discussion

Almond species present enormous variability for many characteristics as a result of their origins, including both multiplication by seed and breeding (Gradziel and Martínez-Gómez, 2013). Flowering time is probably the most notable example of a characteristic displaying such variability (Dicenta et al., 2017b; Martínez-Gómez et al., 2017). This is an advantage for almond cultivation, which can thus be established in a wide range of environmental conditions by regulating the chilling and heat requirements.

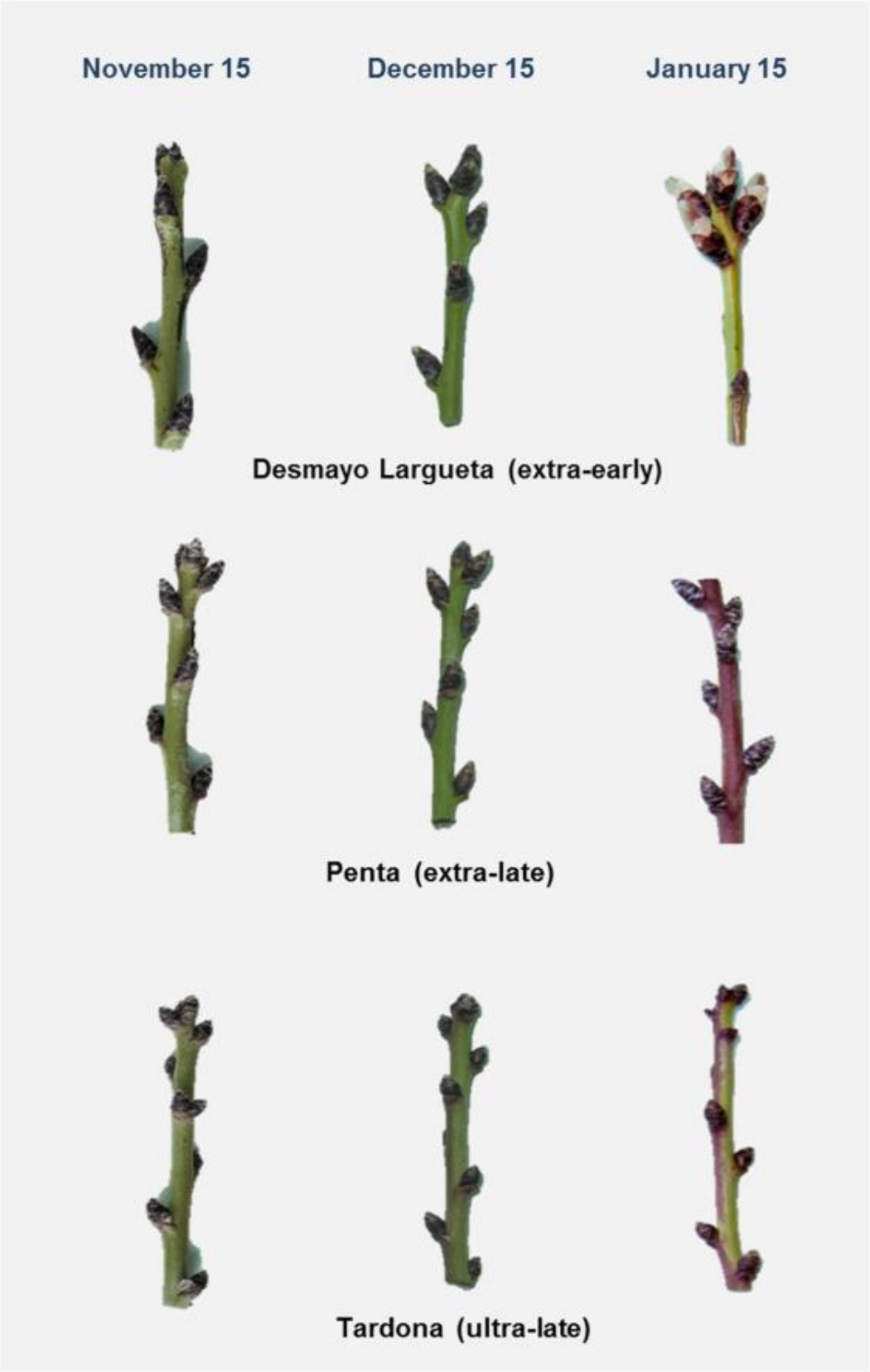


Figure 3.2. Detail of 5cm flower bud twigs phenologic evolution between November 15th of 2016 and January 15th of 2017 in the almond cultivars studied.

3.3.1. Chill and heat accumulation in the field

Figure 3.3 shows the chill and heat accumulation according to the Richardson and Dynamic models during the three seasons of study. Both models estimated that the chill accumulated in the field during the 2015-2016 season was the lowest (308 CU and 49 CP) in comparison to the other two seasons 2014-2015 (843 CU and 61 CP) and 2016-2017 (798 CU and 66 CP).

According to the Richardson model, the initial dates for chill accumulation were December 3rd, November 22nd and November 7th in 2014, 2015 and 2016, respectively. In 2016-2017 the starting date of CU accumulation was the earliest, as shown, but this season was not the coldest in terms of chill accumulation.

On the other hand, the heat accumulated during 2015-2016 season was the highest (36,087 GDH) in comparison with 2014-2015 and 2016-2017 (32,225 and 32,980 GDH, respectively). Results highlight the fact that the winter of 2015-2016 was strangely warm in comparison to the other two seasons, and much warmer than average values in this area.

Our results showed that the climatic conditions were quite different over the period of this study, as shown by the chill and heat accumulated annually. Total chill accumulation in the first and third season was similar to previous evaluations in 2009, when Sánchez-Pérez et al. (2012) described an accumulation of 800 CU. Furthermore, the heat accumulation observed during the three seasons of study was also similar to that found in previous evaluations of Sánchez-Pérez et al. (2012), with an annual accumulation of around 30,000 GDH.

3.3.2. Evaluation of chilling and heat requirements of almond cultivars using Richardson and Dynamic models

Endodormancy (CU/CP) plus ecodormancy (GDH) determined the flowering time of the cultivars (Figure 3.1). Table 3.1 shows the chilling requirements for dormancy release and the heat requirements for flowering of 'Desmayo Largueta', 'Penta' and 'Tardona' during the three seasons of study, according to the Richardson and Dynamic models. The flowering times were as follows in 2015, 2016 and 2017 respectively: February 7th, January 28th and January 27th for 'Desmayo Largueta'; 22, 25 and 12 March 22nd, 25th and 12th for 'Penta' and March 30th, April 7th and March 21th for 'Tardona'. In general 2016-2017 was the earliest flowering season and 2015-2016 the latest.

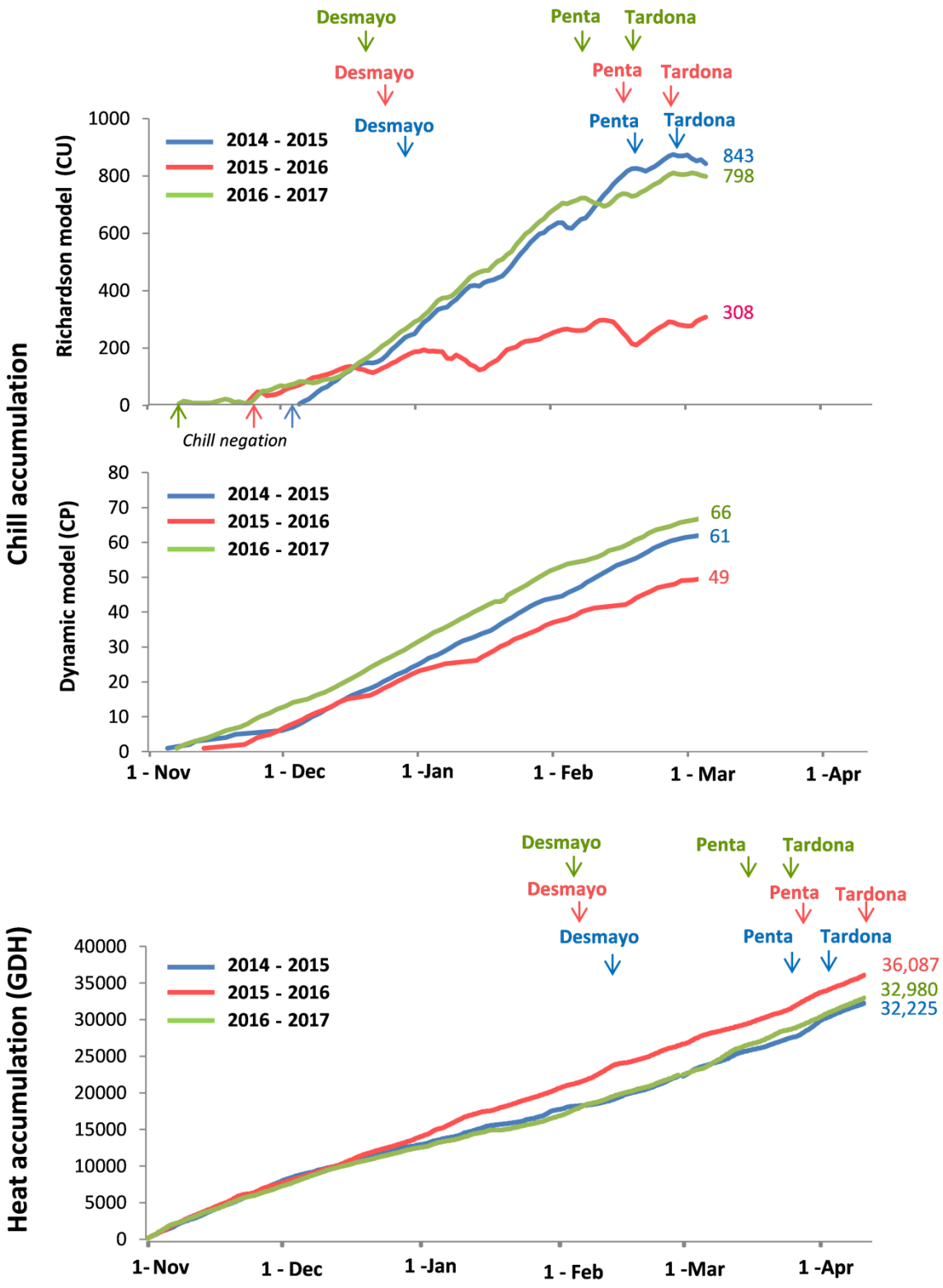


Figure 3.3. Chill accumulated in CU (Chill Units) and CP (Chill Portions) and heat accumulated in Growing Degree Hours (GDH) in field conditions during the seasons 2014-2015, 2015-2016 and 2016-2017. Dormancy release (evaluated in a growth chamber) and flowering dates (evaluated in the field) of the assayed cultivars during the three seasons of the study are also indicated.

We found a narrow relationship between flowering time and the endodormancy period in the three assayed cultivars, while the ecodormancy period was similar (close to 40 days) in all cultivars (Table 3.1). This fact is supported by comparing the coefficients of variation (CVs) of CU and GDH (44% versus 16%) over the seasons. If flowering time is expressed in Julian days, 'Desmayo Largueta', 'Penta' and 'Tardona' flowered on days 30, 79 and 89, on average, with an endodormancy period of 31, 81 and 90 days, respectively.

These results substantiate the previously mentioned notion that chilling requirements are more important than heat requirements for flowering also corroborating previous works in different *Prunus* species including almond (Egea et al., 2003), apricot (Ruiz et al., 2007) or sweet cherry (Alburquerque et al., 2008).

During the second season of study, the cultivars behaved according to their chilling requirements under low chill conditions. 'Desmayo Largueta' fulfilled its low chilling requirements, and the warm conditions promoted flowering on nearly the same date as usual. The extra-late 'Penta', however, and the ultra-late 'Tardona', for the most part, had a delayed flowering time, since the chill accumulation was lower than usual.

This lack of chill produced a more extended flowering period, with large differences in phenological states within each tree. This situation was similar for hundreds of extra- or ultra-late-flowering seedlings in our breeding programme in this season (data not shown).

Looking into detail, according to the Richardson model, chilling requirements of the early flowering cultivar Desmayo Largueta was similar in the second and third seasons (130 and 137 CU) and higher in the first season (234 CU). The Dynamic method, however, produced contradictory results, since first and last seasons were similar (22 and 24 CP); while the second was lower (16 CP).

The anomalous situation observed during the second season of study (2015-2016) is likely due to the fact that chill accumulation started earlier in the third season of study (Figure 3.3), so dormancy release could occur earlier, mainly affecting the earlier flowering cultivar. Heat requirements were quite different along the different studied seasons, reaching values of 6,134, 7,770 and 4,933 GDH for the three seasons. The highest heat requirements were observed during the second season of study (2015-2016).

Table 3.1. Dormancy release date, endodormancy period, Chill Requirements (CR), full flowering time, Heat Requirements (HR) and flowering intensity of 'Desmayo Largueta', 'Penta' and 'Tardona' almond cultivars during 2014-2015, 2015-2016 and 2016-2017 seasons.

Season	Dormancy release	Endo-dormancy[1]	CR		Flowering time	Eco-dormancy[2]	HR	Flowering intensity	Productivity
			CU	CP			(GDH)	(scale 1-5)	(scale 1-5)
'Desmayo Largueta'									
2014/2015	December 26	23	234	22	February 7	43	6134	3	3
2015/2016	December 21	30	130	16	January 28	38	7770	3	3
2016/2017	December 15	39	137	24	January 27	43	4933	2	2
Mean		**31**	**167**	**21**		**41**	**6279**	**2.7**	**2.7**
CV (%)		**26**	**35**	**20**		**7**	**23**	**22**	**22**
'Penta'									
2014/2015	February 17	74	825	55	March 22	33	7103	5	4
2015/2016	February 10	82	274	41	March 25	43	9166	4	2
2016/2017	February 2	87	723	54	March 12	38	8923	5	5
Mean		**81**	**607**	**50**		**38**	**8397**	**4.7**	**3.7**
CV (%)		**8**	**48**	**16**		**13**	**13**	**12**	**42**
'Tardona'									
2014/2015	February 24	81	870	59	March 30	34	7996	4	3
2015/2016	February 23	95	282	47	April 7	43	9929	4	2
2016/2017	February 17	93	762	61	March 21	32	7789	3	3
Mean		**90**	**638**	**56**		**36**	**8571**	**3.7**	**2.7**
CV (%)		**9**	**49**	**14**		**16**	**13**	**16**	**22**

[1] Endormancy: number of days between start of chill accumulation and dormancy release dates. Chill accumulation started on December 3rd, November 22nd and November 7th in 2014, 2015 and 2016, respectively. [2] Ecodormancy: number of days between dormancy release and full flowering dates.

Regarding the extra-late flowering cultivar 'Penta', CUs and CPs were similar in the first and last season (825 CU / 55 CP and 723 CU / CP 54) and much lower (274 CU / 41 CP) in the second. Heat requirements were lower in the first season (7,103 GDH) and higher in the second and third seasons (9,166 and 8,923 GDH, respectively).

Finally, the latest flowering cultivar 'Tardona', showed a similar pattern to 'Penta' cultivar in terms of CUs and CPs: CUs and CPs were higher in the first and last seasons (870 CU / 59 CP and 762 CU / CP 61) and much lower (284 CU / 47 CP) in the second season. Heat requirements were similar in the first and last seasons (7,996 and 7,789 GDH) and higher in the second season (9,929 GDH).

Important differences were observed in the variation of chilling requirements of each cultivar depending on the method applied for calculations (Richardson or Dynamic). Inter-annual variability was always higher with the Richardson than with the Dynamic model, which is shown by the CV of CU and CP. Using the Richardson model (CU), the CV was higher when chilling requirement was estimated in the extra-late cultivar Penta (48%) and the ultra-late 'Tardona' (49%) than in the early flowering cultivar 'Desmayo Largueta' (34%). In general, CVs were smaller in the estimation of chilling requirement using the Dynamic model (20%, 16% and 14% for 'Desmayo Largueta', 'Penta' and 'Tardona', respectively).

The Dynamic model therefore seems to be more accurate in the evaluation of chilling requirements in extra-late and ultra-late cultivars under warm winter conditions.

The Dynamic model showed lower inter-annual variation, as shown by CV. These results agree with those obtained by other authors in almond (Benmoussa et al., 2017); and cherry (Measham et al., 2017), which propose that the Dynamic model is the most suitable/adaptive model for many species and regions. The Dynamic model takes into account high-temperature periods interspersed with moderate chilling temperatures (Erez and Couvillon, 1987). This pattern of temperatures is frequent in our area of study, where winter is usually mild.

In this context, Luedeling and Brown (2011) stated that the chilling requirement data of stone fruit cultivars obtained using different models should always be supplemented with information on the location, study duration and study conditions under which the requirements were determined.

As expected, the chilling requirements of the cultivars were related to their flowering time. On average, the CUs/CPs were 167/21, 607/50 and 638/56 for 'Desmayo Largueta', 'Penta' and 'Tardona', respectively.

Differences between their average GDHs were observed but smaller (6,279, 8,397 and 8,571, respectively). In general, these values were lower than in previous evaluations performed by our group (Egea et al., 2003; Sánchez-Pérez et al., 2012) due to the anomalous second season of evaluation. Without the second season data, the average values would agree with the previous results referenced (CUs/CPs of 185/23, 774/54 and 816/60 for 'Desmayo Largueta', 'Penta' and 'Tardona', respectively).

The fulfilment of chilling requirements is a signal for dormancy release in the flower buds of woody plants (Figure 3.1). If warm weather occurs before the plant completes its chilling requirements, however, dormant meristems are unable to resume growth under favourable conditions (Rohde and Bhalerao, 2007).

Results obtained by Egea et al. (2003) using the Richardson model indicated that flowering date was a consequence of chilling requirements with a small contribution of heat requirements. Results obtained in this study highlighted the likely need to use an alternative model like the Dynamic model to calculate chilling accumulation in the case of warm winters, like that of 2015-2016.

This necessity has been confirmed by other exhaustive studies performed in almond and other fruit-producing species (Luedeling et al., 2009; Benmoussa et al., 2017).

3.3.3. Flowering density and productivity in the different seasons

Table 3.1 shows the flower density and productivity of the almond cultivars assayed –'Desmayo Largueta', 'Penta' and 'Tardona'- in the three seasons of study, both scored on a scale of 1 (very low) to 5 (very high). A certain amount of logical variation between seasons for flowering density and productivity was observed. 'Penta' was the most floriferous and productive cultivar followed by 'Desmayo Largueta' and 'Tardona'.

High temperatures during the second season (2015-2016) greatly affected the productivity of the extra-late ('Penta') and ultra-late-flowering ('Tardona') cultivars more than that of the early-flowering 'Desmayo Largueta'. In fact, 'Desmayo Largueta' had normal flowering and productivity behaviour in this warm season. By contrast, the productivity of 'Penta' and 'Tardona' dropped during 2015-2016 season, despite high density (yet spread-out) flowering. This situation was similar for most of extra- and ultra-late-flowering seedlings of our breeding programme (data not shown).

In the current scenario of climate change (Ponti et al., 2014; IPCC, 2018), several *Prunus* species, including almond, have demonstrated special sensitivity when facing environmental challenges such as warmer winters (Benmoussa et al., 2017; Gannouni et al., 2017). Such events lead to serious damage in fruit production, causing disorders such as altered cold accumulation. Cold accumulation during winter (endodormancy) is fundamental for suitable flowering intensity and, consequently, kernel and fruit productivity in *Prunus* species (Viti et al., 2010; Andreini et al., 2012).

According to our results, there is risk in growing cultivars with high chilling requirements like 'Penta' or 'Tardona' in very warm areas, since the chilling temperatures needed to dormancy release may not be reached. This may not only affect dormancy release and flowering dates, but it is also likely to affect the quantity and quality of the production (Dicenta et al., 2016).

A recent three-season study on sweet cherry in Northern Africa has also shown that warm winters have a greater impact on high-chill cultivars than on low-chill local cultivars (Gannouni et al., 2017). These authors discuss the better adaptation of low-chill cultivars with higher fruit set. Luedeling (2012) has indicated expected global chilling declines, and for the stronger warming scenarios, he anticipates that the losses are likely to impact production. Formerly, the same author recommended that growers explore ways to artificially break dormancy and consider introducing lower-chill cultivars (Luedeling et al., 2009).

3.4. Conclusions

- Our results confirmed that flowering time in almond is a complex process involving the chilling and heat requirements and the genetic background of each cultivar.
- The estimation of chilling and heat requirements under different climatic conditions showed that the Dynamic model is more accurate than the Richardson model, especially under warm conditions.
- If the chilling requirements of the cultivars are not satisfied, productivity could be negatively affected, showing the risk of growing extra-late and ultra-late flowering almond cultivars in warm areas.
- This fact demonstrates the importance of growing each cultivar in the climatically suitable area and the important effect of climatic change on flowering time and productivity of almond.

4. MODULATION OF DORMANCY RELEASE AND FLOWERING TIME IN ALMOND

4. MODULATION OF DORMANCY RELEASE AND FLOWERING TIME IN ALMOND

4.1 Introduction

Dormancy in fruit tree species is described as a rest period determined firstly by endogenous factors (the endodormancy phase) and later by exogenous factors (the ecodormancy phase). The length of endodormancy period is dependent on chilling requirements of each cultivar, whereas ecodormancy period is dependent on heat requirements to flower (see Chapters 1.2 and 3). Both, endodormancy and ecodormancy determine the flowering time.

Late flowering advances in almond implies a qualitative shift for this culture, since extra-late flowering cultivars have spread to colder areas, never considered before because of the frost risk. In this sense, the CEBAS-CSIC Almond Breeding Program has achieved great goals, by the release of cultivars like 'Penta', 'Makako' and 'Tardona'. 'Tardona' flowers 60 days later than early traditional cultivars like 'Desmayo Largueta' (see Chapter 2).

However, as we indicated in Chapter 3 negative effects of the extreme delay of flowering time on tree productivity have been observed (Grasselly, 1978; Kester, 1965). Extra- and ultra-late flowering cultivars can exhibit bud extinction, apical dominance, poor branching, floral bud abortion, irregular flowering and poor fruit set (Oukabli et al., 2003; Petri and Leite, 2003; Atkinson et al., 2013). Low fruit set could be due to the improper development of flower buds during the previous autumn or the competition with the sprouting vegetative buds (Dicenta et al., 2017b). Additionally, higher temperatures occurring during flowering and early fruit development of extra-late flowering cultivars can affect the stigma receptivity, shortening the "Effective Pollination Period".

On the other hand, for cold areas it is important to breed early ripening cultivars, to avoid the harvest in October, when wet and cold weather conditions difficult fruit maturation. Extra-late flowering time could delay ripening time, although in almond, ripening time and flowering time are apparently independent traits. Dicenta and García (1992) found low correlation coefficient between flowering and ripening time, and Sánchez-Pérez et al. (2007) did not found significant correlation between these traits.

In fact, 'Desmayo Largueta' is extra-early flowering but extra-late ripening. However, 'Tardona', which is the latest flowering cultivar released up to date, shows the same ripening time than 'Desmayo Largueta' (Figure 4.1).

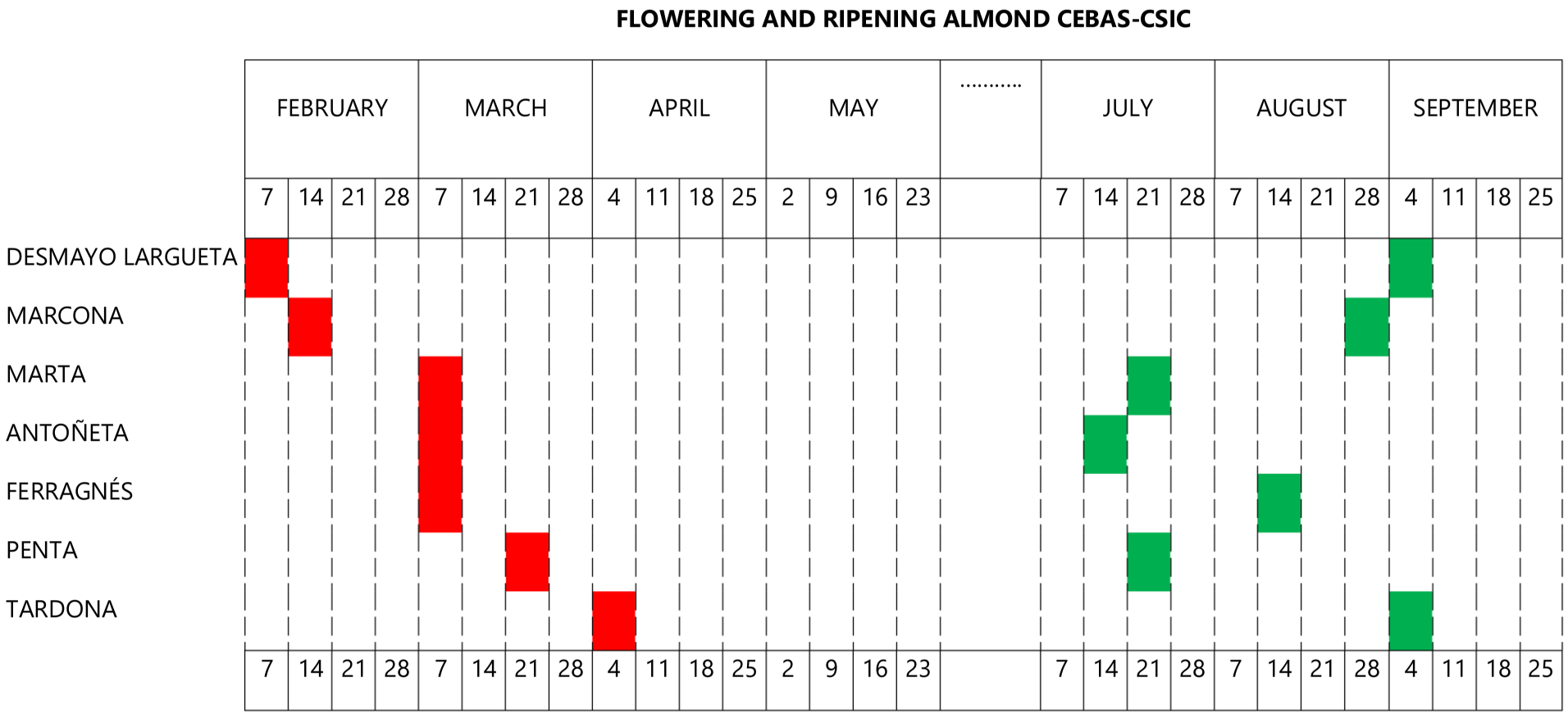


Figure 4.1. Almond cultivars released from CEBAS-CSIC breeding program ('Marta', 'Antoñeta', 'Penta' and 'Tardona') that show the progress on flowering (red) and ripening (green) times, compared to that of the traditional almond cultivars Marcona and Desmayo Largueta. Dates recorded in Santomera (Murcia, Spain).

Regarding the seed size, it has been observed that some extra-late flowering cultivars with early ripening time produce small seeds. This could be related to the short fruit development period or due to the endocarp hardening by high temperatures that limits the seed growth.

To deepen into the genetic basis of endodormancy release and flowering time in almond, the objective of this second experimental chapter was to determine the effect of controlled temperature conditions on flowering time, fruit set, fruit characteristics and maturity time in almond cultivars with different flowering times. Almond cultivars were cultured in big containers and subjected to controlled temperatures in controlled chambers during endodormancy and ecodormancy periods. These assays in controlled conditions complement the results in natural conditions obtained in the Chapter 3 of this Thesis.

4.2 Material and methods

4.2.1 Plant material

Cultivars 'Desmayo Largueta' and 'Tardona' (described in Chapter) were grafted in 'GF677' rootstock clones and established in 40 L containers. Containers were placed outdoors in the experimental field "Tres Caminos" of Santomera (Murcia, Southeast Spain) and were cultured, drip-irrigated, until they came in bearing in 2015, when the assays were initiated. The assay was carried out for three seasons: Season 1 (2015-2016), Season 2 (2016-2017) and Season 3 (2017-2018).

4.2.2 Temperature control for chill and heat accumulation

Plants entered in dormant state in the field, and before the temperature fell, in early autumn, containers were taken to a temperature-controlled chamber (Autumn chamber) to avoid the chill accumulation. Later, containers were moved to another chamber (Winter chamber) for chill accumulation. Once cold treatment was finished, plants were taken to a greenhouse (Spring chamber) for heat accumulation and flowering. Finally, all trees were carried to a shade shelter, for vegetative growth and fruit development (Figure 4.2).

For each cultivar, 10 treatments (T1-T10) with two replicates (two containers) were applied. Cold treatments for endodormancy release and greenhouse conditions for flowering were programmed to provoke flowering in T1 to T10 in a range from natural flowering time of 'Desmayo Largueta' (February 1st) to that from 'Tardona' (April 1st) in our experimental field in Santomera.

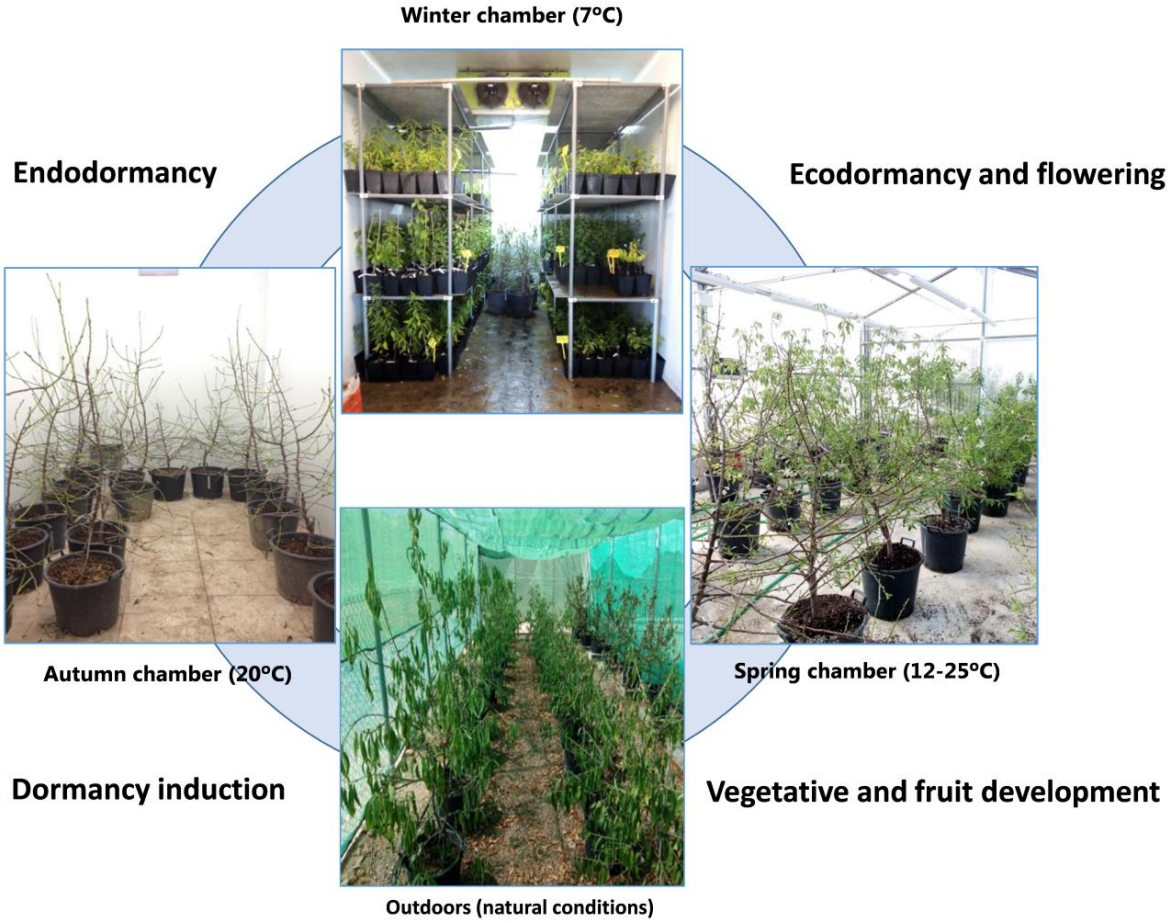


Figure 4.2. Experimental design for flowering time modulation by control of temperature.

Experimental conditions for chill and heat accumulation were adjusted in each season, based on the results obtained in the former season (Figure 4.3).

Autumn chamber

Temperature in the Autumn chamber was raised from 12-15ºC in Season 1, to 20ºC in Season 2 and Season 3. The date of entry in the Autumn chamber was advanced from every season of study to the next (November 17th, October 14th and October 3rd in Seasons 1, 2 and 3, respectively) to allow longer cold treatments and to prevent chill accumulation in the field (Figure 4.3).

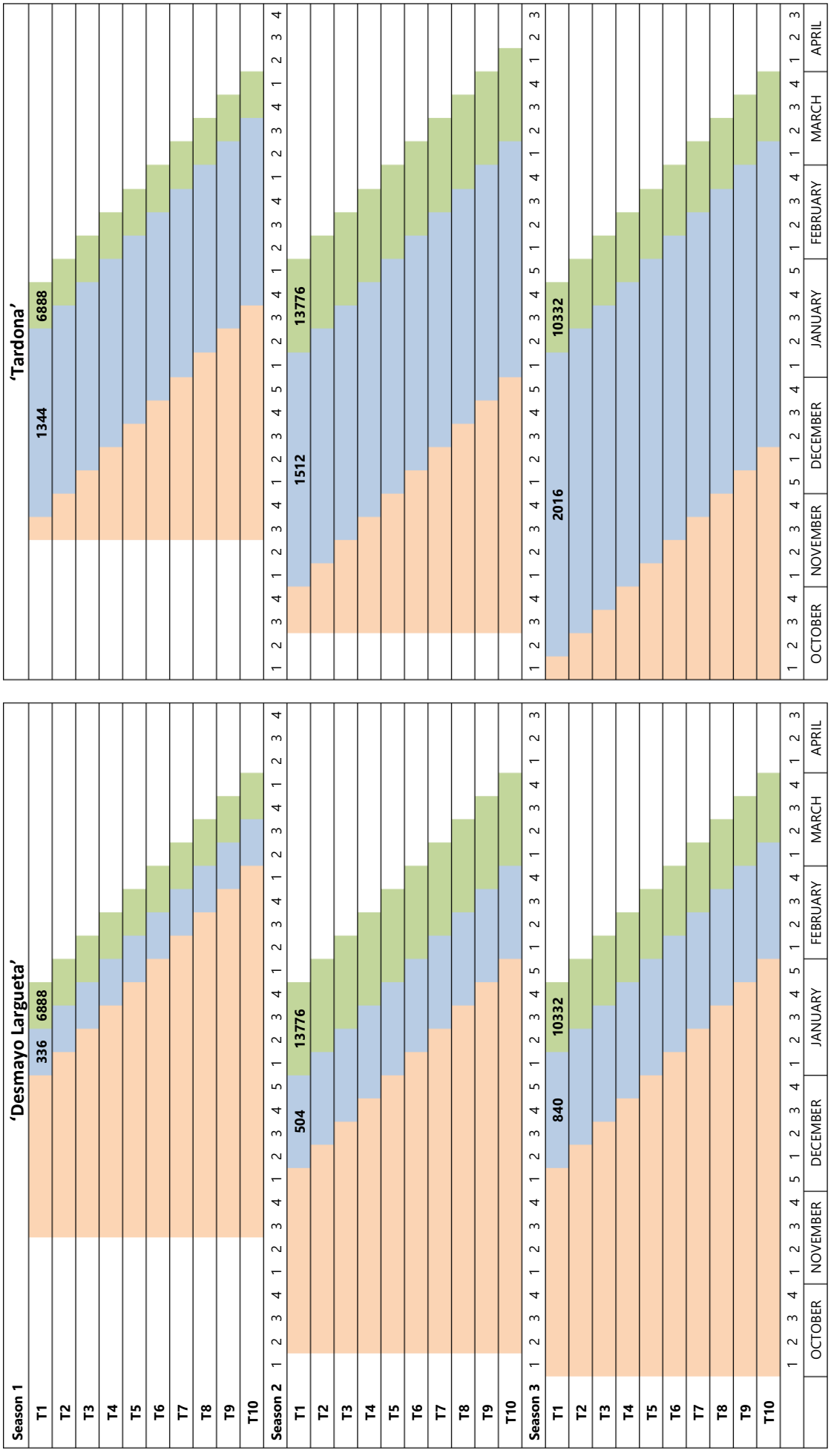


Figure 4.3. Experimental design of temperature treatments (T1-T10) in the three controlled chambers, during the three seasons studied. Autumn chamber (orange bars), Winter chamber (blue bars, with chill units accumulated), Spring chamber (green bars with Growing Degree Hours programmed). Months and weeks along treatments are indicated.

Chill accumulation

'Desmayo Largueta' and 'Tardona' treatments were weekly introduced in the Winter chamber at 7ºC on dates indicated in Figure 4.3. Cold treatment was estimated for fulfilling the chilling requirement of each cultivar, based on data obtained in the experimental field of Santomera (Egea et al., 2003). Treatments differed one from another in the moment of application and thus, in the date of endodormancy release (Figure 4.3). Chill accumulation was estimated as Chill Units by applying the Richardson model (Richardson et al., 1974). Cold treatments increased from one year to another. During the first season, 336 CU (two weeks) were applied to 'Desmayo Largueta' and 1,344 CU (8 weeks) to 'Tardona'. During the second and third year cold treatment was increased to 672 and 840 CU for 'Desmayo Largueta' (3 and 5 weeks) and to 1,512 and 2,016 CU for 'Tardona' (9 and 12 weeks) (Figure 4.3). This was made to assure that there was no problem of insufficient chilling for each cultivar during the trials.

Heat accumulation

Temperature in the Spring chamber were set to 12ºC-25ºC althought fluctuations out of this range were recorded (Annex 1). Heat requirements for flowering were calculated as Growing Degree Hours (GDH) according to Richardson et al. (1975).

Dates of T1 entry to Spring chamber were earlier in Season 2 (December 28th) and Season 3 (January 2nd) respect Season 1 (January 19th). Consequently, the rest of treatments were entered weekly to the Spring chamber. Ecodormancy period estimated was increased in Season 2 and Season 3 in one week and two respect Season 1, respectively (Figure 4.3).

Full flowering time was registered for each tree when the 50% of flowers were opened.

4.2.3. Pollination and fruit evaluation

Flowers were pollinated with pollen previously obtained by manual extraction of flower buds in D state (see Chapter 2, Figure 2.6) from 'Achaak' almond cultivar. Flowers were not emasculated to avoid flower damage and to promote fruit set. In Season 1 trees were not pollinated.

Once all trees had been pollinated (a week after the last pollination), all containers were carried to a shade shelter for tree and fruit development. Fruit set was estimated as the percentage of pollinated flowers that become fruits.

Ripening time was registered when the mesocarp opened, and mature fruits were harvested for evaluation. In shell and kernel weight were estimated and the percentage of kernel calculated.

4.3. *Results and discussion*

4.3.1. Flowering time modulation

Season 1

'Desmayo Largueta' progressed from endodormancy to ecodormancy and flowered inside the Autumn chamber, so for this season flowering data of 'Desmayo Largueta' were no valid. According to Richadson et al. (1974), temperatures between 12-15ºC are low enough to let chill accumulation.

The effect of temperatures on endodormancy depends on the cultivar (Champagnat, 1983) and low chilling requirements cultivars may do not enter into endodormancy state under high temperatures in autumn (Erez, 2000) or may acquire a superficial dormancy state, easy to overcome by moderate temperatures.

'Tardona' treatments flowered gradually according to the date of entry in the Spring chamber, between March 1st to April 12th. These dates were delayed respect programmed dates (February 1st to April 1st). Delay was mainly in the first treatments, T1-T3, which flowered grouped in the same week (data not shown). Heat requirements for flowering ranged between 9,170 and 15,037 GDH, with fluctuations around 10,000 GDH and an average value of 11,328 GDH, but without a clear trend to increase or decrease from T1 to T10. However, a certain trend to reduce the ecodormancy period in the 'Spring' chamber was observed from T1 (41 days) to T10 (21 days) (Figure 4.4).

Season 2

Temperature of the Autumn chamber (20ºC) prevented 'Desmayo Largueta' trees to flower inside the Winter chamber. 'Desmayo Largueta' treatments flowered between February 7th and March 25th, close to dates expected (Figure 4.5).

Ecodormancy period decreased gradually from 41 to 27 days between T1 and T10 treatments. GDH accumulated for flowering were highly stable, around 11,000 GDH, with an average of 10,851 GDH. This means that lower temperatures during the ecodormancy period of T1 are related to a longer ecodormancy period for flowering. GDH accumulated were lower than the expected 13,776 (Figure 4.4).

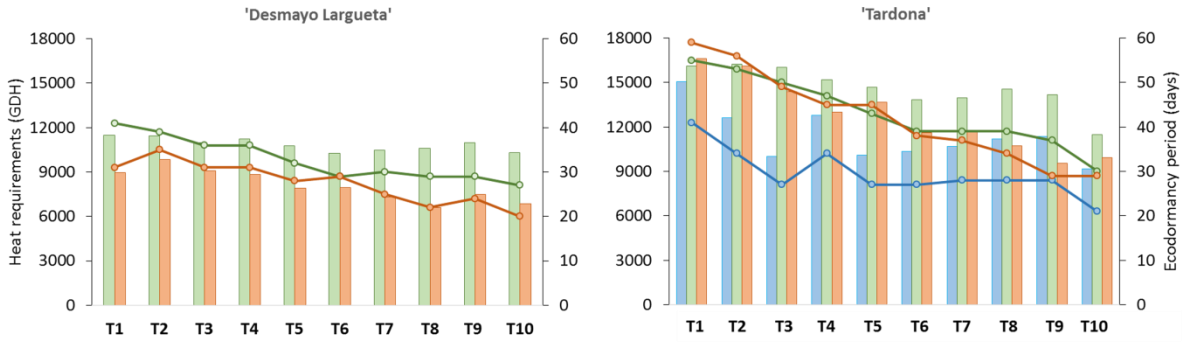


Figure 4.4. Heat requirements (bars) and ecodormancy period (lines) during Season 1 (blue), Season 2 (green) and Season 3 (orange) of treatments. Flowering data of 'Desmayo Largueta' in the Autumn chamber during Season 1 was discarded for analysis.

In case of 'Tardona' treatments, flowering took place between February 21st and March 28th with an important delay of T1 and T2 respect the expected flowering time (February 1st-February 7th) (Figure 4.5). Ecodormancy period also decreased from 55 to 30 days. It supposed a variation of 16,100 to 11,488 GDH, higher than those accumulated during Season 1. The average was 14,623 GDH (Figure 4.4).

In field conditions, GDH accumulated for flowering was 5,492 (Egea et al., 2003) and 6,279 (see Table 3.1 in Chapter 3) in case of 'Desmayo Largueta' and 8,571 GDH for 'Tardona' (see Table 3.1 in Chapter 3). Thus, GDH accumulated for flowering in the Spring chamber was higher than in the field in both cultivars.

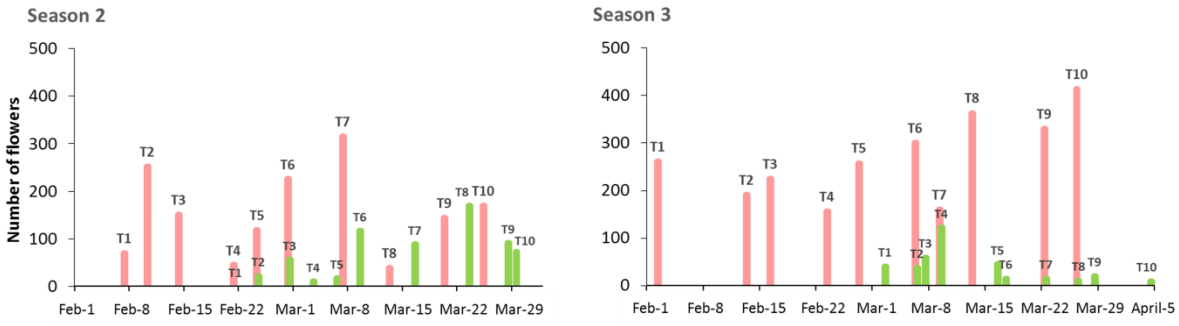


Figure 4.5. Flowering time and number of flowers of 'Desmayo Largueta' (pink) and 'Tardona' (green) treatments during Seasons 2 and 3.

Season 3

'Desmayo Largueta' flowering occurred gradually as observed in Season 2, between February 2nd and March 26th, so results fitted our experimental design (Figure 4.5). GDH ranged from 8,949 in T1 to 6,865 in T10, with an ecodormancy period of 31 and 20 days respectively (Figure 4.4). These results are similar to those obtained in natural conditions (Egea et al., 2003) (see Table 3.1 in Chapter 3). 'Tardona' flowered between March 2nd and April 4th, with an important delay of the first treatments, similarly to which happened in Season 1 and Season 2 (Figure 4.5). Heat requirements and ecodormancy period gradually decreased from 16,626 to 9,940 GDH and from 59 to 29 days, respectively (Figure 4.4). In this case, last treatments were more similar to heat requirements and ecodormancy period registered in the field (see Table 3.1 in Chapter 3).

Other factors apart from chilling requirement could be limiting the flowering in 'Tardona', as photoperiod, that was not present in the Winter chamber (continuously dark) nor in the Spring chamber (natural photoperiod).

4.3.2 Fruit set, ripening time and fruit characteristics

Fruit set

Generally, fruit set ratio was lower than values usually obtained when trees are pollinated in the field, in spite of the high number of flowers pollinated (Figure 4.6).

In Season 2, 'Desmayo Largueta' trees rendered 98 fruits from 1,532 pollinated flowers (6%). Fruit set tended to decrease along treatments. In case of 'Tardona', 116 fruits were obtained from 645 pollinated flowers (15%).

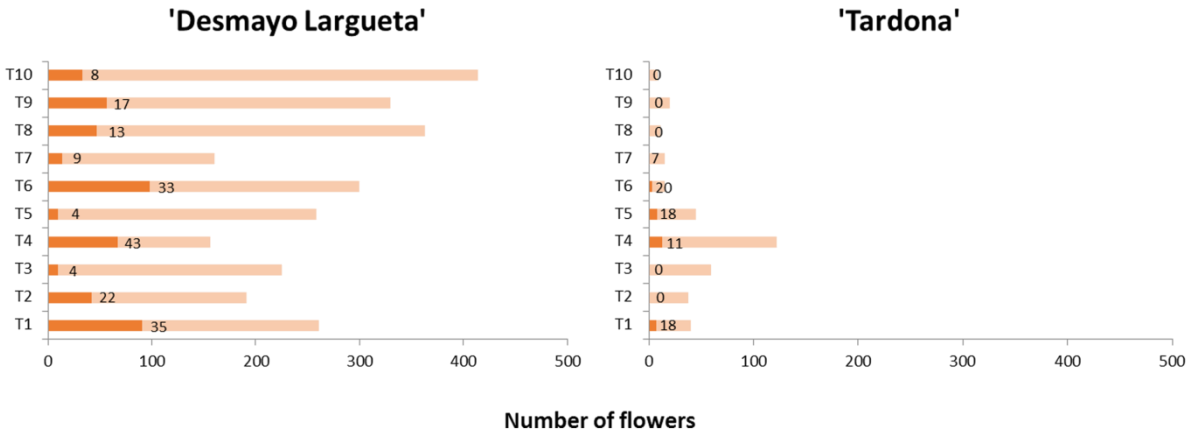


Figure 4.6. Number of flowers pollinated per treatment (orange), number of fruits obtained (dark orange) and fruit set (%) for treatments T1 to T10, for Season 3.

Fruit set slightly increased along treatments (data not shown). In Season 3, fruit set increased to 19% in case of 'Desmayo Largueta' (469 fruits from 2658 flowers). The trend to decrease observed was less clear than in Season 2. 'Tardona' fruit set decreased to 7% in the Season 3 (32 fruits from 374).

Poor flowering was observed during all seasons of study in the ultra-late flowering cultivar 'Tardona', compared to 'Desmayo Largueta' (Figure 4.4). Anomalous flower buds and buds abscission due to sprouting of vegetative buds was observed (Figure 4.7).

Figure 4.7. 'Tardona' flower bud failure. Dried flower buds in different phenological states (A-B) and anomalous flower buds surrounded by leaves (C-D).

This could be explained by a different dormancy state between vegetative and flowering buds. Endodormancy completion is needed for xylem vessels differentiation that allows the rehydration of the developing bud (Bartolini et al., 2006). Vegetative buds released from endodormancy could be in a dominant state and quickly grow during ecodormancy under high temperatures (El Yaacoubi et al., 2016). This could inhibit the growth of other buds by correlative inhibitions and lead to very short ecodormancy (Petri and Leite, 2003). Additionally, it is possible that low chilling requirements cultivars like 'Desmayo Largueta' could better tolerate high temperatures during ecodormancy (El-Yacooubi et al., 2016).

Ito et al. (2015) showed that when chilling is applied too early in Japanese pear (*Pyrus communis* L.) flower buds, the effect on endodormancy release was small, although increasing cold. Moreover, it was confirmed the higher chilling requirement of flower buds compared to vegetative buds (Ito et al., 2015).

Finally, multipistil formation was commonly found in 'Tardona' flowers under experimental conditions, a phenomenon that is not observed in the field (Figure 4.8). This can be related to high temperatures registered in the Spring chamber (Wang et al., 2019) (Annex 1).

Figure 4.8. Double and multipistil formation observed in 'Tardona' flowers.

Ripening time

In Season 2, fruits from four treatments of 'Desmayo Largueta' were obtained. T1 showed an early ripening time (July 13th) compared to the observed in the experimental field of Santomera (first week of September). A progressive delay of ripening time in the following treatments was observed (T2-July 21st; T4 and T7-August 10th) (data not shown). Fruit development period was similar in all treatments, around 155 days. In Season 3, fruits from all treatments of 'Desmayo Largueta' were obtained. The advancement of ripening time (from July 10th) and stability of the ripening period (160 days) was confirmed (Figure 4.9).

Fruits from 'Tardona' T3 and T5 were obtained in Season 2, and the ripening time for both of them was August 10th, similar to ripening time observed in the field (August 17th) (data not shown). In Season 3, 'Tardona' fruits from five treatments were obtained and ripening time was on August 7th. Fruit development period was of 156 and 163 days in Season 2 and from 138 to 158 days in Season 3 (Figure 4.9).

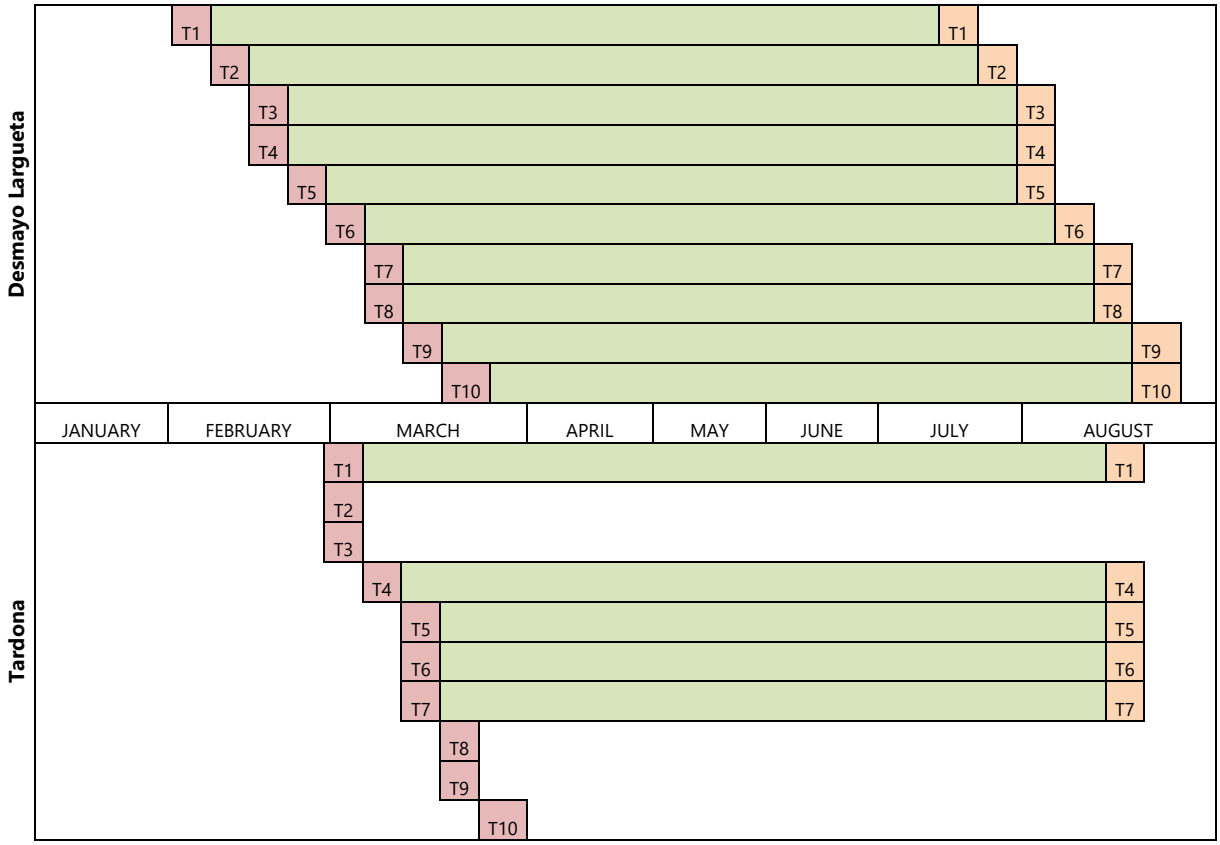


Figure 4.9. Flowering time (pink boxes) and ripening time (orange boxes) of almond treatments (T1-T10) during Season 3. Fruit development period is represented by a green bar.

Fruit weight, seed weight and yield

Generally, fruits and seeds obtained from treatments were smaller than those obtained from trees growth in the field. In Season 3, average fruit and seed weight was of 2.83g and 0.80g for 'Desmayo Largueta'; and 2.45g and 0.63g for 'Tardona' (Figure 4.10). Values obtained in Season 2 was slightly lower (data not shown). Experimental conditions limited the size of the fruits obtained, probably by high temperatures and low relative humidity in the Spring chamber during the earliest phases of fruit development and to the experimental system of trees cultured in containers (Annex 1).

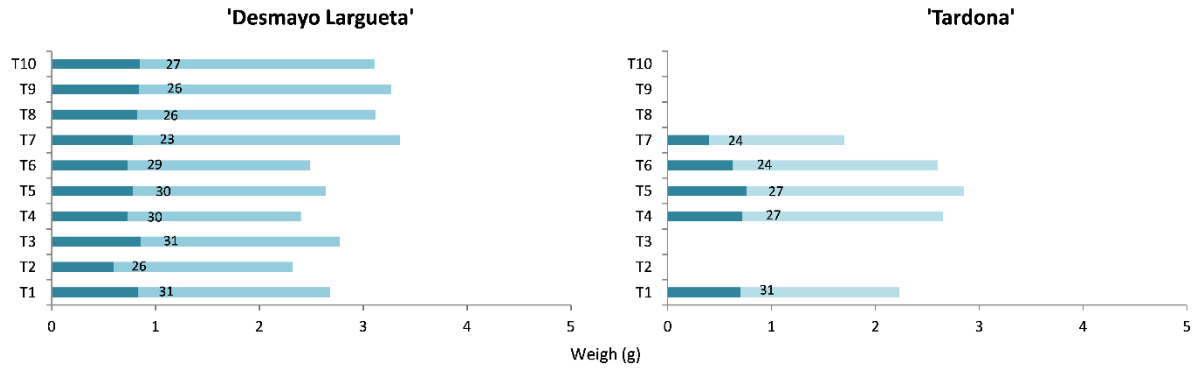


Figure 4.10. Mean weight of fruits (soft blue) and seeds (dark blue) per treatment in Season 3. Fruit yield (%) is indicated in each case.

4.4. Conclusions

- Controlled conditions in Autumn, Winter and Spring chambers successfully modulated flowering time from first February to first April in the extra-early almond 'Desmayo Largueta'. For the ultra-late 'Tardona', flowering could not be advanced to the first week of February, even with the application of enough chill in winter chamber.
- 'Desmayo Largueta' has lower heat requirements than 'Tardona', which were variable depending on the treatment and season, without any clear relationship along the treatments. Since the numbers of days to overcome ecodormancy decrease with the treatments, other unknown factors could be involved.

- Ripening time was much earlier in controlled conditions than in natural conditions for both cultivars, probably consecuence of artificial culture in pots. The delay of flowering time, consequence of treatments, shifted progressively the maturation time in 'Desmayo Largueta' but not in 'Tardona'.
- Fruit set was negatively affected by culture conditions, being lower than in natural conditions, mainly in 'Tardona'. No relationship was observed along the treatments applied.
- Nut and seed weight was smaller than that observed in natural conditions.
- The behaviour of both cultivars under the different treatments shows the complex nature of endodormancy release and flowering time in almond linked to temperature, but probably to other uncontrolled factors.

5. CANDIDATE GENE ANALYSIS DURING ENDODORMANCY RELEASE

5. CANDIDATE GENE ANALYSIS DURING ENDODORMANCY RELEASE

5.1. Introduction

Genetic association studies are useful for studying the inheritance of complex agronomic traits as flowering time (see Chapter 1). Therefore, it is a promising strategy to analyze genes located close to QTL regions identified. In case of almond, Linkage Groups (LG) 1, 2 and 4 are enriched in variants that explain the inheritance of flowering time (see Chapter 1.2.4) (Ballester et al., 2001; Sánchez-Pérez et al., 2012). In addition, cloning studies of specific genes related to dormancy, especially those involved in cold stress have been performed in almond (Barros et al., 2012; 2017).

On the other hand, from a molecular and transcriptomic point of view, discovering the candidate genes whose expression varies as a cause or consequence of endodormancy release and studying the biological role of those genes are priorities for breeding programs (Mazzitelli et al., 2007; Hedley et al., 2010). The differential transcription regulation between early and late flowering cultivars and the genes related to chilling requirements must therefore be identified.

Differentially expressed genes between dormant and non dormant buds were identified by Suppression Subtractive Hybridization (SSH) and microarray in peach (Leida et al., 2010; Leida et al., 2012a). Among them, *DORMANCY ASSOCIATED MADS-BOX 5* (*DAM5*), *ABI FIVE-BINDING PROTEIN (AFP)*, ABA-INDUCED WHEAT PLASMA MEMBRANE 19 (*AWPM19*), *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2c* (*DREB*2c) and a gene coding for Class III peroxidase (Prupe.1G114700) were analyzed by qRT-PCR (Leida et al., 2012b). These genes belong to LG1, excepting *DREB2c* (LG2). *DAM5-6* transcription factors are downregulated during endodormancy progression in peach (Yamane et al., 2011). *DREB2C* and *AFP* are regulators of ABA sensitivity and transduction (López-Molina et al., 2003; Lee et al. 2010), whereas *AWPM19* codes for a membrane protein that enables freezing tolerance (Koike et al., 1997), and transcription of this gene is downregulated by cold treatment (Habu et al., 2014).

On the other hand, candidate genes from LG4 associated to flowering time, chilling requirements and heat requirements were genetically or *in silico* mapped by Castède et al. (2015) in sweet cherry. Genes coding for components of epigenetic complexes were highly represented: *PIE1*, *LYSINE-SPECIFIC HISTONE DEMETHYLASE 1 HOMOLOG 2* (*LDL2*), *EMF2*, *AUXIN RELATED PROTEIN 4* (*ARP4*); and also *GA2ox*, involved in GAs inactivation (see Chapter 1.2.4). EMF2 has been described as a subunit of the PRC2 complex in herbaceous species (Yoshida et al., 2001).

This complex act as a repressor of *FLOWERING LOCUS C* (*FLC*) in response to cold (Bastow et al., 2004; Jiang et al., 2008). In this sense, EMF2 could be considered a flowering activator.

In addition, ARP4 and PIE1 act together in the chromatin remodeling complex SWR1, keeping high levels of *FLC* transcription (Deal et al., 2007; Noh and Amasino, 2003). Finally, *LDL2* is a demethylase that inactive the chromatin state of *FLC,* during dormancy release of flower buds (Jiang et al., 2007).

Recently, endodormancy release in fruit trees was proposed to be due to transcription reprogramming in response to environmental cues, in this case, cold accumulation (Halaly et al., 2008; Horvath, 2009) and may be mediated by oxidative stress (Pérez et al., 2008; Sudawan et al., 2016; Beauvieux et al., 2018).

Regarding the molecular basis of endodormancy release and flowering time in almond at expression level, the objective of this chapter was the expression analysis of candidate genes linked to endodormancy release and flowering and the evaluation of their use as potential markers for monitoring endodormancy release. For that purpose, we have cloned a candidate peroxidase gene related to endodormancy release and analysed its sequence and expression by qRT-PCR from endodormancy to ecodormancy in three almond cultivars from extra-early to ultra-late flowering.

5.2. Material and methods

5.2.1. Plant material

In this work, flower buds from endodormancy (November 10th) to ecodormancy (endodormancy release) were assayed from the almond cultivars 'Desmayo Largueta', 'Penta' and 'Tardona' (see Chapter 2).

5.2.2. Evaluation of endodormancy release

Experiments to evaluate the endodormancy release of the cultivars assayed were conducted in the experimental field of CEBAS-CSIC, in Murcia (south-east Spain), during the 2015-2016 and 2016-2017 seasons according to explained in Chapter 3.

5.2.3. RNA isolation and cDNA synthesis

Flower buds were sampled and processed according to Chapter 2.3. Total RNA was extracted from 15 flower buds according to Le Provost et al. (2007) and treated with DNAseI (Ambion, ThermoFisher Scientific, Waltham, Massachusetts, USA). After checking RNA samples in an RNAse-free agarose gel, cDNA was synthetized using SSIII Reverse Transcriptase (ThermoFisher Scientific, Waltham, Massachusetts, USA) for subsequent amplification by standard and quantitative RT-PCR (qRT-PCR).

5.2.4. Expression analysis by relative qRT-PCR

Relative qRT-PCR experiments were carried out with a One Step Plus real-time PCR system (Applied Biosystems, Foster City, California, USA). Peach, sweet cherry or almond specific primers used are listed on Annex 2. Specific primers based on previously obtained almond sequences were designed using Primer3 (Tm: 60°C, 18-20 bp long, PCR product: 80-120 bp). The qRT-PCR efficiency was checked by the standard curve method. For all real-time qRT-PCR reactions, a 10 µl mix was made including 5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 10 to 20 ng of cDNA, and 2.5 µM of each primer. The qRT-PCR conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The melting temperature in these experiments was set to 60 °C ~ 95 °C, increasing by 0.3 °C/s. Each sample was implemented in three technical replicates. *RPII* was used as reference gene for data normalization (Tong et al., 2009), and the levels of relative expression were calculated by the comparative method (Pfaffl, 2001), using November 10^{th} sample as the reference sample for calculations.

5.2.5. Determination of peroxidase activity

Flower bud samples (100mg) were homogenized with an extraction medium (1/10, w/v) containing 50 mM Tris acetate buffer (pH 6.0); 0.1 mM EDTA; 1 % (w/v) PVP; 1 % (w/v) PVPP; and 0.2 % (v/v) Triton X-100. Total peroxidase activity was analyzed following the oxidation of 4-methoxy-α-naphtol at 593 nm according to Ros-Barceló (1998). Each sample was implemented in at least two biological replicates, except for those indicated in Figure 5.1 by a cross. Peroxidase activity was measured at least twice.

5.2.6. Full-length cDNA amplification and sequence analysis

Full-length cDNA was amplified by standard PCR from the cDNA of the flower buds of the 'Desmayo Largueta', 'Penta' and 'Tardona´ cultivars using 3'-RACE strategy and primers from peach sequences available in databases (Forward primer: 5´-ATG GCC ATG AAG AAG TTG GC-3´; Reverse primer: 5´- TCT CTC CCT TCG TCC CAG TA-3´). High-fidelity PCR was performed using KOD Hot Start DNA polymerase (Novagen, New York, USA), and the product was cloned into *E. coli* using a Zero Blunt Topo PCR Cloning Kit (Life Technologies, Carlsbad, California, USA) for sequencing. PCR was performed in a 30 µl mix according to the manufacturer's instructions with 150ng of cDNA from each almond cultivar and 10µM primers.

The PCR reaction was incubated at 94ºC for 2 min for the initial denaturation step, followed by 35 cycles of 94ºC for 30 s, 62ºC for 1 min and 68ºC for 1 min. A final extension step at 68ºC was set for 10 min. Full-length sequences were aligned and translated using a SeqMan tool within Lasergene software. A BLAST search in the NCBI site and GDR Rosacaeae databases (https://www.rosaceae.org/) was performed using the predicted protein from the three cultivars' sequences in order to study the molecular function of the protein and to collect homologous proteins from *Prunus* and other genera with high identity percentages.

Phylogenetic analysis was performed with phylogeny.fr online software developed by Dereeper et al. (2008): protein sequences were aligned with MUSCLE, and the alignment was curated with Gblocks. The phylogenetic tree was drawn based on the bootstrapping procedure, using the Dayhoff substitution model.

5.2.7. In silico *protein analysis*

Predicted protein domains were identified by homology analysis at the InterPro database (www.ebi.ac.uk/interpro/sequencesearch). A search on SignalP 4.0 (Petersen et al., 2011) was performed to find a potential signal peptide. Physical and chemical protein properties were predicted using a ProtParam tool from ExPASY (https://web.expasy.org/protparam/). Secondary structure analysis was performed with PsiPred (http://bioinf.cs.ucl.ac.uk/psipred/), and the 3D structure was determined by sequence homology analysis (Biasini et al., 2014). The primary amino acid sequence was blasted against the SWISS-MODEL template library (SMTL, last update: 2018-02-28, last included PDB release: 2018-02-23). The templates with the highest quality were then selected for model building. The protein 3D structure model selected for publication was built based on the Arabidopsis peroxidase 1qo4.1a crystal structure

(3Å) (Nielsen et al., 2001). The global and per-residue model quality was assessed using the QMEAN (Qualitative Model Energy Analysis) scoring function (Benkert et al., 2011).

5.3. Results and discussion

5.3.1. Endodormancy release date

According to results obtained in Chapter 3, 'Desmayo Largueta' broke its dormancy state on December 21st in 2015 and on December 15th in 2016. On the other hand, 'Penta' was estimated to break its dormancy state on February 10th in 2016 and on February 2nd in 2017. Finally, 'Tardona' was estimated to break its dormancy state on February 23rd in 2016 and on February 17th in 2017 (see Chapter 3, Table 3.1).

5.3.2. Expression dynamics of dormancy candidate genes during endodormancy release

The initial screening of candidate genes led to the identification of a transcript whose expression pattern was consistent along both years of study and cultivars, showing an induction prior to endodormancy release in all cases, followed by a decrease in most cases (Figure 5.1). This transcript corresponded to the peach peroxidase gene Prupe.1G114700. Thus, further characterization of this gene was performed.

5.3.3. Total peroxidase activity before and after endodormancy release

In the current study, the total peroxidase activity of flower buds from a subset of samples from the same that used for expression analysis were analyzed to determine if the total peroxidase activity pattern correlated with the observed expression dynamic of *PdP40*. In general, endodormancy release is preceded by an increase in total peroxidase activity, as was observed for *PdP40* transcript expression. We observed an increase in the total peroxidase activity of flower buds before endodormancy release, but this was not synchronic with the transcriptional peak (Figure 5.1). This could be explained by the fact that the expression analysis was performed on a single gene, so the results are much more specific than the total peroxidase activity assay, which involves all enzymes with peroxidation activity.

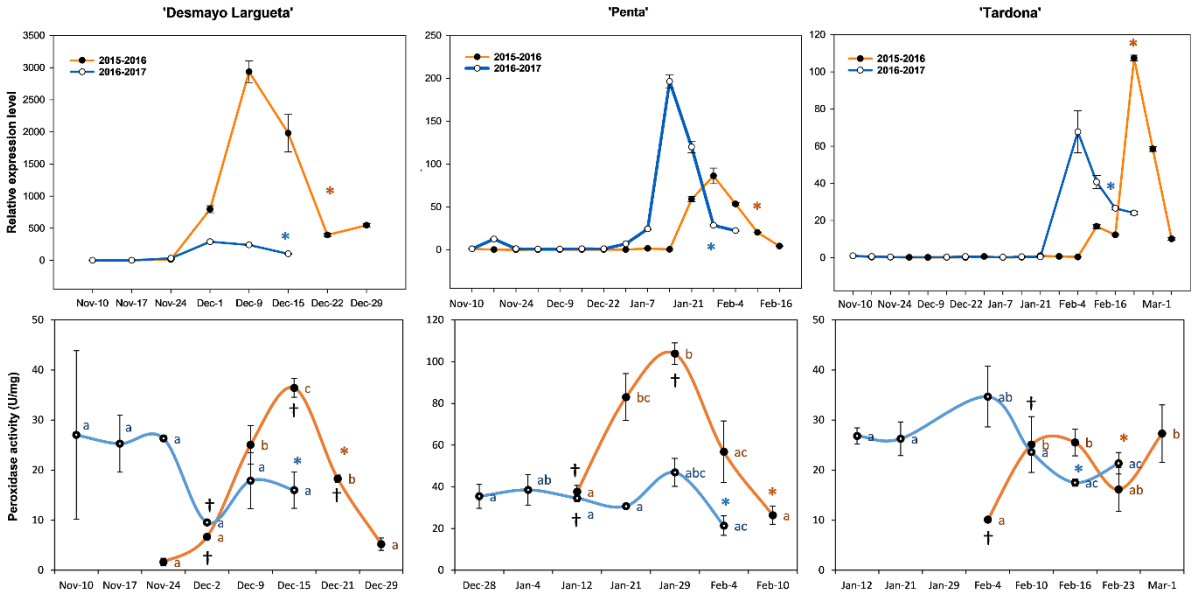


Figure 5.1. Gene expression analysis of *PdP40* and peroxidase activity in the flower buds of 'Desmayo Largueta', 'Penta' and 'Tardona' almond cultivars during the 2015-2016 and 2016-2017 seasons. Endodormancy release (transition from endodormancy to ecodormancy) is indicated by an asterisk in each case. Relative expression values are represented by means of technical replicates ± SE. Peroxidase activity are represented by means of biological replicates ± SD, excepting those samples indicated by a cross, in which SD of technical replicates was applied. Different letters indicate statistical significant differences based on Tukey´s HSD test ($P < 0.05$).

Total peroxidase activity could be another good biochemical marker for monitoring bud dormancy in almond. Several studies indicate that the levels of antioxidant enzymes increase in plants under chilling stress conditions, in order to prevent a toxic accumulation of Reactive Oxygen Species (ROS), such as H_2O_2 (Foyer et al., 1997; Baldermann et al., 2018; Beauvieux et al., 2018). Furthermore, other studies indicate that bud dormancy release coincides with an up-regulation of the antioxidant system (Wang and Faust, 1994; Pérez and Lira, 2005).

By using a proteomic approach, Takemura et al. (2015) analyzed the protein expression during the endodormancy release state in Japanese pear buds. The majority of these proteins were primarily involved in the oxidation-reduction process, such as catalase, peroxidase (POX) and ascorbate peroxidase (APX), all enzymes that can control the H_2O_2 levels in buds. However, only APX activity increased prior to endodormancy release, whereas POX activity did not show statistical changes, and catalase activity progressively declined. In two walnut cultivars with different chilling requirements, the POX activity pattern was somewhat different to that observed in

almond. In walnut, there was an increase in POX activity prior to endodormancy release, and both walnut cultivars showed a POX peak three months before bud dormancy release (Gholizadeh et al., 2017). However, APX activity progressively increased, as occurred in Japanese pear (Takemura et al. 2015), suggesting that the APX increase might play a role in the transition from the endodormancy to the ecodormancy state (Takemura et al., 2015). In addition to H_2O_2 elimination, POXs are involved in cell wall metabolism and plant growth and development. In particular, POXs are responsible for stiffening the cell wall and for lignin and suberin deposition (Pomar et al., 2002).

In pea seedlings and in *in vitro* peach plants, for instance, a correlation between POX increase and growth was observed, suggesting that POX may participate in plant growth regulation (Díaz-Vivancos et al., 2010; Barba-Espín et al., 2010; Clemente-Moreno et al., 2011).

Measuring gene or enzyme activity in fruit trees to monitor bud dormancy is of great interest for fruit production and breeding (Fadón and Rodrigo, 2018). In the case of gene activity, change is first, followed by enzyme activity. These changes define important tipping points in dormancy release and flowering. Monitoring the transition from endodormancy to ecodormancy through gene or enzyme activity can help us understand the plant response to climatic conditions, including spring frost. This knowledge could help limit damage during almond cultivation in the future (Martínez-Gómez et al., 2017).

In addition, knowledge gained through this monitoring should be of great interest in terms of the use and optimization of biostimulants to promote flowering in fruit tree species (Ionescu et al., 2017a; 2017b) in the present climate change and warming context. The moment of application of these biostimulants is critical for success and depends on the endodormancy state of the bud and its transition to ecodormancy (Erez, 1995). Treatments with these biostimulants should be applied at the optimum time for endodormancy release, as they can be null or even toxic depending on the state of the bud (Erez, 1995).

5.3.4. Cloning and sequence analysis of PdP40 (P. dulcis PEROXIDASE40)

Almond consensus sequences from at least three recombinant plasmids of each cultivar cDNA were uploaded to the GeneBank database with the following accessions: MH121042 for 'Desmayo Largueta', MH121043 for 'Penta' and MH121044 for 'Tardona'. The full-length cDNA clone consisted of 1189-1200 bp, depending on the 3´UTR length of the cultivar considered, with an open reading frame of 1029 bp (Figure 5.2). The presence of a poly (A) stretch established that the 3´terminal was complete.

Between 1150 and 1200 bp from the 3´UTR, there is high variability between the sequences from the three cultivars assayed.

Regarding its relative position in genetic maps, *PdP40* locus is close to the QTL associated with chilling requirement qCR1b-2009 (Fan et al., 2010) and with the flowering time marker qFD1 (Hernández Mora et al., 2017). Moreover, the locus is within the QTL associated to the period between endodormancy and ecodormancy release (Romeu et al., 2014).

Sequence analysis showed that the PdP40 protein sequence from 'Desmayo Largueta', and 'Penta' cultivars were identical, whereas the 'Tardona' cultivar PdP40 sequence showed two single amino acid changes on positions 9 and 325 that were not observed in the other *Prunus* genus proteins analyzed (Figure 5.2). These substitutions correspond to L9 instead of F9 and V325 instead of G325. F9 belongs to the predicted signal peptide, so the mutation cannot have structural or functional implications. In contrast, G325V is not a frequent change along the course of evolution, according to BLOSUM62. However, two homologous protein sequences were found to match the same V325 residue, that from olive (*Olea europaea* var. *sylvestris* (Mill.) Lehr) and the species *Nicotiana attenuata* Steud (Figure 5.3). The presence of such a substitution in other sequences supports the non-negative effect of the mutation for these kinds of peroxidase proteins, althought the residue 325 is close to the ligand Ca^{2+} (Figure 5.4). These results indicating the differences between 'Tardona' on the one hand and 'Desmayo Largueta' and 'Penta' on the other are likely due to the different origin of this cultivar (see Figure 2.1 in Chapter 2).

Homology analysis identified the full-length cDNA clone as a coding gene for a Class III plant peroxidase, specifically coding for peroxidase-40, thus it was named *PdP40* (*P. dulcis PEROXIDASE40*). Additionally, a phylogenetic tree was built with the proteins homologous to the predicted PdP40 from woody species and two reference herbaceous species, *Arabidopsis lyrata* (L.) O'Kane & Al-Shehbaz and *Gossypium hirsutum* L. (Figure 5.3).

.

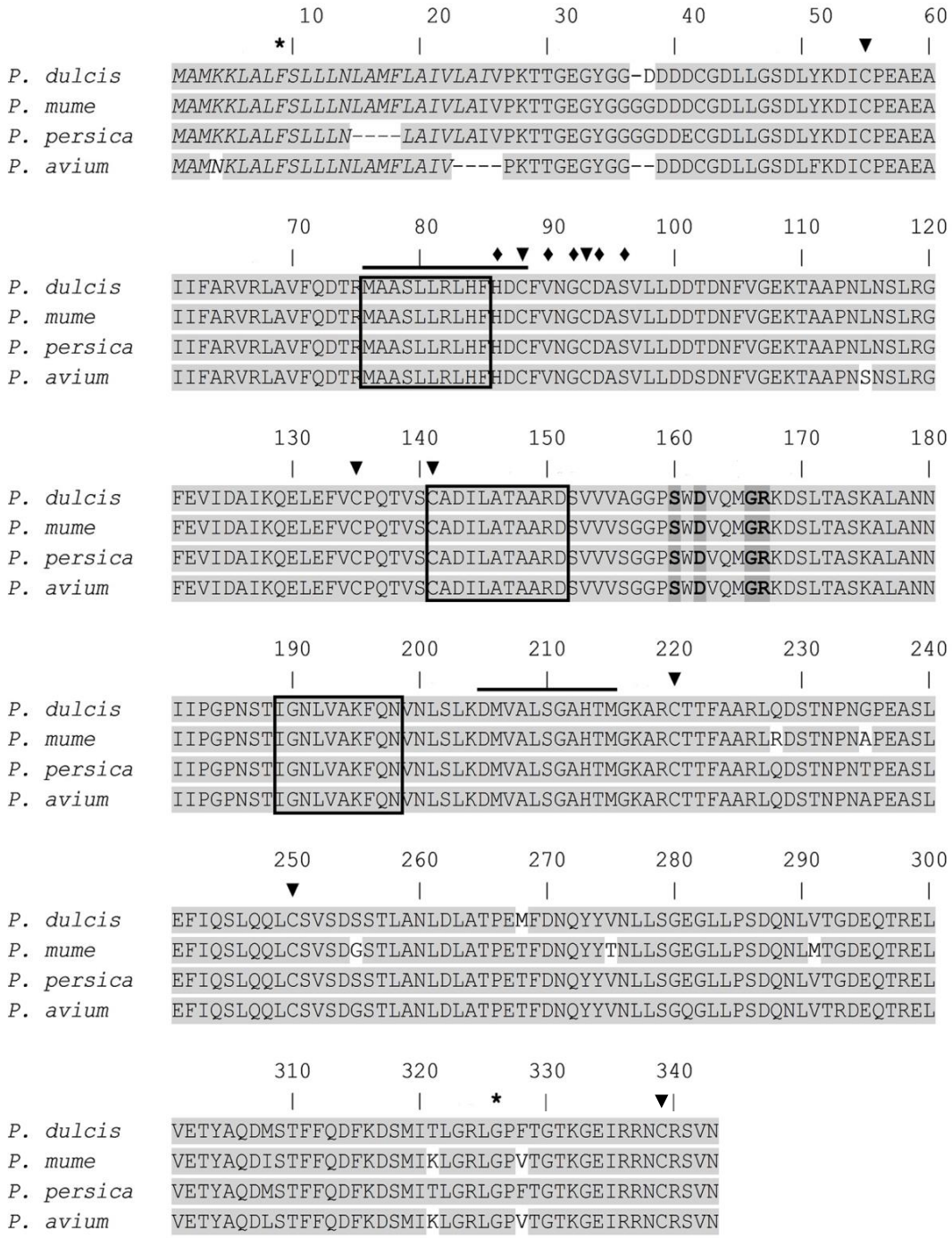


Figure 5.2. Alignment of almond (*P. dulcis*) `Desmayo Largueta´ `Penta´ and 'Tardona' cultivar consensus PdP40 protein sequence with the hypothetical protein sequences of *P. mume* (XP_008223386), *P. persica* (Prupe.1G114700) and *P. avium* (Pav_sc0000028). Identical amino acids are shaded in grey. The star symbol (*) represents amino acids that change in the almond cultivar `Tardona´. Predicted signal peptides are italicised. Conserved helices B, D and F in peroxidases are boxed. Conserved active site residues (76-88: MAASLLRLHFHDC) and a proximal heme-ligand signature (206-215: DMVALSGAHTM) are marked with a line on the top. Eight conserved cysteine residues are marked with an arrowhead symbol (▼). The black diamond symbol (♦) represents residues involved in calcium binding (H86; V90; G92; D94 and S96). Residues involved in a salt bridge are in bold and shaded in dark grey (S160; D162; G166

and R167). The unique residues in almond are M268 and D38. Position 235 is variable between species.

Protein analysis performed in the InterPro and SWISSPROT databases showed that predicted PdP40 contains conserved domains within the Class III peroxidases: the active site and calcium binding residues; a proximal heme-ligand signature; eight conserved cysteine residues, which yielded four disulphide bridges (C54-134, C87-C92, C140-C338 and C219-C249); and amino acids involved in the conserved salt bridge (Figure 5.2). A 24-amino-acid signal peptide was predicted to be cleaved between Ala24 and Ile25. The molecular mass of this PdP40 deduced protein was estimated to be 36.95 kDa, with a pI of 4.57. On the other hand, secondary structure analysis by PsiPred indicated that PdP40 contains the B, D and F conserved helix domains (Figure 5.4A).

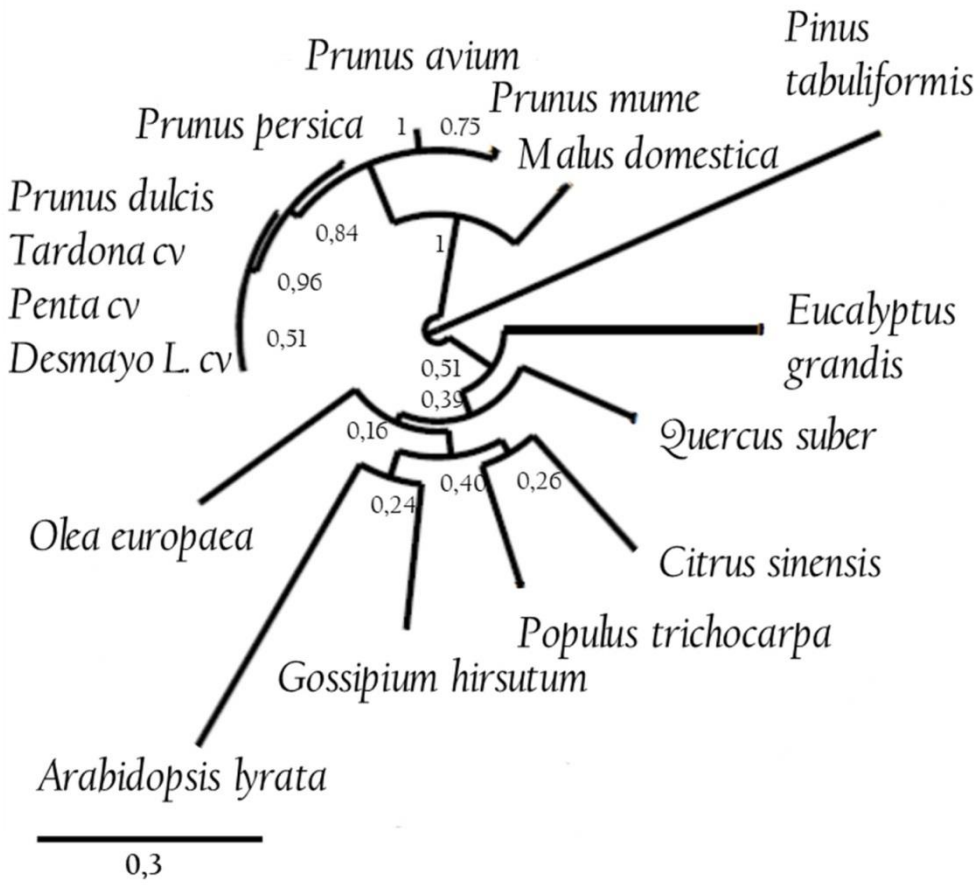


Figure 5.3. Phylogenetic tree based on the alignment of homologous sequences to the PdP40 predicted protein. Bootstrap values are indicated in each branch. Accessions of proteins represented by species name are as follows: *M. domestica* (XP_008390425), *P. mume* (XP_008223386), *P. avium* (Pav_sc0000028), *P. persica* (Prupe.1G114700), *P. dulcis* cv. 'Tardona' (MH121042), *P. dulcis* cv. 'Penta' (MH121043), *P. dulcis* cv. 'Desmayo Largueta' (MH121044), *O. europaea* (XP_022849329), *A. lyrata* (XM_002870120), *G. hirsutum* (NP_001313929), *P.*

trichocarpa (XP_024461812), *C. sinensis* (XP_015388973), *Q. suber* (XP_023870557), *E. grandis* (XP_010027112), and *P. tabuliformis* protein MCE1 (AJP06326).

The PdP40 3D structure model was built based on the *Arabidopsis thaliana* peroxidase 1qo4.1a crystal structure (3Å) (Nielsen et al., 2001) (Figure 5.4B). The PdP40 3D structure was modelled as a monomer, displaying 50.67% sequence identity and 0.44 similarity with 1qo.4.1A. The coverage index was 0.87 (from residue 44 to the end of the protein).

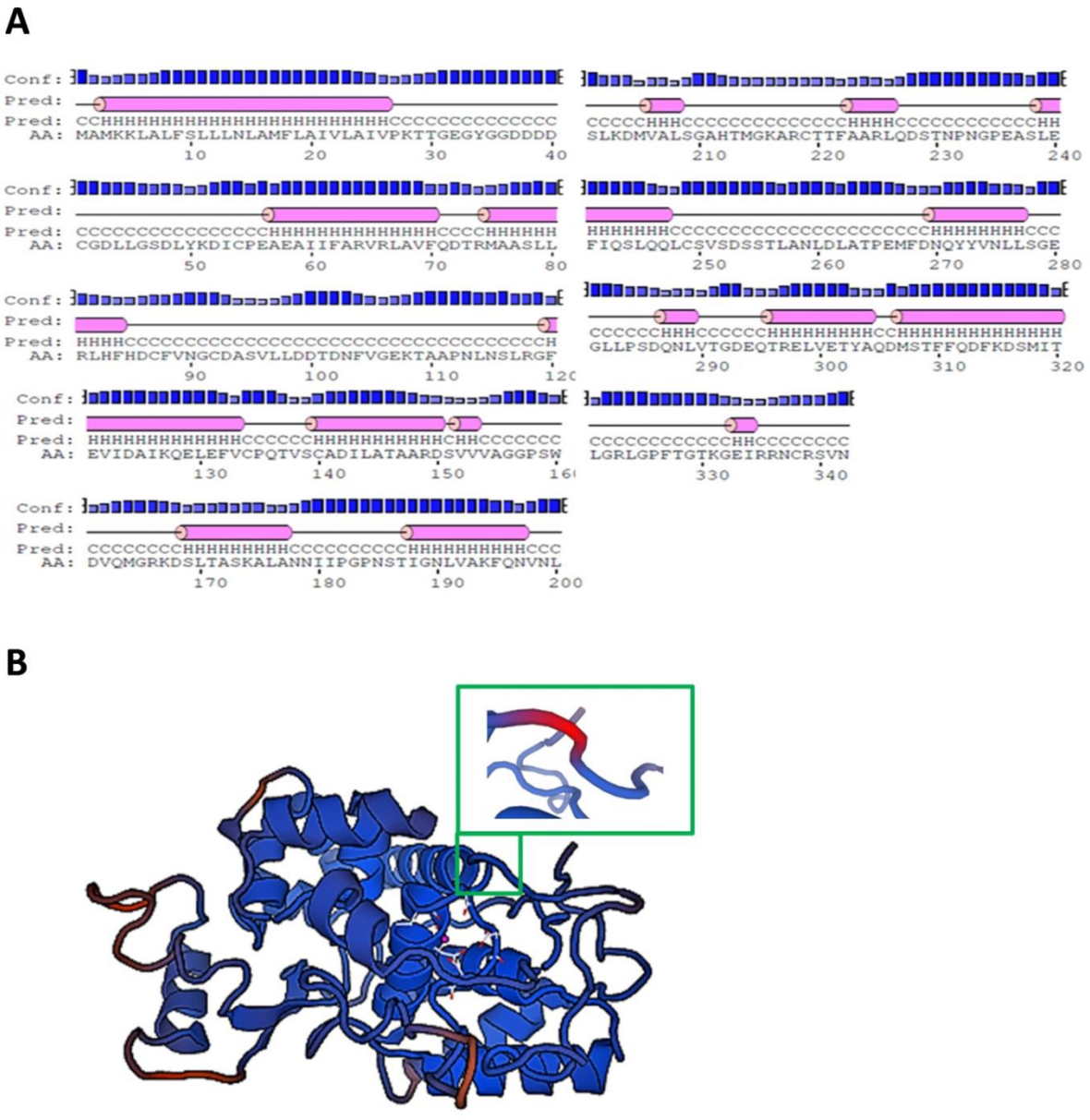


Figure 5.4 A. PdP40 secondary structure predicted by PsiPred software. Blue bars indicate confidence of prediction. Helix domains are represented by rollers. **B.** Predicted 3D structure model of the PdP40 protein obtained by homology between a PdP40 consensus sequence from 'Desmayo Largueta' and 'Penta' cultivars and an Arabidopsis 1qo4.1[a] crystal structure obtained by Nielsen et al. (2001). The conserved calcium binding site is shown. The G325

residue, which is substituted by V325 in the PdP40 sequence of the 'Tardona' cultivar, is boxed and zoomed in.

The Global Model Quality Estimate (GMQE) and QMEAN parameters validated the model, as values were estimated to be 0.72 and (-2.54), respectively. One calcium ion was identified by this method as a ligand in contact with D86, V89, G91, D93 and S95, marked on Figure 5.2. The use of the 'Tardona' PdP40 predicted protein sequence instead of the consensus from 'Desmayo Largueta' and 'Penta' did not disturb the result of the model building, i.e., it did not affect the identified ligand binding site (Figure 5.4B).

The peroxidase superfamily (EC 1.11.1.X) includes three classes according to Welinder (1992). The peroxidases belonging to Class III (EC 1.11.1.7), named plant or fungi peroxidases, are of the secretory type and share the following structural features: a heme group (consisting of an Fe (III) photoporphyrin); two calcium ions (Ca^{2+}); 4 disulphide linkages; and a buried salt bridge (Hiraga et al., 2001).

The PdP40 predicted protein identified in this work contained all the conserved domains mentioned (Figure 5.2). Furthermore, the homology analysis showed that *PdP40* corresponds to a Class III plant peroxidase and is homologous to the flower-specific peroxidase gene *GhPOD* from *G. hirsutum* (Chen et al., 2009). These results indicate the key role this specific Class III peroxidase gene plays in the regulation of bud dormancy in plant species from different families.

5.4. Conclusions

- Results highlighted an important increase in the transcript levels of *PdP40* gene before endodormancy release in the three cultivars with different chilling requirements. The candidate transcript *PdP40* is a member of the flower-specific Class III peroxidase encoding family.
- This increase, during the transition from endodormancy to ecodormancy, was independent of the endodormancy period length, so *PdP40* is a good RNA marker to monitor the release of endodormancy.
- Total peroxidase activity in flower buds shared a common pattern with the *PdP40* expression, and it could be a good biochemical marker for monitoring bud dormancy release in almond.
- Understanding the transition from endodormancy to ecodormancy through gene or enzyme activity is of great interest for the development and use of biostimulants to modulate flowering time.

6. RNA-SEQ MONITORING DURING ENDODORMANCY RELEASE

6. RNA-SEQ MONITORING DURING ENDODORMANCY RELEASE

6.1. Introduction

Because of its economic importance (clearly indicated in the Chapter 1), endodormancy release is being studied in model and cultivated tree species (Ding and Nilsson, 2016) and molecular processes linked to cold accumulation have been associated. After endodormancy, cell to cell symplasmic connection is reestablished by callose degradation at plasmodesmata, a process triggered by cold-induced *FT* in poplar (van der Schoot et al. 2011; Rinne et al., 2011). At the subcellular level, cell wall loosening and ABA synthesis and signaling have been highlighted (Wang et al., 2015; Li et al., 2018). Sugar complexes as raffinose, stachyose and galactinol are induced upon endodormancy for tree protection from chilling, drought and oxidative stress (Nishizawa et al., 2008; Ibáñez et al., 2013). Carbohydrates act not only as a source of carbon and energy upon dormancy release, but they also function as developmental signals (Anderson et al., 2005; González-Rossia et al., 2008; Rabot et al., 2012). These changes are thought to be orchestrated by the activity of transcription factors (see Chapter 1.2.4).

Transcriptional reprogramming leads developmental transitions in plants (Kaufmann et al., 2010). In the last decade, high-throughput sequencing technology has allowed the transcriptomic approach to decipher which gene networks are working upon dormancy onset and release (Bai et al., 2013; Howe et al., 2015; Ionescu et al., 2017b; Zhang et al., 2018). In this sense, the availability of a wide range of flowering time almond cultivars (Martínez-Gómez et al., 2017) offers valuable plant material for dormancy studies. Results obtained in Chapter 5 allowed the identification of a key intermediate state, between endodormancy (A) and ecodormancy state (B), evidenced by the induction of *PdP40* gene (see Figure 5.1 in Chapter 5).

To deepen into the molecular basis of endodormancy release and flowering time in almond at transcriptomic level, complementing the candidate gene analysis of the Chapter 5, the objective of this chapter was the identification of new expression markers by whole transcriptome sequencing of endodormant and ecodormant flower buds of early, extra-late and ultra-late almond cultivars. In addition, new candidate genes associated to endodormancy and ecodormancy obtained from the RNA-seq results were validated using qRT-PCR, and transcriptome changes during endodormancy and ecodormancy of the three almond cultivars were compared. This expression analysis allowed the identification of "early" and "late" flowering time

genes that could give a cue about differential chilling response or other cultivar-specific traits in almond.

6.2. Material and methods

6.2.1 Plant material

In this work, flower bud samples from endodormancy (November 10th) to ecodormancy (endodormancy release) were used from early, extra-late and the ultra-late flowering almond cultivars ('Desmayo Largueta', 'Penta' and 'Tardona') (see Chapter 2). Experiments to evaluate the endodormancy release of the cultivars assayed and flowering time were conducted in the experimental field of CEBAS-CSIC, in Murcia (south-east Spain), during the 2015-2016 (Season 1) and 2016-2017 (Season 2) seasons according to explained in Chapter 3.2.2. Chill accumulation as Chill Portions (CPs) and heat accumulation as Growing Degree Hours (GDHs) during the two winter seasons were considered according to results obtained in Chapter 3 (Figure 6.1).

6.2.2. RNA extraction and sequencing

Flower buds (15 flower buds per sample) were collected and processed according to Chapter 2.3. Samples were classified according to their natural phenological state at the moment of sampling in the field. Season 1 samples were selected for RNA sequencing and classified as: endodormant flower buds, in phenological state A, in which the flower bud is small and enclosed by brown scales; and ecodormant flower buds, in phenological state B, in which the flower bud is swollen but still enclosed by its brown scales (Figure 2.7 and 2.8). The A samples were taken the first day of sampling (November 10th of 2015) for all cultivars assayed. The B samples were taken at the date at which every cultivar had fulfilled its chilling requirements. The AB samples were considered when *PdP40* started to increase its expression levels in all the three cultivars (see Chapter 5, Figure 5.1). In this moment, at least every cultivar had achieved the 40% of their chilling requirements (Figure 6.1).

For experimental design, it was considered that at certain time point between AB and B samples endodormancy release may take place (Figure 6.1). Total RNA was extracted according to a standardized protocol (Le Provost et al., 2007). RNA samples were treated with DNaseI (AMBION) and purified with PowerClean Pro RNA Clean-Up kit (MOBIO). Among 2-5 µg per RNA sample were sent to Sistemas Genómicos (Valencia, Spain) for library preparation and RNA sequencing.

All cDNA libraries were prepared according to Illumina protocols and subsequently sequenced (125 bp paired end) using the Illumina HiSeq 2000 platform.

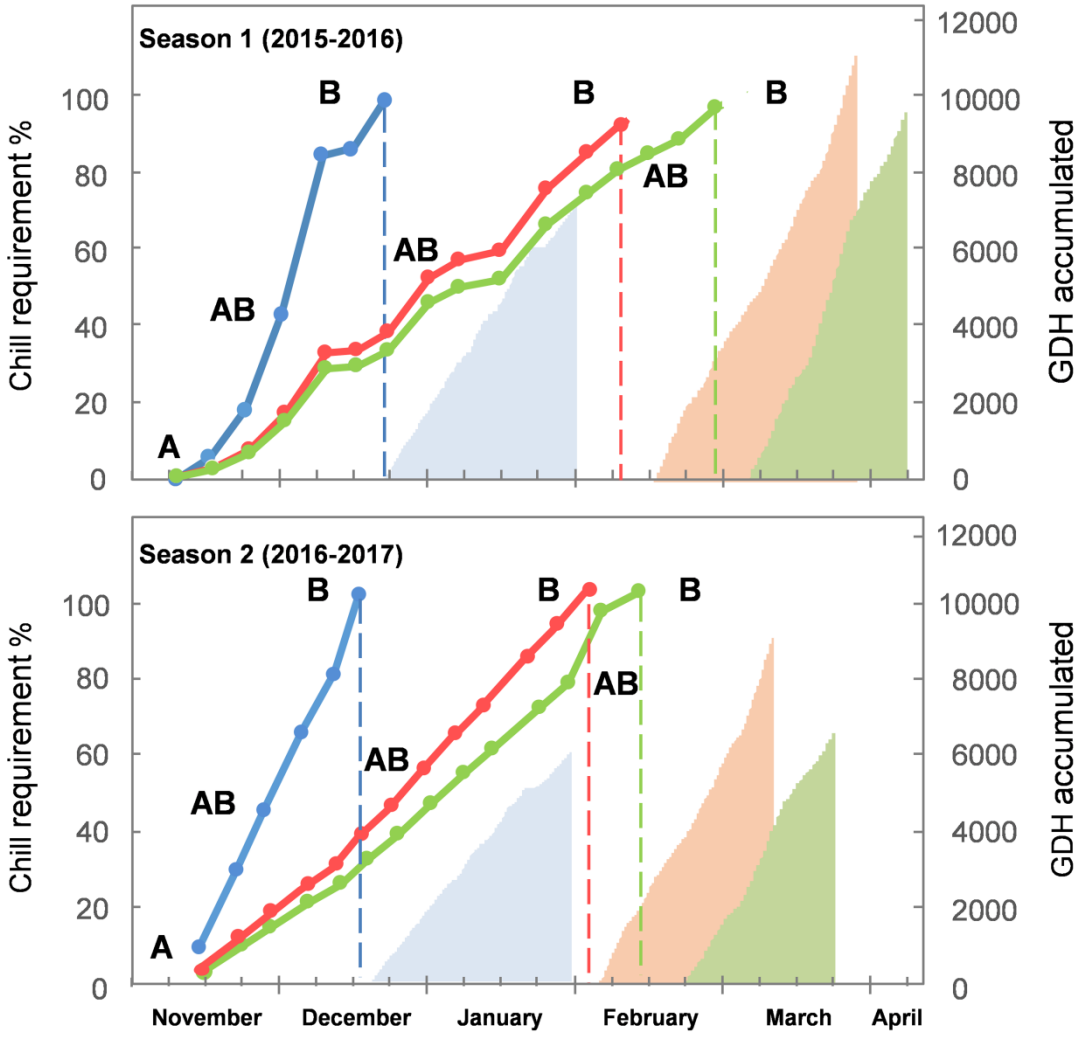


Figure 6.1. Sampling schedule of the two seasons of study of Desmayo Largueta (blue), Penta (red), and Tardona (green) almond cultivars. Chilling requirements (CR) % completion is indicated for each cultivar by lines and Growing Degree Hours (GDHs) for flowering is indicated by areas. Flower bud samples harvested in different phenological states are represented by points. Samples sequenced (A, AB and B states) are from Season 1.

6.2.3. De novo *transcriptome assembly, read mapping, and gene annotation*

RNA-seq generated 934 million reads, approximately 100 million reads per sample. Quality control of generated sequences (reads) was done using FastQC software. Next, reads were trimmed for quality. A custom-made assembly consisting of 68,361 gene fragments (contigs) from almond, was obtained by *de novo* assembly of over 628 million 125 bp paired end reads (after duplicate removal) coming from all

sequenced samples. Average contig length was 854 bp. For gene expression analysis, all sequence reads were mapped (per sample) to this reference. Between 73 and 77% of all reads could be mapped to this reference. Trimming, mapping and de novo assembly were done using CLC Bio Genomics Workbench software (Qiagen, Hilden, Germany).

To determine gene expression levels, Reads Per Kilobase per Million reads (RPKM, read counts corrected for library size and transcript length) values were calculated using CLC Bio software. Only contigs with more than 100 reads in at least one of the 9 samples were used for downstream analysis (40,757 of the 68,361 available contigs).

To functionally categorize all almond contigs in the assembled reference, Gene Ontology (GO) terms were assigned to each contig using Blast2GO software. GO terms provide a controlled vocabulary to describe the functions of genes across species. Blast2GO is an automated tool for the assignment of GO terms based on sequence similarity (Conesa et al., 2005).

Data concerning number of reads and reads counted per sample, transcriptome contigs sequence and Blast2GO annotation is found available at https://mega.nz/#F!oVVTGYQL (encryption key: B5RDXKKusiec-kothvtZoQ).

6.2.4. Differential expression analysis and cluster analysis

Two sets of DEGs were generated according to different criteria: 1) 'Dormancy' DEGs by dormancy state (A-AB-B) comparison including all samples from the three cultivars and 2) 'Cultivar' DEGs by almond cultivar comparison. Contigs of candidate DEGs were mapped in the almond genome (Sánchez-Pérez et al., 2019) using CLC Genomics Workbench 5 (Qiagen, Hilden, Germany) and those not found were searched in the Texas almond genome v.2 (https://www.rosaceae.org/analysis/295). Hierarchical clustering (Euclidean distance, average linkage) were performed using TM4 software (Saeed et al., 2003). Mean centered log2 (RPKM) values were used.

All DEGs included in clusters with adjusted RPKM values are available at https://mega.nz/#F!kE9nnYbS (encryption key: jkK6dsV39IoNFkd6zSOitQ).

6.2.5. Identification of orthologous genes

Orthologous genes were identified by comparing 43,673 protein sequences from the sweet cherry genome Prunus_avium_v1.0 (Shirasawa et al., 2017), and the translated 68,361 contigs from abovementioned almond assembly to a collection of

250 translated kiwifruit contigs (Balk et al., 2017) using OrthoMCL software (Li et al., 2003). For the IntraBlast analysis, a threshold E-value of 1x10-5 was used, the OrthoMCL inflation factor used was 1.8.

6.2.6. Quantitative Real Time PCR

Sequenced samples (A, AB and B) from Season 1 together with intermediate samples collected between them (A2, AB2 and B2) were used to validate the obtained RNA-seq data by qRT-PCR. A2 and AB2 samples are the previous and next states to AB sample. In addition, samples from Season 2 were classified following the same criteria and they were also analyzed by qRT-PCR (Figure 6.1).

Dormancy candidate DEGs, based on A-AB-B states comparison, were used for RNA-seq validation (Table 6.1). Primers were designed using CLC Genomics Workbench 5, preferably on exon-exon junctions to ensure no genomic DNA amplification. Following adjustment was used for primer design: PCR product size 75-160 bp; Primer melting temperature (Tm): 54-60°C, and the other adjustments were kept in the default mode. Primers used for qRT-PCR are listed in Annex 3. Only primers with an efficiency >80% were used. Primer efficiency was tested by the standard curve method. The qRT-PCR was performed in 10 µl reactions of 2xSYBR GREEN PCR mix using the StepOnePlus® PCR system of Applied Biosystems. 5 ng cDNA and 2.5 µM of each set of primers were added. Relative expression was determined taking into account the efficiency of each primer pair (Pfaffl, 2001) using *60S* and *OEP16* as housekeeping genes for data normalization. Relative expression was calculated taking samples in A state (November 10th for Season 1 and November 17th for Season 2) as the reference sample. Each sample was implemented in three technical replicates. Correlation index was calculated between relative expression levels and RNA-seq reads (RPKM values).

6.3. Results and discussion

RNA sequencing technology allowed the study transcriptomic changes in flower buds developing from the endodormant to ecodormant state. Moreover, this analysis shows the comparison between cultivars with different chilling requirements and flowering times. In this sense, we can discuss results obtained regarding endodormancy release in the three cultivars assayed and also the identification of which genes have cultivar-associated expression patterns.

Table 6.1. Dormancy candidate genes mapped onto 'Lauranne' almond v.1 genome. Selection of genes was done by the presence in clusters or by orthology to kiwifruit and sweet cherry transcripts.

***P. dulcis* code**	**Cluster**	**OrthoMCL**	**Gene name**	**Gene annotation**
Prudu.08G187400	1	Yes	*XET*	*XYLOGLUCAN ENDOTRANSGLUCOSYLASE 2*
Prudu.08G242500	1	No	*4CL*	*4-COUMARATE:CoA LIGASE*
Prudu.01G134200	2	No	*NIP7*	*AQUAPORIN, NODULIN-LIKE INTRINSIC PROTEIN 7*
Prudu.01G175100	2	Yes	*STR13*	*STRICTOSIDINE SYNTHASE-LIKE 13*
Prudu.01G238900	2	Yes	*β-1,3-GLUCOSIDASE*	*GLUCAN ENDO-β-1,3-GLUCOSIDASE*
Prudu.05G129900	2	Yes	*β-1,4-GLUCANASE*	*ENDO-β-1,4-GLUCANASE*
Prudu.05G142100	2	Yes	*SWEET10*	*BIDIRECTIONAL SUGAR TRANSPORTER SWEET10*
Prudu.05G071900	3	No	*AIP2*	*ABI3 INTERACTING PROTEIN 2, E3 LIGASE*
Prudu.01G481600	4	No	*DAM1*	*DORMANCY ASSOCIATED MADS BOX 1*
Prudu.01G481600	4	No	*DAM2*	*DORMANCY ASSOCIATED MADS BOX 2*
Prudu.03G036100	4	No	*LOX3-1*	*LINOLEATE 13S-LIPOXYGENASE 3-1, CHLOROPLASTIC*
Prudu.03G257900	4	Yes	*RS5*	*RAFFINOSE SYNTHASE 5*
Prudu.01G25465394	-	Yes	*MIZ1*	*MIZU-KUSSEI 1*
Prudu.02G12667860	-	Yes	*PBL15*	*PROBABLE SERINE/THREONINE-PROTEIN KINASE 15*
Prudu.02G194600	-	Yes	*ATHB12*	*ARABIDOPSIS THALIANA HOMEOBOX-LEUCINE ZIPPER PROTEIN 12*
Prudu.04G01017920	-	Yes	*S40*	*SENESCENCE REGULATOR 40*
Prudu.04G070600	-	Yes	*NCED5*	*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 5, ABA BIOSYNTHESIS*
Prudu.04G098100	-	Yes	*EXO7*	*EXORDIUM-LIKE 7*

6.3.1. Chilling requirements for endodormancy release and heat requirements for flowering time

Chilling requirements for endodormancy release and heat requirements for flowering of 'Desmayo Largueta', 'Penta' and 'Tardona' during the two seasons of study are shown in Figure 6.1, as well as heat requirements for flowering. The advanced phenology observed during the second season of study, together with the lower and higher chilling and heat requirements respectively are discussed in Chapter 3.

6.3.2. Identification and annotation of dormancy related genes

Dormancy-related differentially expressed genes (DEGs) were identified by using a coefficient of variation (CV) selection criterion during the progression from endodormancy to ecodormancy (A to AB to B). The CV was calculated as the ratio of the standard deviation over all three measurements and the absolute value of the average expression over the time series.

A total of 1,164 genes were differentially expressed with a CV > 0.8 in the 'Desmayo Largueta', 'Penta' and 'Tardona' cultivars, and were subsequently subjected to hierarchical average linkage clustering. This resulted in clusters 1 to 4. Clusters 1 (13 DEGs), 2 (385 DEGs), and 3 (170 DEGs) contained DEGS that were upregulated during the endodormant (A) to the ecodormant (B) transition. Cluster 4 included 290 DEGs that were downregulated during this period (Figure 6.2A).

6.3.3. DEGs annotation and GO enrichment analysis

Generally (in all clusters), GO terms belonging to primary metabolic and oxidation-reduction processes were the most enriched biological processes. This has been corroborated in other RNA-seq studies about endodormancy release (Bai et al., 2013; Ionescu et al., 2017; Zhang et al., 2018). Oxidative burst triggers the endodormancy release, derived from treatment or under natural conditions (Gholizadeh et al., 2017; Ionescu et al., 2017b). On the other hand, metabolic reprogramming is needed both for dormancy release signaling and also for growth resumption for flowering (Zhang et al., 2018). However, specific categories to "up" and "down" clusters were found (Figure 6.2B; Annex 4). In this sense, and considering the number of DEGs/cluster, it is important to highlight that during endodormancy release there were more DEGs upregulated than downregulated.

Moreover, categories involved in pollen development and metabolic and structural changes are found in clusters 1, 2, and 3, whereas there are DNA transcription regulation categories specific to cluster 4, the downregulated cluster (Figure 6.2B; Annex 4).

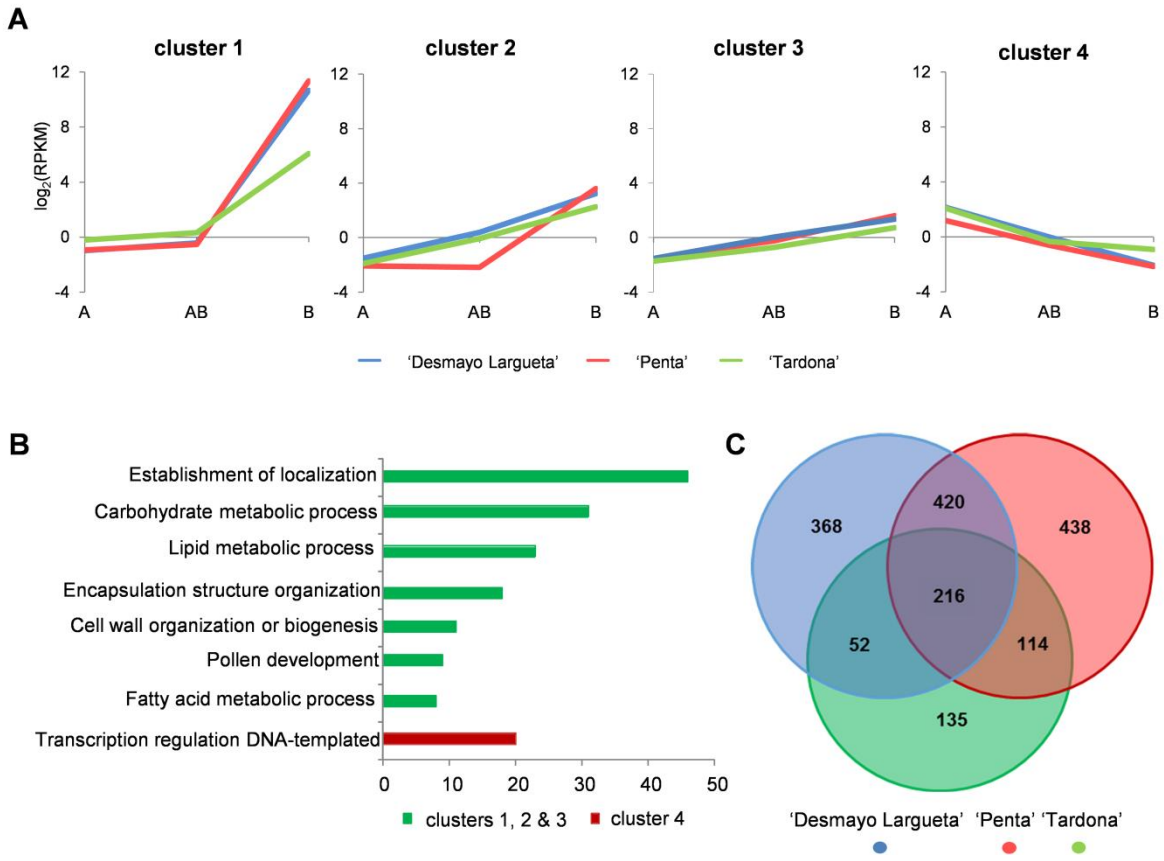


Figure 6.2. Almond DEGs identified over dormancy release. **A:** Dormancy genes clusters patterns represented by log2 (RPKM) average of genes included within each cluster. **B:** GO terms related to "Biological process" enriched and specific to "up" clusters (1, 2 and 3) or the "down" cluster (4). **C:** Almond cultivar DEGs represented by Venn diagram.

6.3.4 Candidate genes selected for qRT-PCR validation

A number of dormancy-related DEGs, whose expression varied within the three cultivars series, were selected from clusters 1 to 4, mapped onto the almond genome, and used for qRT-PCR validation (Table 6.1). In addition, genes were selected by identifying almond genes orthologous of dormancy-related genes from kiwifruit and sweet cherry using OrthoMCL (Li et al., 2003).

By comparing a collection of 250 unique dormancy-related contigs from kiwifruit (Balk et al., 2017) to the sweet cherry genome and our *de novo* assembled almond transcriptome, 147 protein groups that contained at least 1 sequence from each species were generated. In total, 11 almond genes having orthologous sequences in kiwifruit and sweet cherry and showing comparable expression pattern in dormancy-related RNA-seq experiments (Balk et al., 2017) were selected (Table 6.1). Relative expression of all genes during Season 1 and Season 2 is shown in Figure 6.3 and Annex 5 and 6, respectively.

- Endodormancy-associated genes

The first selection of genes was classified as endodormancy-associated genes as they displayed the highest expression during endodormancy (A to AB) and lower expression during ecodormancy (AB to B).

In cluster 4, *RAFFINOSE SYNTHASE 5* (*RS5*) and *LINOLEATE 13S-LIPOXYGENASE 3-1* (*LOX3.1*), involved in carbohydrate and lipid metabolism respectively, decreased their expression over the endodormancy release period (Figure 6.3A). In fact, primary metabolism and carbohydrate metabolism were among the most represented functional categories across the DEGs identified in this work (Figure 6.2B). In chestnut, *RAFFINOSE SYNTHASE* (*RS*) expression increased under low temperatures and raffinose was accumulated in vegetative tissues under drought stress (Nishizawa et al., 2008; Ibañez et al., 2013). Thus, the *RS5* expression pattern observed in almond cultivars during endodormancy release is in line with former observations. Lipid peroxidation is also a process that supplies energy and carbon skeleton. Moreover, fatty acid synthesis occurred in the last phase of dormancy or during dormancy release (Gabay et al., 2019). *LOX3.1* (cluster 4) encodes a plastidial peroxygenase able to catalyze membrane galactolipid peroxidation and free fatty acid chains as linolenic and linoleic acid (Pilati et al., 2014).

Expression decrease of *LOX3.1* was observed during potato meristem tuberization (Viola et al., 2007), which is considered an analog process to dormancy release (Rodríguez-Falcón et al., 2006). Habu et al. (2014) observed that a *LOX2* transcript was downregulated under cold treatment of Japanese apricot vegetative buds.

Moreover, upstream regulators of floral integrators *DORMANCY ASSOCIATED MADS-BOX* (*DAM1-2*) genes (cluster 4), were also analyzed by qRT-PCR and their expression levels were downregulated concomitantly with endodormancy release

(Figure 6.3A). This evidence in line with the dormancy induction and maintenance role associated to DAM1-2 (Falavigna et al., 2019; Hao et al., 2015).

Several orthologs of ABA-related genes were also analyzed (Table 6.1). Among the, two endodormancy-associated genes were found:

The almond ortholog to *NINE CIS EPOXYCAROTENOID DIOXYGENASE 5* (*NCED5*) and *ARABIDOPSIS THALIANA HOMEOBOX-LEUCINE ZIPPER PROTEIN 12* (*ATHB12*). *NCED5* codes for an enzyme involved in ABA biosynthesis and the expression decreased over dormancy release (Figure 6.3A). Dormancy maintenance role associated to ABA has been reported by different studies (Li et al., 2018; Wang et al., 2015; Zhao et al., 2018). In this sense, biosynthesis and signaling by ABA metabolism related genes are expected to be downregulated during endodormancy release.

On the other hand, *ATHB12* codes for a transcription factor that is induced by drought conditions and ABA signal in Arabidopsis (Valdés et al., 2012). In almond, *ATHB12* ortholog expression is downregulated during endodormancy release, excepting the case of 'Desmayo Largueta' in Season 1 (Figure 6.3A) and 'Penta' in Season 2 (Annex 5). These observations point out to a temporary repression of *ATHB12* towards endodormancy release, probably by ABA signal.

- Ecodormancy-associated genes

A second set of genes was classified as ecodormancy-associated genes as they displayed the highest expression during ecodormancy (AB to B) and lower expression during endodormancy (A to AB).

From cluster 3 (see Table 6.1) *ABI3 INTERACTING PROTEIN 2* (*AIP2*) ortholog was selected. Expression was induced in AB to B samples and therefore considered as an ecodormancy-associated gene. *AIP2* codes for a RING-type E3 ligase that targets ABI3 to 26S proteasomes for proteolysis regulation, which leads to inhibition of the ABA response (Zhang et al., 2005). A role of this protein has been described for seed dormancy in Arabidopsis (Liu et al., 2010). In wheat, in H_2O_2-treated seeds, a transient increase of expression in *AIP2* was observed (Gao et al., 2012).

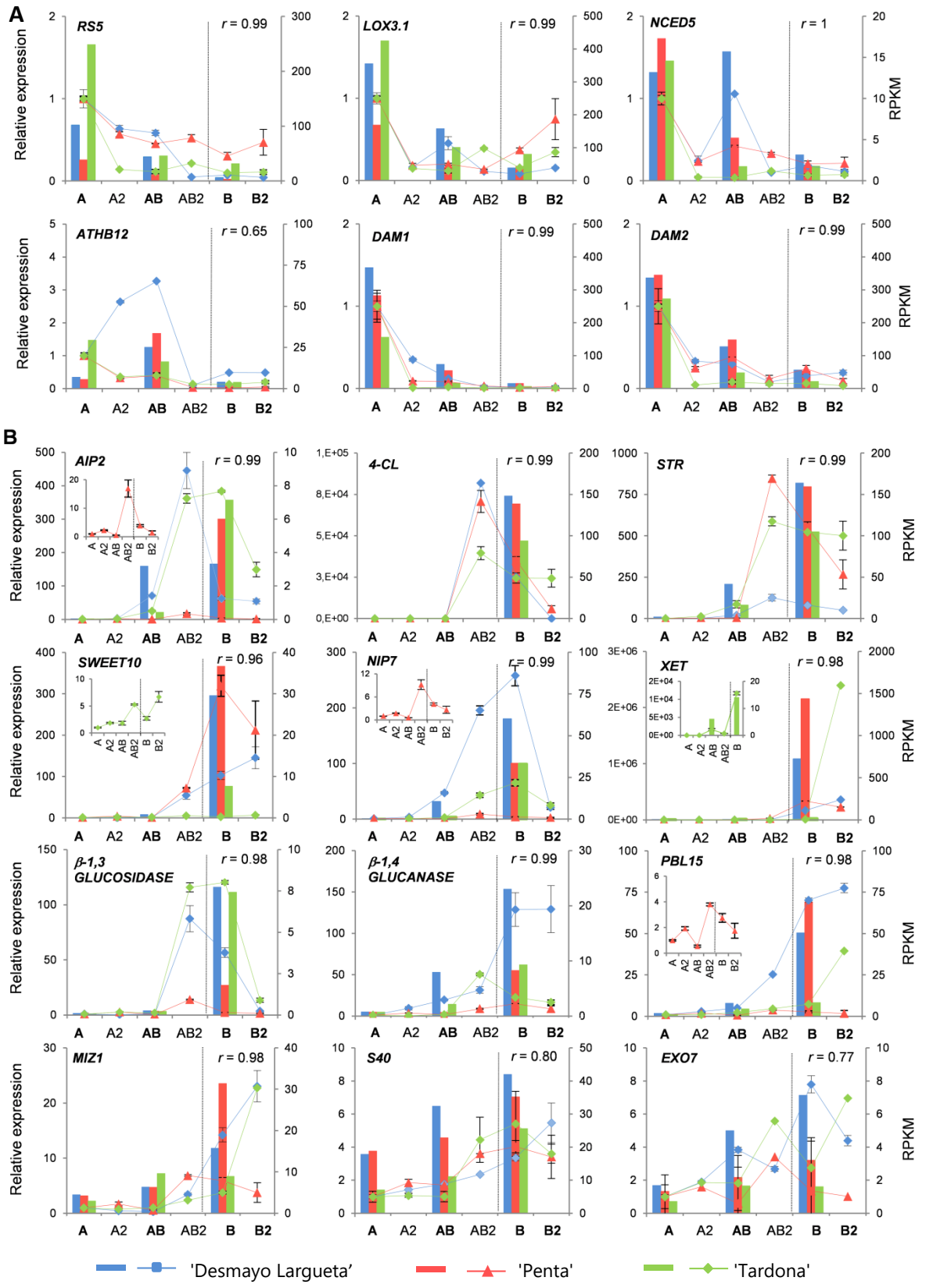


Figure 6.3 Expression dynamics of candidate DEGs by qRT-PCR in Season 1 (**A**). Endodormancy genes. (**B**) Ecodormancy genes. Endodormancy release (between AB2 and B state) is indicated by a dashed line. Sequenced samples by RNA-seq are in bold (A, AB, B). Relative expression of genes is represented by means of technical replicates ± SE. Pearson correlation mean between relative expression to A state and RPKM values of every cultivar is indicated on the right side at the top.

Two genes from clusters 1 and 2 showed an induction period peaking in AB2: 4-*COUMARATE-CoA LIGASE* (*4CL*) and *STRICTOSIDINE SYNTHASE-LIKE 13*, (*STR13*) orthologs (Table 6.1). These genes code for enzymes involved in the phenylpropanoid and indole alkaloid biosynthetic pathways, respectively. Phenylpropanoid compounds as coumaric acid are accumulated during cold treatment in peach seeds (Leida et al., 2012c).

Once the chilling accumulation is over, coumarate could be transiently targeted for CoA ligation and downstream reactions. Moreover, phenylpropanoid compounds accumulation dropped during ecodormancy (Conrad et al., 2019). Strictosidine is a key intermediate in the indole alkaloid biosynthetic pathway (Stöckigt et al., 2008) and *STR* expression has already been associated to peach late flowering flower buds (Rios et al., 2013). STR has been related to pollen exine biosynthesis during microsporogenesis in Arabidopsis (Dobritsa et al., 2009). In our work, pollen development is one of the most enriched up-regulated processes (Figure 2b). In fact, meiosis event during microsporogenesis has been associated to the end of endodormancy in apricot (Julian et al., 2011).

The rest of dormancy DEGs from clusters 1 and 2 are involved in cell to cell transport and cell wall remodeling, showing upregulation from the AB to B state (Table 6.1). This was confirmed by qRT-PCR (Figure 6.3B):

Sucrose transporters are up-regulated during dormancy release in walnut (Decourteix et al., 2008), leafy spurge (Chao and Serpe, 2010) and in Japanese apricot (Zhang et al., 2018). Moreover, sucrose is mobilized and accumulated in grapevine buds following Hydrogen Cyanamide (HC) treatment for endodormancy release (Ben Mohamed et al., 2012). All this evidence supports the dormancy release induction of *BIDIRECTIONAL SUGAR TRANSPORTER SWEET10* (*SWEET10*) almond ortholog. However, this increase peaked rather late, at B or B2 state (dormancy released or after released buds), which indicates the sugar utilization capacity of buds as metabolic sinks (Figure 6.3).

NODULIN-LIKE INTRINSIC PROTEIN 7 (*NIP7*) belongs to a subfamily of the aquaporin proteins that are predominantly expressed in developing flowers in Arabidopsis. NIP7 has been proposed as boric acid channel to pollen cell wall building in tapetal cells (Routray et al., 2018). The narrow developmental expression pattern observed in Routray et al. (2018) is also observed in almond from AB/AB2 state to B2 (Figure 6.3B). This further supports the pollen development genes role as one of the main expression markers of endodormancy to ecodormancy transition.

Glucosyl Hydrolases (GH) encoding genes are highly represented in this analysis: 1,3-β-Glucosidases, also referred to as GH17, are callose-degrading enzymes promoted by GAs (Rinne et al., 2011; Gai et al., 2013). 1,4-β-Glucanases (GH9) and xyloglucan endotransglusylases (GH16) are well-described cell wall loosening enzymes (Fry et al., 1992; Baumann et al., 2007; Huang et al., 2019) with an active role in the cell wall remodeling coupled to endodormancy release. Thus, the up-regulation of the almond orthologs of *GLUCAN ENDO-β-1,3-GLUCOSIDASE* (*β -1,3-GLUCOSIDASE*), *ENDO- β -1,4-GLUCANASE*, (*β-1,4-GLUCANASE*) and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE 2* (*XET2*), is in line with their biological function during dormancy release (Figure 6.3B).

On the other hand, we found other almond orthologs by the OrthoMCL analysis (Table 6.1). *PROBABLE SERINE/THREONINE-PROTEIN KINASE 15* (*PBL15*) ortholog expression was associated with the active state of flower buds (B) (Figure 6.3B). PBL15 is considered a Ser-Thr kinase. The kinase protein KINβ1 encoding gene increased its expression upon endodormancy release in Japanese apricot (Zhang et al., 2018). In this case, KINβ1 promotes sugar metabolism and cell division, characteristics from a metabolically active flower bud, which could be applicable to almond PBL15.

Expression of almond orthologs of genes *MIZU-KUSSEI 1* (*MIZ1*) and *SENESCENCE REGULATOR 40* (*S40*) was highly induced from AB2 to B state (Figure 6.3B).

The role of *MIZ1* in flower buds endodormancy release remains unknown. Functional studies with MIZ1 in Arabidopsis are related to the establishment of root hydrotropism (Kobayashi et al., 2007; Yamazaki et al., 2012). MIZ1 is an endoplasmic reticulum-associated protein whose biological function is the generation of Ca^{2+} cytosolic signal in the phloem tissue for water tracking (Shkolnik et al., 2018). Although it has been reported that MIZ1 expression is induced by ABA (Moriwaki et al., 2012), probably generated by drought stress, this does not seem to be the case in endodormancy release. MIZ1 overexpression enhances osmotic tolerance (Miyazawa et al., 2012) and whatever would be its functional involvement in dormancy release, it is likely related to water foraging after endodormancy release. Another possibility would be the participation in calcium wave generation for rapid systemic signaling (Shkolnik et al., 2018), even for symplasmic re-connection (van Bel et al., 2014). From our results, it is clear that MIZ1 expression is necessary when metabolism of the flower bud is re-activated. Therefore, functional implication of MIZ1 in the endodormancy to ecodormancy shift would be interesting to unveil, as it could be a new expression marker.

S40 has not been characterized in fruit trees to date. In herbaceous species, the expression of this gene is known to be responsive to senescence in leaves (Krupinska et al., 2002; Gregersen and Holm, 2007; Fischer-Kilbienski et al., 2010; Jehanzeb et al., 2017), and induced by darkness, pathogen attack, ABA and Salicylic Acid (SA) in Arabidopsis, and a DNA binding motif in its sequence was identified (Fischer-Kilbienski et al., 2010). A new role for S40 gene in dormancy of flower bud meristem in deciduous tree species such almond is revealed by the progressive increase from AB2 state. The progressive increase of S40 over chill accumulation and dormancy release is not explained by its transcriptional ageing-dependent accumulation. However, leaf senescence during autumn and winter dormancy are interrelated processes. Perennial plants mutants affecting MADS-box genes as peach *evergrowing* (*evg*) (Rodríguez et al. 1994; Bielenberg et al. 2004) and poplar overexpressing the birch *FRUITFUL* (*FUL*) (Hoenicka et al., 2008) maintain their leaves photosynthetically active during the autumn/winter season.

Finally, almond ortholog of *EXORDIUM 7* (*EXO7*) expression increased and decreased alternatively over chill accumulation and dormancy release period (Figure 6.3B). EXO7 is thought to regulate the brassinosteroids (BRs) response by transcriptional control in Arabidopsis (Coll-Garcia et al., 2004) and its expression is repressed by cold in grapevine (Kim et al., 2016). However, an *EXO* transcript appeared to be upregulated under cold treatment in the microarray analysis performed by Habu et al. (2014) in Japanese apricot vegetative buds.

In this work, the expression pattern of *EXO7* could be explained by the role of BRs in the control of cell-cycle for meristem maintenance (Farrar et al., 2003; Schröder et al., 2009; Gai et al., 2013). Furthermore, GAs biosynthesis is regulated by BRs in Arabidopsis (Unterholzner et al., 2015). In poplar, a gene-set associated to BRs appeared as upregulated from endodormancy to ecodormancy transition of vegetative buds (Howe et al., 2015).

6.3.5 Early versus late flowering candidate genes

Another group of DEGs was selected. For this, DEGs were defined for each cultivar using a CV threshold of 1.4. Instead of looking for overlap among the three varieties, we looked for genes that were differentially expressed in the early flowering cultivar 'Desmayo Largueta' but not in the late flowering cultivars Penta and Tardona (368 DEGs), or vice versa (114 DEGs). Together, this resulted in 482 DEGs that we named early and late flowering candidate genes (Figure 6.2C). RPKM values and Blast Top Hit description of these DEGs are available at https://mega.nz/#F!ccUijY5D (encryption key: fK6b3AV0bwM8vgeX41gIdA).

From the early and late flowering genes, we removed those contigs which were absent in the early flowering cultivar 'Desmayo Largueta' and present in the late flowering cultivars 'Penta' and 'Tardona' and vice versa. These contigs were mapped onto the almond genome and almond genomic code was assigned (Table 6.2 and Table 6.3).

Some of the early cultivar genes (Table 6.2) are orthologs of *SPHINGANINE C4-MONOOXYGENASE 1* (*SBH*) isoforms, *WALLS ARE THIN-1 RELATED PROTEIN AT2G39510-LIKE, WAX ESTER SYNTHASE/DIACYLGLYCEROL ACYLTRANSFERASE 1* (*WSD1*) and disease resistance genes as *TMV-RESISTANCE PROTEIN* and *RESISTANCE GENE ANALOGUE 1* (*RGA1*). SBH enzymes are involved in the sphingolipids synthesis pathway; while AT2G39510-LIKE is an auxin transporter (Ranocha et al., 2013) and WSD1 is involved in drought tolerance (Li et al., 2008; Patwari et al., 2019)

Regarding the genes only expressed in the late flowering cultivars we found among others: orthologs of the *RICESLEEPER* family of transcription factors, and of *SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG PROTEIN 5* (*SUVH5*), which codes for a histone methyl-transferase; together with orthologs of other disease resistance genes (Table 6.3).

Table 6.2. DEGs identified expressed in the early flowering almond cultivar Desmayo Largueta and not in the late flowering cultivars Penta and Tardona. "Prudu." genomic code came from *P. dulcis* Genome (DDBJ database: AP019297-AP019304) annotation and "Prudul" gene code came from *P. dulcis* Texas Genome v2.0 (https://www.rosaceae.org/analysis/295).

***P. dulcis* code**	**Blast Top Hit Description**
-	gi|694319684|ref|XP_009348116.1|PREDICTED: uncharacterized protein [*Pyrus x bretschneideri*]
Prudu.01G515900	Not hits found
Prudu.02G068100	gi|645251927|ref|XP_008231892.1|PREDICTED: TMV resistance protein N-like [*Prunus mume*]
Prudu.02G227100	gi|1220055291|ref|XP_021813732.1|WAT1-related protein At2g39510-like [*Prunus avium*]
Prudu.03G265800	Not hits found
Prudu.04G144200	gi|645263101|ref|XP_008237074.1|PREDICTED: putative disease resistance protein RGA1 isoform X2 [*Prunus mume*]
Prudu.04G270000	Not hits found
Prudu.05G175300	Not hits found
Prudu.05G190200	gi|1162557061|ref|XP_020419344.1|SNAP25 homologous protein SNAP33
Prudu.07G197300	gi|1139762772|gb|ONH97869.1|hypothetical protein PRUPE_7G215100 [*Prunus persica*]
Prudu.08G024300	gi|1162572451|ref|XP_020424465.1|uncharacterized protein LOC109950319 [*Prunus persica*]
Prudu.08G109600	gi|1220093990|ref|XP_021833862.1|TMV resistance protein N-like [*Prunus avium*]
Prudu.1102S000200	Not hits found
Prudu.1494S000500	Not hits found
Prudul26A004395	gi|1027109451|ref|XP_016651093.1|PREDICTED: serine-rich adhesin for platelets-like [*Prunus mume*]
Prudul26A006096	gi|595791755|ref|XP_007199626.1|O-acyltransferase WSD1 [*Prunus persica*]
Prudul26A016423	gi|1220057083|ref|XP_021814681.1|uncharacterized protein LOC110757394 [*Prunus avium*]
Prudul26A017504	Not hits found
Prudul26A01834T1	Not hits found
Prudul26A027853T1	Not hits found
Prudul26A030734	gi|1162573887|ref|XP_020424972.1|sphinganine C4-monooxygenase 1 isoform X2 [*Prunus persica*]
Prudul26A030734	gi|1162573885|ref|XP_007204865.2|sphinganine C4-monooxygenase 1 isoform X1 [*Prunus persica*]
Prudul26A032938	Not hits found

Table 6.3. DEGs identified expressed in the late-flowering almond cultivars Penta and Tardona and not in the early flowering cultivar Desmayo Largueta. "Prudu." genomic code came from *P. dulcis* Genome (DDBJ database: AP019297-AP019304) annotation and "Prudul" gene code came from *P. dulcis* Texas Genome v2.0 (https://www.rosaceae.org/analysis/295).

***P. dulcis* code**	**Blast Top Hit Description**
-	Not hits found
-	Not hits found
Prudu.01G108400	Not hits found
Prudu.01G507600	gi|1027091638|ref|XP_016646926.1|PREDICTED: disease resistance protein RML1A-like [*Prunus mume*]
Prudu.02G102500	gi|1027109346|ref|XP_016651059.1|PREDICTED: zinc finger BED domain-containing protein RICESLEEPER 2-like [*Prunus mume*]
Prudu.03G111600	gi|1162511259|ref|XP_020411395.1|zinc finger BED domain-containing protein RICESLEEPER 2-like [*Prunus persica*]
Prudu.03G150700	Not hits found
Prudu.04G243300	Not hits found
Prudu.06G136600	gi|1220097986|ref|XP_021800895.1|rust resistance kinase Lr10-like [*Prunus avium*]
Prudu.07G041200	gi|1162572394|ref|XP_020424433.1|probable disease resistance protein At4g27220 [*Prunus persica*]
Prudu.07G196000	gi|1220042646|ref|XP_021807146.1|pre-mRNA-splicing factor ATP-dependent RNA helicase DEAH1-like [*Prunus avium*]
Prudu.08G110100	gi|1139755909|gb|ONH91366.1|hypothetical protein PRUPE_8G110000 [*Prunus persica*]
Prudu.08G110100	Not hits found
Prudu.08G110100	Not hits found
Prudu.08G230000	gi|1139758210|gb|ONH93667.1|hypothetical protein PRUPE_8G245400 [*Prunus persica*]
Prudu.08G230000	gi|1027106472|ref|XP_016650367.1|PREDICTED: uncharacterized protein LOC103333928 [*Prunus mume*]
Prudu.23S000300	gi|1220076702|ref|XP_021824944.1|histone-lysine N-methyltransferase, H3 lysine-9 specific SUVH5-like [*Prunus avium*]
Prudul26A009021	gi|1139774370|gb|ONI08842.1|hypothetical protein PRUPE_5G203500 [*Prunus persica*]
Prudul26A016169	gi|1162519146|ref|XP_007225342.2|putative disease resistance protein RGA3 isoform X2 [*Prunus persica*]
Prudul26A017168	gi|1139760708|gb|ONH95805.1|hypothetical protein PRUPE_7G090600 [*Prunus persica*]
Prudul26A017516 T1	Not hits found
Prudul26A017872	gi|645221735|ref|XP_008246111.1|PREDICTED: cyclic phosphodiesterase-like [*Prunus mume*]
Prudul26A018365 T1	Not hits found
Prudul26A022440 T1	Not hits found

6.4. Conclusions

- The natural progression from endodormancy to ecodormancy of an early flowering versus two late flowering almond cultivars was monitored by RNA sequencing of flower buds.
- This monitoring allowed the identification of candidate dormancy-associated genes and cultivar-associated almond genes:
    - DEGs related to metabolic switches, cell-to-cell transport, cell wall remodeling, phytohormone signaling and pollen development were commonly found in the general endodormancy release process.
    - DEGs related to pathogen-resistance and transcription factors were identified between early and late flowering cultivars, including "early" flowering genes *SBH1-2*, *WSD1*, *TMV-RESISTANCE PROTEIN* and *RGA1* and "late" flowering genes as *RICESLEPPER* and *SUVH5*.
- These transcriptomic results agree with the complex nature of the genetic determination of endodormancy release and flowering time previously evidenced in Chapters 3 and 4 of this Thesis.
- The obtained information may be used for the development of dormancy release molecular markers and the improvement of breeding programs efficiency, in a climate-change context.

7. METHYLATION ANALYSIS OF ALMOND FLOWER BUDS DURING ENDODORMANCY RELEASE

7. METHYLATION ANALYSIS OF ALMOND FLOWER BUDS DURING ENDODORMANCY RELEASE

7.1. Introduction

The dormancy release process involves the sensing of environmental cues (such as temperature), signal transduction and gene expression regulation to establish a suitable response according to the stimuli received (Cooke et al., 2012; Abbot et al., 2015). Epigenetic changes are part of the transcriptional regulation machinery of genomes. The dynamic but heritable character of such modifications make them interesting regulators mediating adaptive responses to environmental changes, such as seasonal cycles and, in the long term, climate change (Lämke and Bäurle, 2017). Transcription reprogramming leading to dormancy release may thus be mediated by epigenetic mechanisms (Yaish et al., 2011; Ríos et al., 2014).

Epigenetics are chemical modifications affecting DNA or structural proteins (histones) within the chromatin. Two types of epigenetic modifications have been described: DNA methylation (in plants 5´-cytosine methylation, 5mC) and PostTranslational histone Modifications (PTMs), which include the acetylation and methylation of histones (H2A, H2B, H3 and H4) (Saze, 2008; Feng and Jacobsen 2011). DNA methylation is associated with cell status stability and regulation of expression. It occurs in three sequence contexts: CG and CHG, which are found in promoter and coding regions, and CHH (where H = A, C or T), found in non-coding regions and Transposable Elements (TEs) (Pascual et al., 2014).

Several works have described the role of epigenetics in the regulation of dormancy in deciduous plant species. Santamaría et al. (2009), for instance, described a methylation decrease concomitant with H4 deacetylation and the progress of dormancy release in chestnut (*Castanea sativa* Mill.). In peach, De la Fuente et al. (2015) identified a genome-wide pattern of the PTM H3K27me3 during bud dormancy release, and Lloret et al. (2017) found a relationship between gene expression, PTMs and sorbitol synthesis during bud dormancy progression and release.

Rothkegel et al. (2017) showed that DNA methylation is one of the mechanisms participating in the regulation of MADS-box genes controlling bud dormancy in sweet cherry. In apple, genome methylation patterns have been linked to chilling acquisition during dormancy (Kumar et al., 2016b).

In the case of almond, results from the transcriptome sequencing (Chapter 6) of endodormant and ecodormant flower buds showed differential expression in a DNA methyltransferase gene and in the *S-ADENOSYL METHIONINE SYNTHASE* (*SAM*) gene responsible for the synthesis of the molecule S-Adenosyl Methionine (SAM), which donates the methyl group to the DNA molecule.

In addition, DNA methylation phenomena have also been associated with floral self-incompatibility (Fernández i Martí et al., 2014) and with bud falling phenomena (Fresnedo-Ramírez et al., 2017) in this species.

Genome-wide analysis of DNA methylation can be done by bisulfite sequencing, which consists of the Next Generation Sequencing of digested and bisulfite-treated DNA samples. The epi-GBS technique has been developed to represent a small part of the genome for cost-effective exploration and comparative analysis of DNA methylation and genetic variation in hundreds of *de novo* samples. Furthermore, this method makes it possible to genotype samples without a prior reference genome (van Gurp et al., 2016).

Regarding the molecular basis of endodormancy release and flowering time in almond at epigenetic level, the objective of this Chapter was to analyze the DNA methylation status and the identification of epigenetic marks in endodormant and ecodormant flower buds of early and extra-late flowering almond cultivars using epi-Genotyping by Sequencing (epi-GBS) analysis.

7.2. Materials and Methods

7.2.1. Plant material and experimental design

Flower buds from 'Desmayo Largueta' and 'Penta' were used (see Chapter 2.1 in Chapter 2). The plant material consisted of flower buds at states A (endodormancy phase) and B (ecodormancy phase) (Figure 2.7). Dormancy release evaluation was performed by the forcing method according to Chapter 3.2.3. Almond flower buds were picked from the experimental field of CEBAS-CSIC during two seasons of study: Season 1 (2015-2016) and Season 2 (2016-2017).

7.2.2. epi-GBS protocol

Every sample ('Desmayo Largueta' state A, 'Penta' state A, 'Desmayo Largueta' state B and 'Penta' state B from Season 1 and Season 2) consisted of a pool of ten flower buds. Genomic DNA was extracted from each sample following the method described by Doyle and Doyle (1987).

The DNA samples were quantified using Qubit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and diluted to 1 µg in 100 µl. A total of 20 µl was digested using PstI restriction enzyme. Adapters consisting of barcoded oligonucleotides were ligated to every sample. Non-phosphorylated hemimethylated adapters were used to reduce costs. Fragmented samples (libraries generated by restriction) were pooled and purified and subsequently subjected to nick translation with C-dNTPs (Zymo Research, Irvine, California, USA) and 7.5 µl of DNA PolI (NEB, Ipswich, Massachusetts) in NEB buffer 2. An EZ DNA Methylation-Lightning kit (Zymo Research) was used for bisulfite treatment, and fragments were selected by size with a ThermoScientific Size Selection kit. Libraries were amplified using the Kapa HiFi HotStart Uracil+ ReadyMix and purified with Magjet NGS Cleanup. Paired-end Illumina 2500 reads (2x100 bases) were generated by Macrogen (Seoul, South Korea) (van Gurp et al., 2016).

7.2.3. Bioinformatic analysis of DNA methylation

The process_radtags program of the Stacks 1.48 pipeline (Catchen et al., 2013) for demultiplexing and quality filtering with the default settings was disabled with the exception the rad_check. This was necessary because the bisulfite treatment changes unmethylated cytosines in the recognition sequence of PstI, and, as a result, checking the restriction cut site would filter out all fragments. The ustacks program of the pipeline was used to align the fragments into perfectly matching stacks. The default settings were used with the exception of -M, which was set to 4 in order to increase the maximum distance (in nucleotides) between stacks. Finally, cstacks was used to build a catalog of consensus loci. A custom C program was used for the reconstruction of the original sequences of the fragments by comparing the reads with origins in the "Watson" and "Crick" strands of the genomic DNA. The reconstructed DNA fragments were merged by another custom C program to produce one continuous "mock genome". Bismark_v0.19.0 (Krueger et al., 2011) was used to align the original fragments to the mock genome and to extract the methylation information. The Bismark coverage reports were used as input for the methylKit R package (Akalin et al., 2012).

A methyl kit was used to elaborate histograms of C-methylation and coverage and to assess sample similarity and correlation using the default settings. For the hierarchical clustering of the samples, dist was set to "correlation" and method to "ward". Finally we used the calculate DiffMeth function of a MethylKit to search for differentially methylated cytosines with the settings difference = 25, qvalue = 0.01. We looked for both hypermethylated and hypomethylated bases setting type = hyper and = hypo, respectively. The positions of the differentially methylated cytosines were extracted from the MethylKit files. Another custom made C-program was used to identify the original fragments where these differentially methylated cytosines were located.

7.2.4. Gene finding and annotation

The sequence of each fragment was mapped against the peach reference genome (v2.0) (Verde et al., 2017) with Gmap (v2015-06-23) (Wu and Watanabe, 2005). Two different output files, in the gff3 and SAM format, were obtained. The gff3 ouput files were processed to extract the boundary coordinates (start and end positions) of each hit using command line tools. After that, the boundary coordinates were used by a second custom python script to retrieve three different categories of annotations based on gene locations on the peach reference genome: upstream and downstream genes (in a size window of 10,000 bp) and "inside genes" (fragments within gene sequence) (Figure 7.1).

Finally, SAM format files were processed using a custom python script to extract the alignment information (number of exons, percentage of coverage, percentage of identity and amino acid changes) (Figure 7.1). Such data is found available as an excel file at https://mega.nz/#F!ENkWiaJS (encription key: mysiAoluFTdNTKmLTFrmzw).

Functional annotation of genes selected by distance to the mapped fragment was carried out using AgriGO software using Singular Enrichement Analysis (SEA) and Fisher's test following manual recommendation (Tian et al., 2017).

7.3. Results and discussion

7.3.1. Evaluation of the quality of the epiGBS analysis

A total of 9,518 fragments were sequenced (about a 1,244 kb size) with an identified 7,317 methylated or unmethylated fragments. We were able to reconstruct the original sequence of 4,377 fragments.

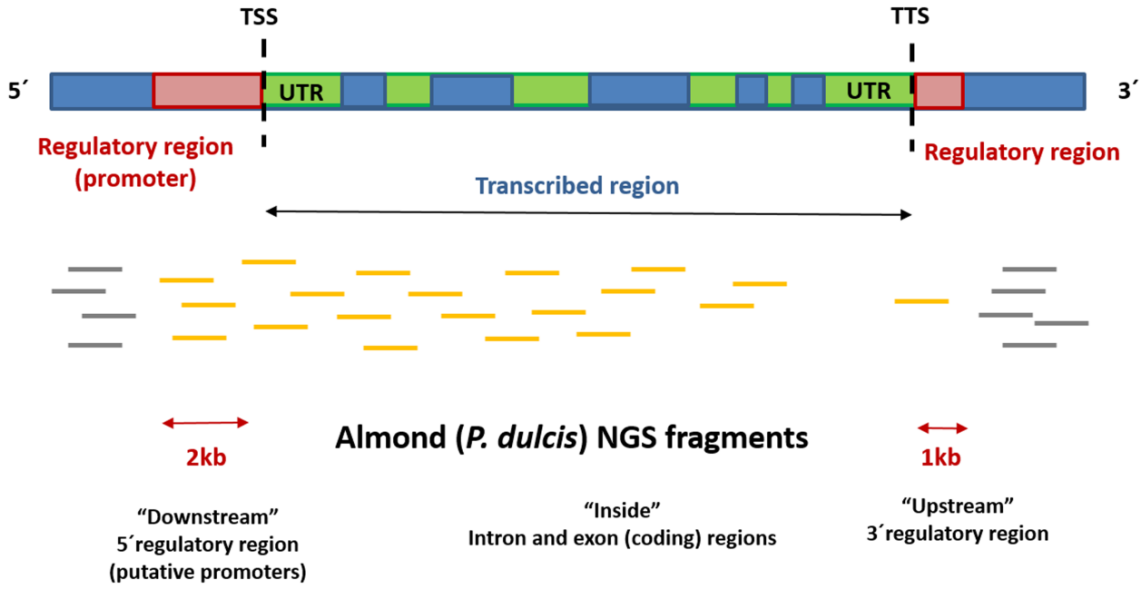


Figure 7.1. Schematic representation of a plant gene and classification of almond DMFs mapped in the peach genome. DMFs mapping from 2kb upstream TSS to 1kb downstream TTS were selected for functional annotation (fragments colored in orange). DMFs were classified according to the gene position –downstream, inside or upstream- respect to the fragment mapping region. Fragments mapped in intergenic regions (colored in grey) were discarded as putative gene regulatory regions. TSS: Transcriptional Start Site, UTR: Untranslated Transcription Region, TTS: Transcriptional Terminal Site. Exon and introns within transcribed region are colored in blue and green respectively.

The total length of the "mock genome" obtained by merging the reconstructed fragments was 662,458 bp (data not shown). Regarding the quality of this epiGBS analysis, the absence of a secondary peak towards the right of the read coverage histograms shows that the data do not suffer from a PCR duplication bias in both years (Figure 7.2; data from the Season 2 sampled flower buds are not shown).

These read coverage results show the uniformity of reads and the correct PCR amplification (good quality) uniformly around the whole genome in both seasons of study and contexts.

The histograms of CpG methylation showed that roughly 70%-75% of the cytosine positions in a CpG context of the mock genome were unmethylated and around 10% of the positions completely methylated with the remaining positions partially methylated to varying degrees in both seasons of study (Figure 7.3; data from the Season 2 are not shown).

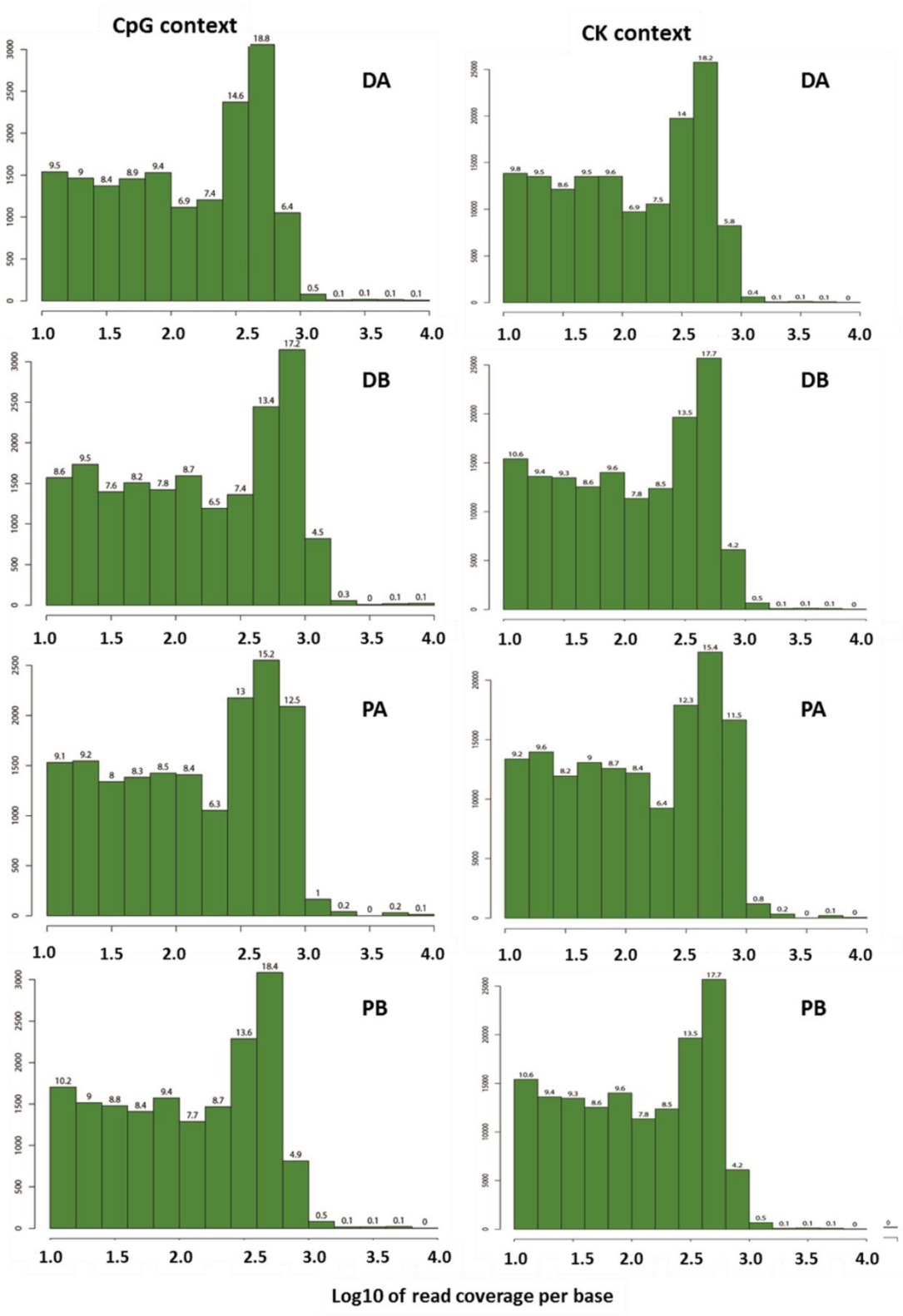


Figure 7.2. Read coverage of the samples tested in the CpG and CK (including CHG and CHH) contexts during Season 1. "D" = 'Desmayo Largueta', "P" = 'Penta'; "A" = endodormant buds, "B" = ecodormant buds.

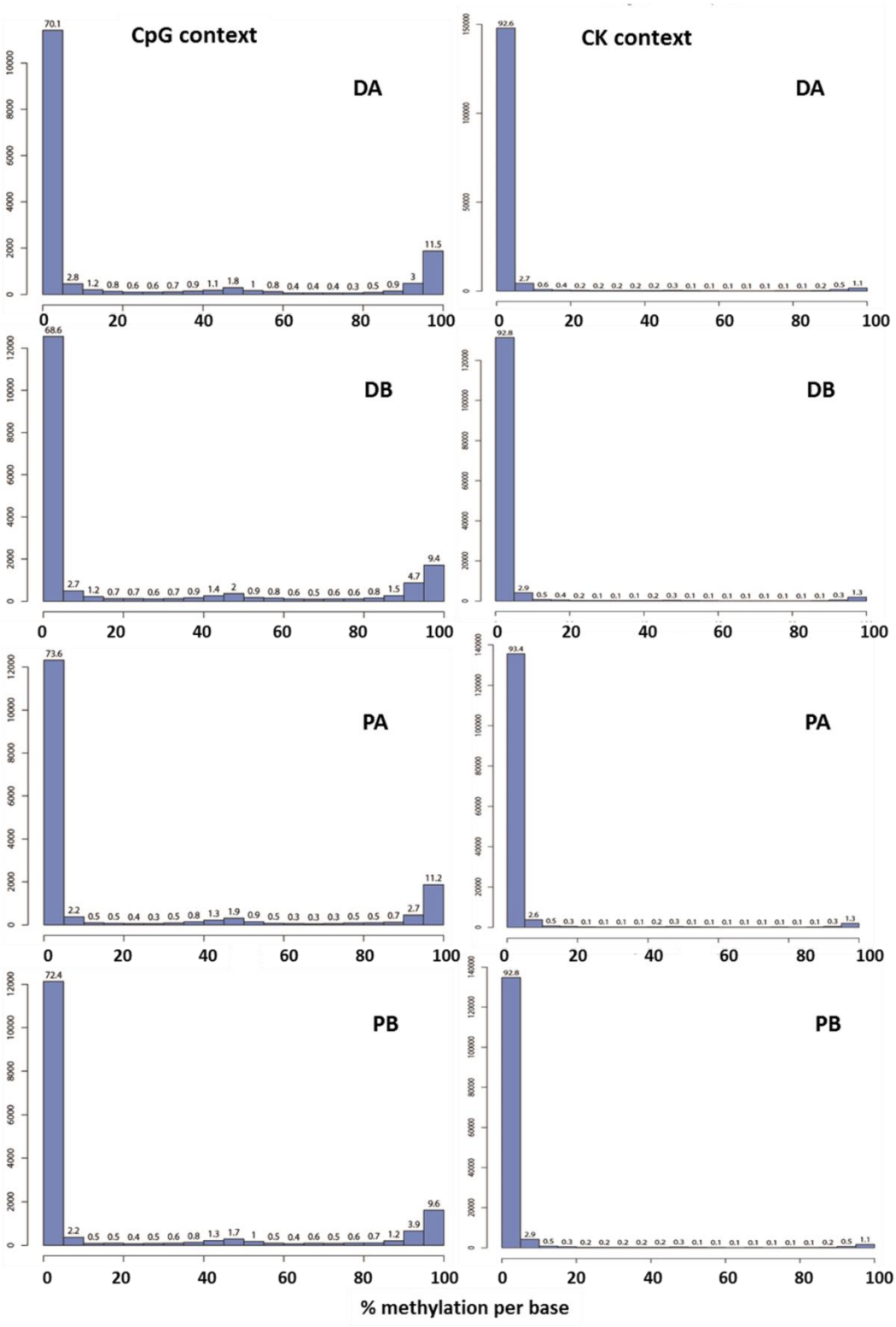


Figure 7.3. . Percentage of DNA methylation of the samples tested in the CpG and CK (including CHG and CHH) contexts during Season 1. "D" = 'Desmayo Largueta', "P" = 'Penta'; "A" = endodormant buds, "B" = ecodormant buds.

On the other hand, the correlation analyses shows clearly that samples of the same cultivar cluster together independently of the developmental state (endodormancy/ecodormancy) (Figure 7.4).

Pearson's correlations coefficient was constantly 0.99 in comparisons within each cultivar and in the range of 0.84 - 0.85 in camparisons between samples of different cultivars (Figure 7.4A). These results were also corroborated after clustering analysis where samples belonging to the same cultivar are closely together while the two cultivars are separated by long branches (Figure 7.4B). Thus, DNA methylation partner is in general cultivar dependent instead of dormancy dependent.

Quantitative analysis showed that 7,317 different fragments were methylated in at least one sample: 5,109 'Cs' were methylated in 'Desmayo Largueta' A samples; 5,089 'Cs' were methylated in 'Desmayo Largueta' B samples; 4,955 'Cs' were methylated in 'Penta' A samples; and 5,003 'Cs' were methylated in 'Penta' B samples (data not shown).

The number of Differentially Methylated Fragments (DMFs) detected was variable depending on the comparison performed (Table 7.1). Furthermore, a total of 677 DMFs were found between 'Desmayo Largueta' and 'Penta' genotype samples in all states analyzed. However, when comparing dormancy state samples (A and B), 23 DMFs were found between 'Desmayo Largueta' state A respect 'Desmayo Largueta' state B samples and 48 in 'Penta' state A samples respect 'Penta' state B samples. Of those DMFs, ten were common between 'Desmayo Largueta' and 'Penta' in the A to B state comparison. The DMFs were divided into hypermethylated or hypomethylated categories using 'Penta' or 'state B' samples as the reference. DMFs sequences are found available at https://mega.nz/#F!xI8SWYSS (encryption key: nbAF-tqOygqmF11Gk2oqRAI).

7.3.2. Differentially methylated genes detected

More than 99% of the identified DMFs were mapped on the peach v2.1 genome (Verde et al., 2017), and those located between 2kb upstream and 1kb downstream from gene coding sequences were selected for subsequent annotation (Figure 7.1). The number of differentially methylated genes (DMGs) thus identified is shown in Table 7.2 and Table 7.3. DMGs were classified according to their position with respect to the fragment mapping region. Fragments mapped most frequently within gene regions ("inside" DMGs) followed by 5´regulatory regions ("downstream" DMGs) and, finally, in 3´ regions ("upstream" DMGs) (Table 7.2).

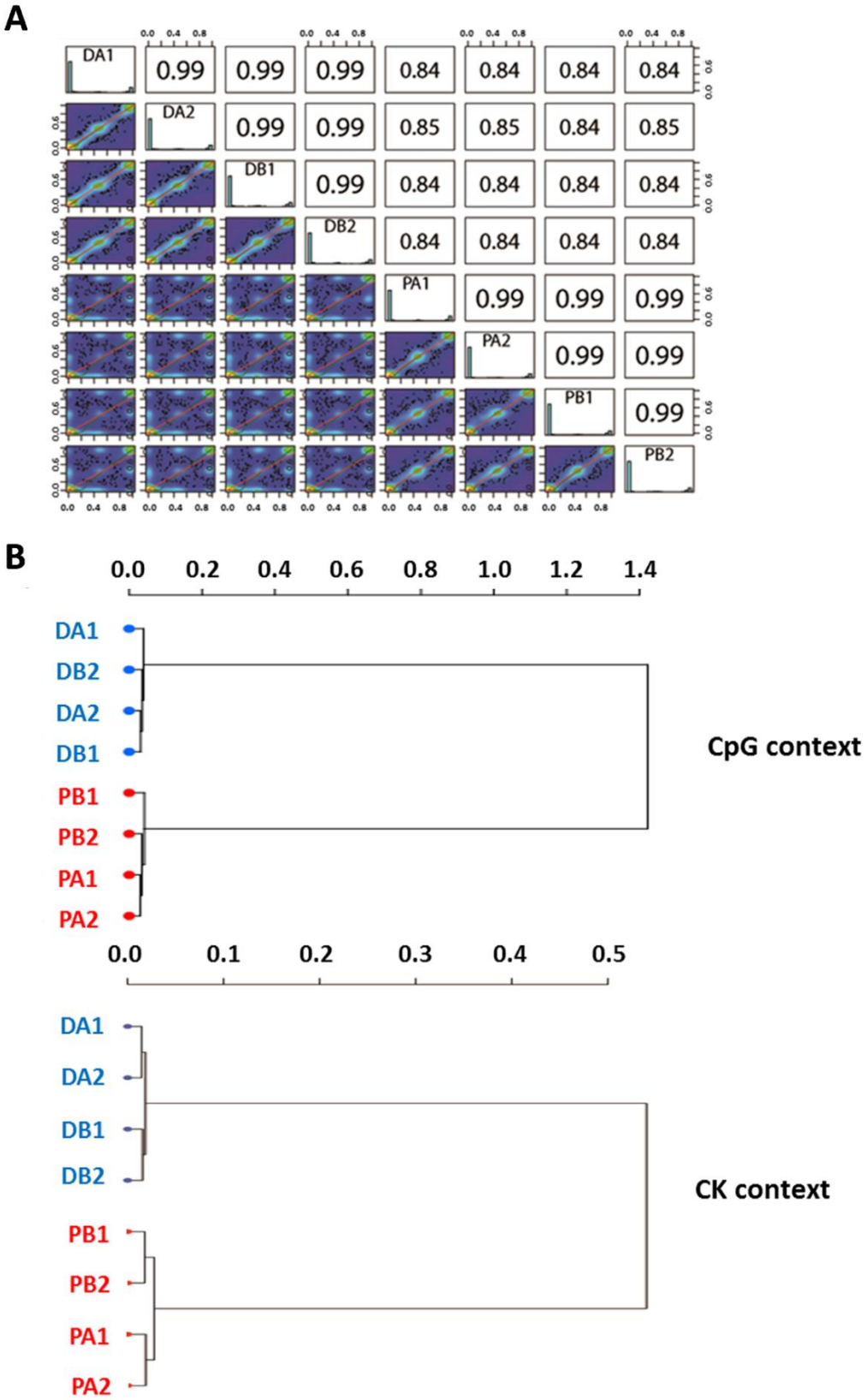


Figure 7.4. Almond correlations (A) and clustering analysis (B) of the methylated fragments in both CpG and CKcontexts. "D" = 'Desmayo Largueta', "P" = 'Penta'; "A" = endodormant buds, "B"=ecodormant buds. "1" samples are from Season 1 and "2" samples are from Season 2.

Table 7.1. Number of differentially methylated fragments (DMFs) detected by epi-GBS according to sample comparisons. "A" = endodormant bud state, "B" = ecodormant bud state.

Differentially Methylated Fragments (DMFs)		
Desmayo L. –Penta cultivar comparison		
Hypo (<)	Hyper (>)	State
307	370	A and B
677 DMFs		
A-B state comparison		
Hypo (<)	Hyper (>)	Cultivar
3	20	Desmayo L.
21	27	Penta
3	7	Desmayo L. and Penta
10 DMFs		

Table 7.2. Number of Differentially Methylated Genes (DMGs) identified from sequenced fragments mapping versus peach genome (v2.1). Methylation state refers to 'Desmayo Largueta' number of 5mCs respect 'Penta' samples. The category "equally methylated" refers to those genes whose number of 5mCs are the same between samples but 5mCs are located in different fragment positions. Gene position is based on gene location respect fragment mapping region ("upstream", "inside" and "downstream").

Differentially Methylated Genes (DMGs)			
Methylation state	**Gene position**	**Gene hits**	**Genes identified**
Hypermethylated	Upstream	36	
	Inside	291	
	Downstream	134	
Total		**461**	**423**
Hypomethylated	Upstream	19	
	Inside	201	
	Downstream	80	
Total		**300**	**281**
Equally-methylated	Upstream	6	
	Inside	41	
	Downstream	8	
Total		**55**	**27**
Total DMGs		**816**	**731**

When comparing DMGs between 'Desmayo Largueta' and 'Penta' flower buds, DMGs were found as hypermethylated in ´Desmayo Largueta´ samples (in both A and B states) to a greater extent than in ´Penta´samples (Table 7.2). Total DMGs found among flower buds of the two different cultivars and functional annotation are found available at https://mega.nz/#F!0l8zSAzA (encryption key: mhvY4trBOs4kJk6DseB3lA).

We found enriched hypermethylated genes in 'Desmayo Largueta' flower bud samples in the following processes related to primary metabolism in the "Biological function" GO category: amino-acid and carbohydrate synthesis and protein phosphorylation. ATP binding and protein kinase and phosphatase activity were the two main "Molecular function" GO terms found (Figure 7.5).

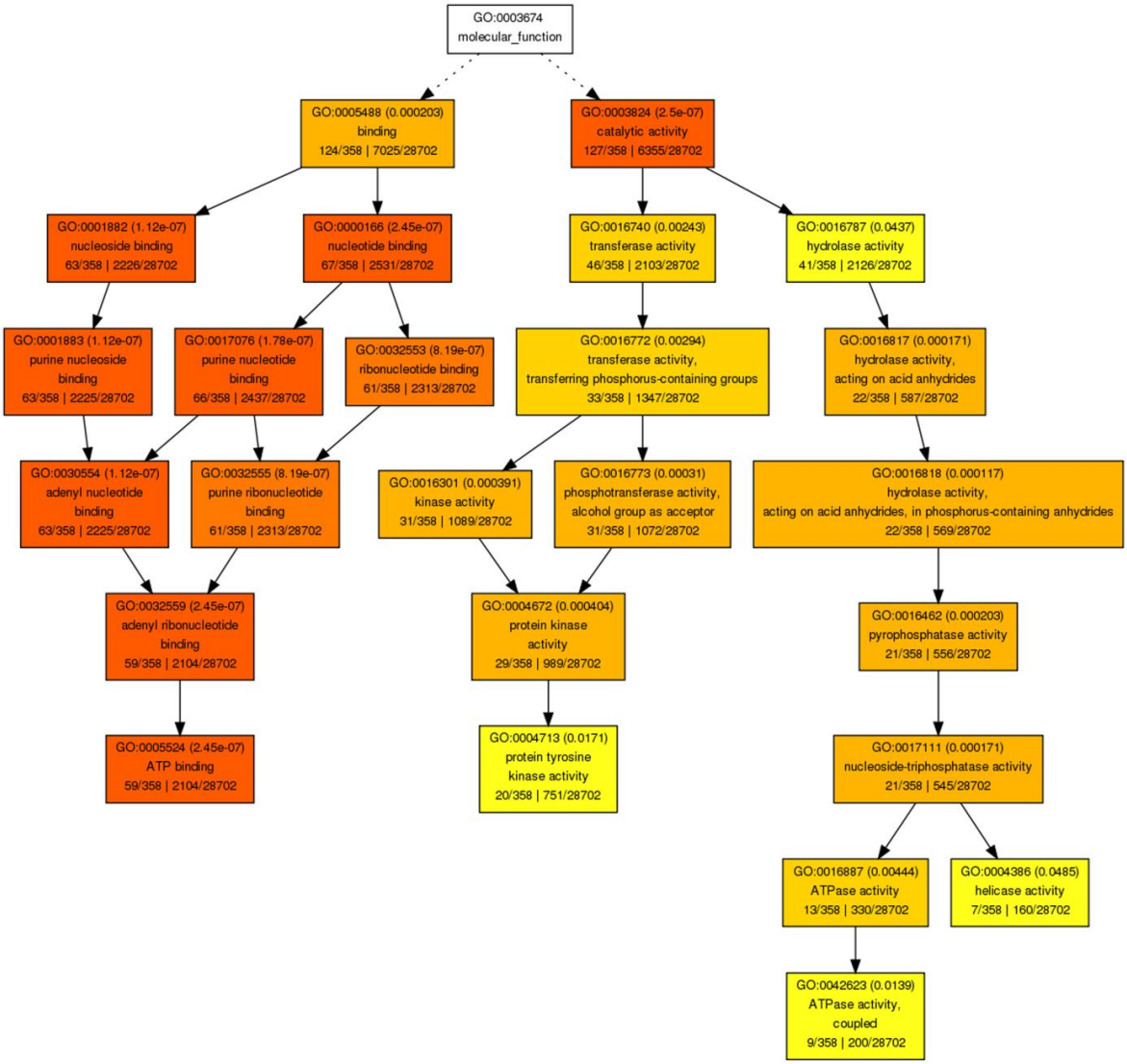


Figure 7.5. GO terms of the "Molecular function" category represented in genes identified as hypermethylated in 'Desmayo Largueta' flower buds in both the A and B dormancy states.

Table 7.3. Number of Differentially Methylated Genes (DMGs) between A and B state flower buds identified from sequenced fragments mapping peach genome (v2.1). Methylation state refers to A state (endodormant buds) samples number of 5mCs respect B state (ecodormant buds) samples. Gene position is based on gene location respect fragment mapping region.

Cultivar	Gene position	*P.persica* code	Functional annotation
Penta	DOWNSTREAM	Prupe.1G099900	Clathrin assembly protein AP180 and related proteins, contain ENTH domain
Desmayo L. and Penta	DOWNSTREAM	Prupe.1G105700	Fcf2 pre-rRNA processing
Desmayo L. and Penta	UPSTREAM	Prupe.1G125600	DUF3511
Desmayo L. and Penta	DOWNSTREAM	Prupe.1G287200	Iron/zinc purple acid phosphatase-like protein C
Desmayo L. and Penta	INSIDE	Prupe.2G019300	GPI anchor biosynthase
Desmayo L.	DOWNSTREAM	Prupe.2G031100	-
Penta	UPSTREAM	Prupe.2G039500	Vacuolar sorting protein VPS1, dynamin and related proteins
Desmayo L.	UPSTREAM	Prupe.2G053300	Family not named
Desmayo L.	INSIDE	Prupe.2G057100	Apoptotic ATPase, signal transduction, LRR, TIR domain
Desmayo L.	INSIDE	Prupe.2G057800	Apoptotic ATPase, signal transduction, LRR, TIR domain
Desmayo L.	UPSTREAM	Prupe.2G074300	Cytochrome P450 CYP2 subfamily
Desmayo L. and Penta	INSIDE	Prupe.2G074400	N-terminal C2 EEIG1 and EHBP1 proteins
Desmayo L.	INSIDE	Prupe.2G146000	Cornichon protein, cell polarity
Desmayo L.	UPSTREAM	Prupe.3G026400	Vacuolar sorting protein VPS1, dynamin and related proteins
Desmayo L. and Penta	DOWNSTREAM	Prupe.3G130700	Apoptotic ATPase, signal transduction, LRR (2 copies), TIR domain
Desmayo L.	DOWNSTREAM	Prupe.3G130700	Apoptotic ATPase, signal transduction, LRR, TIR domain
Penta	DOWNSTREAM	Prupe.4G186400	Glycosyl hydrolase family 1
Penta	DOWNSTREAM	Prupe.4G253800	Class 3 lipase
Desmayo L. and Penta	DOWNSTREAM	Prupe.4G270800	Mitogen-Activated Ser-Tre kinase (MAPK)
Desmayo L.	INSIDE	Prupe.5G036900	-
Desmayo L.	DOWNSTREAM	Prupe.5G038500	CAAX protease self-immunity
Penta	UPSTREAM	Prupe.6G014500	WDSAM1 ubiquitination protein
Desmayo L.	INSIDE	Prupe.6G097800	Mlo protein, defense response, integral to membrane
Desmayo L. and Penta	DOWNSTREAM	Prupe.6G307900	Zinc finger fyve domain, GDSL-like lipase/acylhydrolase
Penta	DOWNSTREAM	Prupe.6G307900	Zinc finger fyve domain, GDSL-like lipase/acylhydrolase
Desmayo L.	UPSTREAM	Prupe.6G331300	Phosphoglycerate mutase, histidine phosphatase superfamily

Candidate genes related to transcription regulation processes including transcription factors (Prupe.1G395600, Prupe.5G088700, Prupe.6G343100), RNA-mediated silencing (Prupe.7G221200), and chromatin remodelling (Prupe.8G221300) and *LATE ELONGATED HYPOCOTYL* (*LHY*) (Prupe.2G200400) appeared as hypermethylated in `Desmayo Largueta´ flower bud samples. The *LHY* protein is a well described flowering time regulator in response to the photoperiod (Fujiwara et al., 2008; Park et al., 2010) (see Chapter 1.2.4) and the gene network controlling this trait has been studied (Park et al., 2016). 'Desmayo Largueta' is a low-chill cultivar whose dormancy period takes place under short photoperiod conditions such as the experimental conditions of this work. It would be interesting to study *LHY* behaviour during dormancy progression in different almond cultivars.

Proteins involved in auxin response were highly represented (Prupe.1G000200, Prupe.1G067400, Prupe.7G048400) including *AUXIN RESPONSE FACTORS* (*ARFs*) (Prupe.3G010900, Prupe.5G217700, Prupe.7G228800). Its known that expression of *ARFs* are subjected to epigenetic regulation (Yaish et al., 2011; Xiao et al., 2006), and *ARFs* transcriptional regulation is required for developmental processes like germination (Xiao et al., 2006).

Accordingly, Zhang et al. (2017) observed a flowering delay in Arabidopsis when *ARF6* and *ARF8* were repressed. Nonetheless, the reason underlying the hypermethylated state of genes participating in the auxin response pathway in both endodormant and ecodormant flower buds of the early flowering genotype 'Desmayo Largueta' has yet to be unravelled.

We also identified DNA repair proteins, such as those encoded by Prupe.1G510000, Prupe.2G013900, Prupe.3G029600, Prupe.3G16000 and Prupe.5G066100.

Finally, proteins participating in oxidoreduction processes, such as *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) proteins encoded by Prupe.4G026900 and Prupe.4G02700, also appeared as hypermethylated in 'Desmayo Largueta' flower bud samples. LEA proteins are involved in osmoprotection, which is activated in response to low temperatures (Bañuelos et al., 2008). In case of hypermethylation reflected a repressed state of expression, in low chilling requirement cultivars like 'Desmayo Largueta', osmoprotection would not be so necessary or may be regulated in a different way. The LEA gene family has been characterized by Du et al. (2013) in Japanese apricot and differential expression has been identified during bud dormancy in this species (Yamane et al., 2006).

Regarding hypomethylated genes in 'Desmayo Largueta' samples, we found cellular protein localization within the "biological function" GO category. ATP-coupled transmembrane transport and ATP-binding activity, on the other hand, appeared in the "molecular function" GO category (Figure 7.6).

We were able to identify a wide range of DNA-binding proteins encoded by hypomethylated genes in 'Desmayo Largueta' samples: histone methyltransferases (encoded by Prupe.1G050800 and Prupe.7G271600); the transcription factor histone-like *NF-Y* (encoded by Prupe.2G47600); DNA topoisomerases (encoded by Prupe.1G173400 and Prupe.1G173500); and *FAR RED IMPAIRED RESPONSE 1* (*FAR1*) (Prupe.1G196400).

Interestingly, a single gene coding for a *HYDROPHOBIC SEED PROTEIN* (*HSP*) also appeared as hypomethylated in 'Desmayo Largueta' samples. It is interesting to note the high degree of differential methylation that seems to be fixed between the two almond cultivars analyzed.

This fact is of practical importance in cultivar improvement for developing epigenetic markers based on methylation variants and taking into account the high flexibility of methylation patterns in relation to external signals in order to identify markers based on methylation polymorphisms.

In contrast to standard sequencing, bisulfite sequencing let to gain information that conditions the phenotype. As a consequence, knowing the methylation state might help us understand the genetic determinism of important agronomic traits more deeply.

Although the methylation patterns are highly variable in response to different external factors, the markers that we have detected in our almond genotypes are conserved in different states of development and in different years and can therefore be considered as stable and conserved epigenetic marks.

In this study, data showed important differences between genotypes, which displayed different phenotypes in terms of breeding traits (chilling requirements for dormancy release, flowering and ripening times, almond production and almond characteristics) (Table 7.1 and Table 7.2). It is remarkable that more hypermethylated than hypomethylated fragments were identified in state A (endodormant flower buds) almond samples of any cultivar (Table 7.2). This is concordant with the general decrease in 5mC during dormancy progression in chestnut (Santamaría et al., 2009).

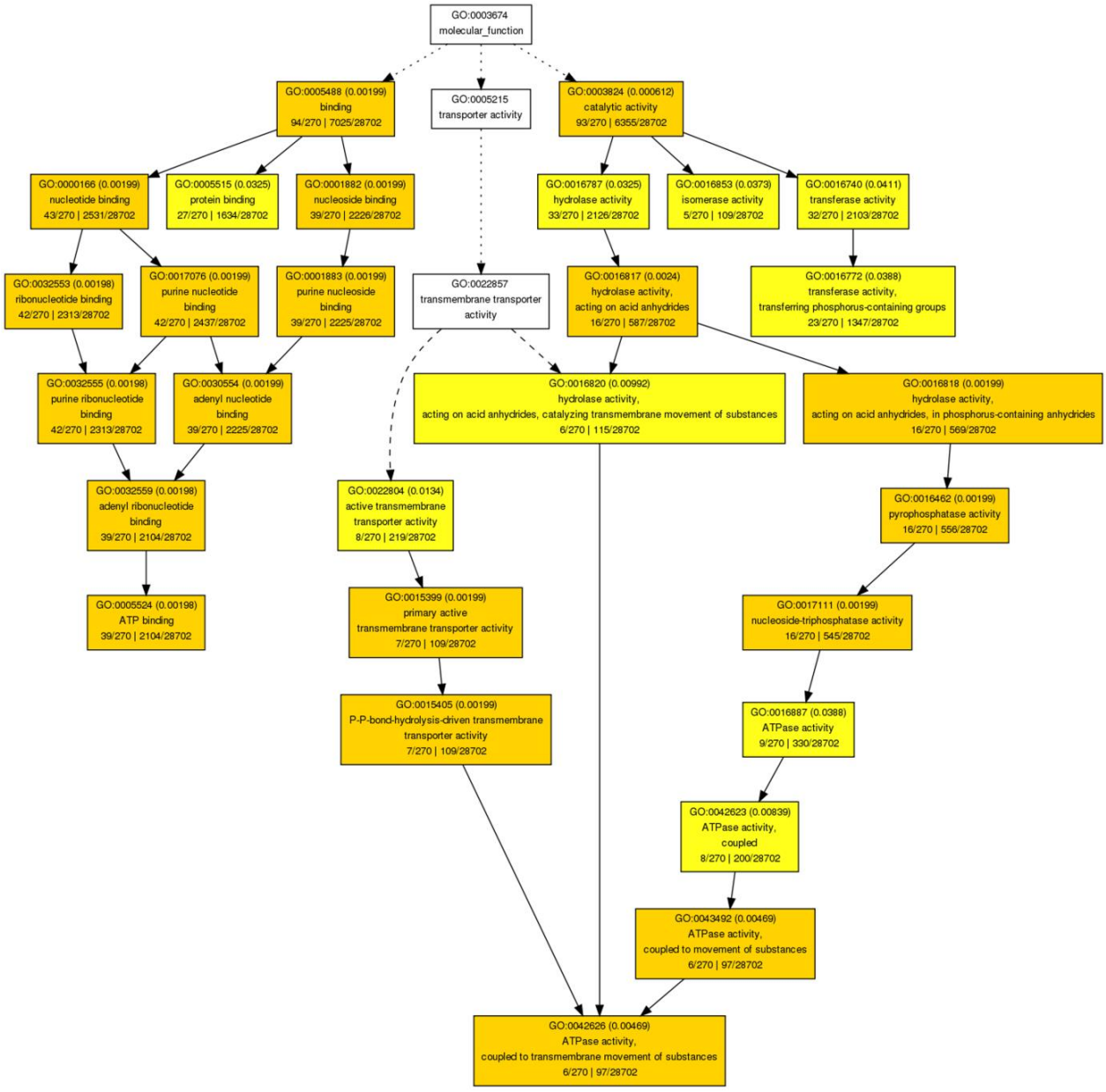


Figure 7.6. "Molecular function" GO terms represented in genes identified as hypomethylated in 'Desmayo Largueta' flower buds in both the A and B dormancy states.

On the other hand, fragments mapped the most frequently within gene regions ("inside" DMGs), followed by 5´regulatory regions ("downstream" DMGs) and, in last place, in 3´ regions ("upstream" DMGs). According to Vining et al. (2012), 5mC in promoters and gene body parts is related to a repressed state of chromatin, a condition that inhibits the accesibility of the transcriptional machinery.

The methylation variants observed may be associated with evolutionary changes related to each cultivar´s features (Varriale, 2017). It will be interesting to distinguish

which variants are related to traits of agronomic interest in order to explore adaptive mechanisms to the environment (Viggiano and de Pinto, 2017). Recently, for instance, Garg et al. (2015) identified conserved methylation polymorphisms distribution of rice varieties with contrasted response to drought resistance.

7.3.3. Differentially methylated genes related to bud dormancy

More DMGs were found as hypermethylated than hypomethylated in state A samples (Table 7.3). Furthermore, just one hypomethylated gene could be functionally annotated, and it was mapped in the 3' regulatory region of the gene Prupe.4G277200, which encodes for a *REGULATION OF CHROMOSOME CONDENSATION* (*RCC1*) protein (Table 7.4).

'Desmayo Largueta' and 'Penta' common hypermethylated genes were found (Table 7.4). MITOGEN-ACTIVATED PROTEIN KINASES (MAPK) and phosphatases have been found to participate in the initial response to cold induced by an increase in Ca^{2+} (Bañuelos et al., 2008). Furthermore, MAPK3 has been shown to be a central regulator of seed dormancy in barley (Nakamura et al., 2016).

On the other hand, LEUCINE RICH REPEAT-TOLL INTERLEUKIN 1 RECEPTOR (LRR-TIR) apoptotic ATPases have been related to a type of Programmed Cell Death (PCD) called Developmental Cell Death (DCD), leading to a differentiation of cells after dormancy release, as occurs in floral morphogenesis or in the pollen tube (Koonin and Aravind, 2002; del Duca et al., 2014).

N-terminal C2 proteins and VACUOLAR SORTING PROTEIN 1 (VSP1) protein are involved in vesicular trafficking from the cell membrane, and this process has been linked to cell wall differentiation and appears to be important in the dormancy release process (Ebine and Ueda, 2015; Kim and Brandizzi, 2014).

Finally, protein glycosylation by GLYCEROPHOSPHATIDYLINOSITOL (GPI) anchoring proteins is involved in intercellular signaling in different processes as flowering transition, as shown in poplar by Rinne et al. (2011). Three additional LRR-TIR apoptotic ATPases, among others Hypermethylated genes were found in 'Desmayo Largueta' samples (Table 7.4). In 'Penta' A samples hypermethylated genes a GLYCOSYL HYDROLASE (GH) 1 were found, among others (Table 7.4). GHs genes from different families were identified by RNA-SEQ in Chapter 6 as ecodormancy associated genes (see Chapter 6.3.3).

7.4. Conclusions

- In this Chapter, we have applied the epi-GBS protocol to almond DNA samples for the first time. The technical potential is evident in the discovery of epigenetic variants, based on 5mC.
- According to the results obtained, the DNA methylation (5mC) pattern was cultivar-dependent, rather than dormancy state-dependent.
- In spite of coverage limitation of the performed sequencing, we were able to identify genes whose methylation state changed between the endodormant and the ecodormant state of flower buds DNA. This was possible in both the early-flowering 'Desmayo Largueta' and the extra-late flowering 'Penta'. Furthermore, common genes arose from the analysis.
- Short term, these results can be complemented with qRT-PCR analysis of DMGs identified between cultivars and dormancy states. On the other hand, long term, it would be interesting to improve the technique coverage to obtain a greater representation of the genome.
- After confirmation of epigenetic and expression results, these observed methylation marks should be used as epigenetic markers for endodormancy release and flowering time in almond.
- Comparative DNA methylation studies of both traditional and almond cultivars released from breeding programs will surely contribute to our knowledge of methylation variants and provide candidate epialleles linked to agronomic traits. Such polymorphisms can be screened in large populations using NGS (New Generation Sequencing) to confirm the locus methylation state associated with a given character of interest.

8. GENERAL CONCLUSIONS

8. GENERAL CONCLUSIONS

Regarding the genetic basis of endodormancy release and flowering time in almond, results of the Chapter 3 confirmed that flowering time in almond is a complex process involving the chilling and heat requirements and the genetic background of each cultivar. The estimation of chilling and heat requirements under different climatic conditions showed that the Dynamic model is more accurate than the Richardson model, especially under warm conditions. If the chilling requirements of the cultivars are not satisfied, productivity could be negatively affected, showing the risk of growing extra-late and ultra-late flowering almond cultivars in warm areas. This fact demonstrates the importance of growing each cultivar in the climatically suitable area and the important effect of climatic change on flowering time and productivity of almond.

Results of Chapter 4 evidenced that controlled conditions in Autumn, Winter and Spring chambers successfully modulated flowering time from first February to first April in the extra-early almond 'Desmayo Largueta', but did not for the ultra-late 'Tardona', even with the application of enough chill in Winter chamber. 'Desmayo Largueta' has lower heat requirements than 'Tardona', which were variable depending on the treatment and season, without any clear relationship along the treatments. Since the numbers of days to overcome ecodormancy decrease with the treatments, other unknown factors could be involved. Ripening time was much earlier in controlled conditions than in natural conditions for both cultivars, probably consecuence of artificial culture in pots. The delay of flowering time, consequence of treatments, shifted progressively the maturation time in 'Desmayo Largueta' but not in 'Tardona'. Fruit set was negatively affected by culture conditions, being lower than in natural conditions, mainly in 'Tardona'. No relationship was observed along the treatments applied. Nut and seed weight was smaller than that observed in natural conditions. The behaviour of both cultivars under the different treatments shows the complex nature of endodormancy release and flowering time in almond linked to temperature, but probably to other uncontrolled factors.

Regarding the molecular basis of endodormancy release and flowering time in almond at transcriptomic level, results of Chapter 5 highlighted an important increase in the transcript levels of *PdP40* gene before endodormancy release in the three cultivars with different chilling requirements. The candidate transcript *PdP40* is a member of the flower-specific Class III peroxidase encoding family. This increase, during the transition from endodormancy to ecodormancy, was independent of the endodormancy period length, so *PdP40* is a good RNA marker to monitor the release of endodormancy. Total peroxidase activity in flower buds shared a common pattern with the PdP40 expression, and it could also be a good biochemical marker for monitoring bud dormancy release in almond. Understanding the transition from endodormancy to ecodormancy through gene or enzyme activity is of great interest for the development and use of biostimulants to modulate flowering time.

At transcriptomic level, results of the Chapter 6 allowed the identification of candidate dormancy-associated genes and cultivar-associated almond genes by RNA sequencing of flower buds from endodormancy to ecodormancy. DEGs related to metabolic switches, cell-to-cell transport, cell wall remodeling, phytohormone signaling and pollen development were commonly found in the general endodormancy release process. DEGs related to pathogen-resistance and transcription factors were identified between early and late flowering cultivars, including "early" flowering genes *SBH1-2*, *WSD1*, *TMV-RESISTANCE PROTEIN* and *RGA1* and "late" flowering genes as *RICESLEPPER* and *SUVH5*. The obtained information may be used for the development of dormancy release molecular markers and the improvement of breeding programs efficiency, in a climate-change context.

Finally, regarding the molecular basis of endodormancy release and flowering time, in the epigenetic analysis performed by epi-GBS in the Chapter 7, the DNA methylation (5mC) pattern was cultivar-dependent, rather than dormancy state-dependent. In spite of coverage limitation of the performed sequencing, we were able to identify genes whose methylation state changed between the endodormant and the ecodormant state of flower buds DNA. This was possible in both the early-flowering 'Desmayo Largueta' and the extra-late flowering 'Penta'. After confirmation of epigenetic and expression results, these observed methylation marks should be used as epigenetic markers for endodormancy release and flowering time in almond.

9. REFERENCES

9. REFERENCES

Abbott, A.G., Zhebentyayeva, T., Barakat, A. and Liu, Z.R. (2015) The genetic control of bud-break in trees. In Plomion, C. and Adam Blondon, A.F., eds. Advances in Botanical Research. Academic Press, London. Pp. 201-228.

Addicot, F.T. (1983) Abscisic Acid. In Addicot F.T., ed. Praeger Scientific, New York.

Ahmad, A., Zhang, Y. and Cao, X.F. (2010) Decoding the epigenetic language of plant development. *Molecular Plant* 3, 719-728.

Akalin, A.; Kormaksson, M.; Li, S.; Garrett-Bakelman, F.E.; Figueroa, M.E.; Melnick, A. and Mason, C.E. (2012) methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology* 13, R87.

Alburquerque, N., García-Montiel, F., Carrillo, A. and Burgos, L. (2008) Chilling and heat requirements of sweet cherry cultivars and the relationship between altitude and the probability of satisfying the chill requirements. *Environmental and Experimental Botany* 64, 162-170.

Aloni, R. and Peterson, C. A. (1997) Auxin promotes dormancy callose removal from the phloem of *Magnolia kobus* and callose accumulation and earlywood vessel differentiation in *Quercus robur*. *Journal of plant research* 110, 37.

Alonso Segura, J.M., Socias i Company, R. and Kodad, O. (2017) Late-blooming in almond: A controversial objective. *Scientia Horticulturae* 224, 61-67.

Amen, R.D. (1968) A model of seed dormancy. *The Botanical Review* 34, 1–31.

An, L., Lei, H., Shen, X., and Li, T. (2012) Identification and characterization of *PpLFL*, a homolog of *FLORICAULA/LEAFY* in peach (*Prunus persica*). *Plant Molecular Biology Reporter*, 30, 1488-1495.

Anderson, J.V., Gesch, R.W., Jia, Y., Chao, W.S., and Horvath, D.P. (2005) Seasonal shifts in dormancy status, carbohydrate metabolism, and related gene expression in crown buds of leafy spurge. *Plant, Cell and Environment* 28, 1567-1578.

Andreini, L., Viti, R., Bartolini, S., Ruiz, D., Egea, J., and Campoy, J.A. (2012) The relationship between xylem differentiation and dormancy evolution in apricot flower buds (*Prunus armeniaca* L.): the influence of environmental conditions in two Mediterranean areas. *Trees* 26, 919-928.

Arias, O. and Crabbé, J. (1975) Gradients morphogenetiques du rameau d'un an des vegetaux ligneux, en repos apparent; donnees complementaires fournies par l'etude de *Prunus avium* L. *Physiologie végétale* 13, 69–81.

Arora, R., Rowland, L.J. and Tanino, K. (2003) Induction and release of bud dormancy in woody perennials: A science comes of age. *HortScience* 38, 911-921.

Artlip, T.S., Wisniewski, M.E., Bassett, C.L. and Norelli, J.L. (2013) *CBF* gene expression in peach leaf and bark tissues is gated by a circadian clock. *Tree physiology* 33, 866-877.

Atkinson, C.J., Brennan, R.M. and Jones, H.G. (2013) Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany* 91, 48-62.

Bacarella, A., Chironi, G., and Barbera, G. (1991) Aspetti tecnici, economici e di mercato del mandorlo in Sicilia.Baggiolini, M. (1952) Stade repères du pêcher. *Revue Romande d'Agriculture, Viticulture et Arboriculture* 4, 29-35.

Bai, S., Saito, T., Sakamoto, D., Ito, A., Fujii, H. and Moriguchi, T. (2013) Transcriptome analysis of Japanese pear (*Pyrus pyrifolia* Nakai) flower buds transitioning through endodormancy. *Plant and Cell Physiology* 54, 1132-1151.

Baldermann, S., Homann, T., Neugart, S., Chmielewski, F.-M., Götz, K.-P., Gödeke, K., Huschek, G., Morlock, G. and Rawel, H. (2018) Selected plant metabolites involved in oxidation-reduction processes during bud dormancy and ontogenetic development in sweet cherry buds (*Prunus avium* L.). *Molecules* 23, 1197.

Balk, P., Hoeberichts, F., Verhoef, N., Schumacher-Strijker, A. and Aanhane, T. (2017) Monitoring dormancy release in fruit trees and ornamentals by RNA sequencing and its implications for horticulture. Paper presented at: International Symposium on Flowering, Fruit Set and Alternate Bearing, 1229.

Ballester, J., Socias i Company, R., Arus, P. and De Vicente, M.C. (2001) Genetic mapping of a major gene delaying blooming time in almond. *Plant Breeding* 120, 268-270.

Bañuelos, M.L.G.; Moreno, L.V.; Winzerling, J.; Orozco, J.A. and Gardea, A.A. (2008) Winter metabolism in deciduous trees: Mechanisms, genes and associated proteins. *Revista fitotecnia mexicana* 31, 295–308.

Barba-Espín, G., Díaz-Vivancos, P., Clemente-Moreno M.J., Albacete, A., Faize, L., Faize, M., Perez-Alfocea F. and Hernandez, J.A. (2010). Interaction between hydrogen peroxide and plant hormones during germination and the early growth of pea seedlings. *Plant Cell and Environment* 33, 981–994.

Barros, P.M., Cherian, S., Costa, M., Sapeta, H., Saibo, N.J.M., and Oliveira, M.M. (2017) The identification of almond *GIGANTEA* gene and its expression under cold stress, variable photoperiod, and seasonal dormancy. *Biologia Plantarum* 61, 631-640.

Barros, P.M., Goncalves, N., Saibo, N.J.M., and Margarida Oliveira, M. (2012). Cold acclimation and floral development in almond bud break: insights into the regulatory pathways. *Journal of Experimental Botany* 63, 4585-4596.

Bartolini, S., Viti, R., and Guerriero, R. (2006) Xylem differentiation and microsporogenesis during dormancy of apricot flower buds (*Prunus armeniaca* L.). *European Journal of Horticultural Science* 71, 84.

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

Baumann, M.J., Eklöf, J.M., Michel, G., Kallas, Å.M., Teeri, T.T., Czjzek, M. and Brumer, H. (2007) Structural evidence for the evolution of xyloglucanase activity from xyloglucan endo-transglycosylases: Biological implications for cell wall metabolism. *The Plant Cell* 19, 1947-1963.

Beauvieux, R., Wenden, B. and Dirlewanger, E. (2018) Bud dormancy in perennial fruit tree species: A pivotal role for oxidative cues. *Frontiers in Plant Science* 9, 657.

Ben Ismail, M.C. (1989) Dormance et reprise de la croissance des bourgeons chez les arbres fruitiers. PhD Thesis, Faculté des Sciences Agronomiques de Gembloux, Belgium.

Ben Mohamed, H., Vadel, A.M., Geuns, J.M.C., and Khemira, H. (2012) Effects of hydrogen cyanamide on antioxidant enzymes' activity, proline and polyamine contents during bud dormancy release in Superior Seedless grapevine buds. *Acta Physiologiae Plantarum* 34, 429-437.

Benkert P., Biasini M. and Schwede, T. (2011) Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics* 27, 343–350.

Benmoussa, H., Ghrab, M., Ben Mimoun, M., and Luedeling, E. (2017) Chilling and heat requirements for local and foreign almond (*Prunus dulcis* Mill.) cultivars in a warm Mediterranean location based on 30 years of phenology records. *Agricultural and Forest Meteorology* 239, 34-46.

Bennett, J., and Skoog, F. (1938) Preliminary experiments on the relation of growth-promoting substances to the rest period in fruit trees. *Plant Physiology* 13, 219.

Bertozzi, T., Bennett, C. and Sedgley, M. (1998) Almond improvement in Australia. *Nucis Newsletter* 7, 10-11.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T.G., Bertoni, M., Bordoli, L., and Schwede, T. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research* 42, 252–258.

Bielenberg, D.G., Wang, Y., Fan, S., Reighard, G.L., Scorza, R., and Abbott, A.G. (2004) A deletion affecting several gene candidates is present in the *evergrowing* peach mutant. *Journal of Heredity* 95, 436-444.

Bielenberg, D.G., Wang, Y., Li, Z., Zhebentyayeva, T., Fan, S., Reighard, G.L., Scorza, R., and Abbott, A.G. (2008) Sequencing and annotation of the evergrowing locus in peach *Prunus persica* (L.) Batsch reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. *Tree Genetics and Genomes* 4, 495-507.

Bielenberg, D. G., Rauh, B., Fan, S., Gasic, K., Abbott, A. G., Reighard, G. L. et al. (2015). Genotyping by sequencing for SNP-based linkage map construction and QTL analysis of chilling requirement and bloom date in peach [*Prunus persica* (L.) Batsch]. *PLoS ONE*, 10(10), e0139406.

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

Bonhomme, M., Rageau, R., Lacointe, A. and Gendraud, M. (2005) Influences of cold deprivation during dormancy on carbohydrate contents of vegetative and floral primordia and nearby structures of peach buds (*Prunus persica* L. Batch). *Scientia Horticulturae*, 105, 223-240.

Byrne, D. H. and Bacon, T. A. (1992) Chilling estimation: its importance and estimation. *The Texas Horticulturist* 18, 5-8.

Calixto, F.S., Bauza, M., Martinez de Toda, F. and Argamenteria, A. (1981) Amino acids, sugars, and inorganic elements in the sweet almond (*Prunus amygdalus*). *Journal of Agricultural Food Chemistry* 29, 509-511.

Campoy, J.A. (2009). Letargo invernal en albaricoquero (*Prunus armeniaca* L.): Análisis de diversos factores que afectan su evolución. PhD Thesis, Universidad Politécnica de Cartagena, Spain.

Campoy, J.A., Ruiz, D. and Egea, J. (2011) Dormancy in temperate fruit trees in a global warming context: A review. *Scientia Horticulturae* 130, 357-372.

Castède, S., Campoy, J.A., Le Dantec, L., Quero-García, J., Barreneche, T., Wenden, B. and Dirlewanger, E. (2015) Mapping of candidate genes involved in bud dormancy and flowering time in sweet cherry (*Prunus avium*). *PLoS ONE* 10, e0143250.

Catchen, J., Hohenlohe, P.A., Bassham, S., Amores, A. and Cresko, W.A. (2013) Stacks: an analysis tool set for population genomics. *Molecular ecology* 22, 3124-3140.

Champagnat, P. (1983) Bud dormancy, correlation between organs, and morphogenesis in woody plants. *Soviet Plant Physiology*,30, 458-471.

Chao, W.S. and Serpe, M.D. (2010) Changes in the expression of carbohydrate metabolism genes during three phases of bud dormancy in leafy spurge. *Plant Molecular Biology* 73, 227-239.

Chen, D., Ding, Y., Guo, W. and Zhang, T. (2009) Molecular cloning and characterization of a flower-specific class III peroxidase gene in *G. Hirsutum*. *Molecular Biology Reports* 36, 461-469.

Cheng, H., Qin, L., Lee, S., Fu, X., Richards, D. E., Cao, D. et al. (2004) Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. *Development*, 131, 1055-1064.

Citadin, I., Raseira, M.C., Herter, F.G. and da Silva, J.B. (2001) Heat requirement for blooming and leafing in peach. *HortScience* 36, 305-307.

Clemente-Moreno, M.J., Piqueras, A. and Hernández, J.A. (2011) Implication of peroxidase activity in development of healthy and PPV-infected micropropagated GF305 peach plants. *Plant Growth Regulation* 65, 359–367.

Coll-García, D., Mazuch, J., Altmann, T. and Müssig, C. (2004) EXORDIUM regulates brassinosteroid-responsive genes. *FEBS Letters* 563, 82-86.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M. and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674-3676.

Conrad, A., Yu, J., E Staton, M., Audergon, J.-M., Roch, G., Decroocq, V., Knagge, K., Chen, H., Zhebentyayeva, T., Liu, Z. et al. (2019) Association of the phenylpropanoid pathway with dormancy and adaptive trait variation in apricot (*Prunus armeniaca*). *Tree physiology* 39.

Cook, N.C., Bellen, A., Cronjé, P.J.R., Wit, I.D., Keulemans, W., Van den Putte, A. and Steyn, W. (2005) Freezing temperature treatment induces bud dormancy in 'Granny Smith' apple shoots. *Scientia Horticulturae* 106, 170-176.

Cooke, J.E.K., Eriksson, M.E. and Junttila, O. (2012) The dynamic nature of bud dormancy in trees: Environmental control and molecular mechanisms. *Plant, Cell and Environment* 35, 1707–1728.

Cordero, E.C., Kessomkiat, W., Abatzoglou, J. and Mauget, S.A. (2011) The identification of distinct patterns in California temperature trends. *Climate Change* 108, 357-382.

Couvillon, G. and Erez, A. (1985a) Influence of prolonged exposure to chilling temperatures on bud break and heat requirement for bloom of several fruit species. *Journal of the American Society for Horticultural Science* 110, 47-50.

Couvillon, G. and Erez, A. (1985b) Effect of level and duration of high temperatures on rest in the peach. *Journal of American Society of Horticultural Sciences* 110, 579-581.

Coville, F.V. (1920) The influence of cold in stimulating the growth of plants. *Journal of Agricultural Research* 20, 151–192.

Crabbé, J.J. (1984) Correlative effects modifying the course of bud dormancy in woody plants. *Zeitschrift für Pflanzenphysiologie* 113, 465-469.

Darbyshire, R., Webb, L., Goodwin, I. and Barlow, E. (2014). Challenges in predicting climate change impacts on pome fruit phenology. *International Journal of Biometeorology* 58, 1119-1133.

De la Fuente, L., Conesa, A., Lloret, A., Badenes, M.L. and Ríos, G. (2015) Genome-wide changes in histone H3 lysine 27 trimethylation associated with bud dormancy release in peach. *Tree Genetics and Genomes* 11, 45.

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

Decourteix, M., Alves, G., Bonhomme, M., Peuch, M., Baaziz K, B., Brunel, N., Guilliot, A., Rageau, R., Améglio, T., Pétel, G. et al. (2008) Sucrose (JrSUT1) and hexose (JrHT1 and JrHT2) transporters in walnut xylem parenchyma cells: their potential role in early events of growth resumption. *Tree physiology* 28, 215-224.

Del Duca, S., Serafini-Fracassini, D., Cai, G. (2014) Senescence and programmed cell death in plants: polyamine action mediated by transglutaminase. *Frontiers in Plant Science* 5, 120.

Denisov, V.P. (1988) Almond Genetic Resources in the USSR and their use in production and breeding. *Acta Horticulturae* 224, 299-306.

Dennis, F.G. (1994). Dormancy—what we know (and don't know). *HortScience* 29, 1249-1255.

Dennis, F.G. (2003) Problems in standardizing methods for evaluating the chilling requirements for the breaking of dormancy in buds of woody plants. *HortScience* 38, 347-350.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M. et al. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* 36, W465-W469.

Díaz-Vivancos, P., Barba-Espín, G., Clemente-Moreno, M.J. and Hernández, J.A. (2010) Characterization of the antioxidant system during the vegetative development of pea plants. *Biologia Plantarum* 54, 76–82.

Dicenta, F. and García, J.E. (1992) Phenotypical correlations among some traits in almond. *Journal of Genetics and Breeding* 46, 241–246.

Dicenta, F. and García, J. (1993) Inheritance of self-compatibility in almond. *Heredity* 70, 313.

Dicenta, F., Egea, J., Cremades, T., Martínez-Gómez, P., Ortega, E., Rubio, M., Sánchez-Pérez, R., Martínez-García, P. and Alcolea, J.L. (2017a) Makako: una variedad de almendro del CEBAS-CSIC de floración extra-tardía y auto-compatible. *Revista de fruticultura*, 6-11.

Dicenta, F., Egea, J., Ortega, E., Martínez-Gómez, P., Sánchez-Pérez, R., Rubio, M., Martínez-García, P., Sánchez-Prudencio, A. and Cremades, T. (2016) Almond Breeding: important issues and challenges for research. *Options Méditerranéennes* 119, 23-28.

Dicenta, F., García, J.E. and Carbonell, E.A. (1993) Heritability of flowering, productivity and maturity in almond. *Journal of Horticultural Science* 68, 113–120.

Dicenta, F., Ortega, E., Egea, J. and Cánovas, J.A. (2000). Hacia la plantación monovarietal de almendro con variedades autocompatibles. *Fruticultura profesional*, 11-18.

Dicenta, F., Ortega, E., Martínez-Gómez, P., Sánchez-Pérez, R., Gambín, M. and Egea, J. (2009) Penta and Tardona: two new extra-late flowering self-compatible almond cultivars. *Acta Horticulturae* 814, 189-192.

Dicenta, F., Sánchez-Pérez, R., Rubio, M., Egea, J., Batlle, I., Miarnau, X., Palasciano, M., Lipari, E., Confolent, C. and Martínez-Gómez, P. (2015) The origin of the self-compatible almond 'Guara'. *Scientia Horticulturae* 197, 1-4.

Dicenta, F., Sánchez-Pérez, P., Batlle, I. and Martínez-Gómez, P. (2017b) Late-blooming cultivar development. In Socias i Company, R. and Gradziel, T.M. eds., Almond: Botany, Production and Uses. CABI, Boston. Pp. 168-187.

Ding, J., and Nilsson, O. (2016) Molecular regulation of phenology in trees—because the seasons they are a-changin'. *Current Opinion in Plant Biology* 29, 73-79.

Dobritsa, A. A., Nishikawa, S. I., Preuss, D., Urbanczyk-Wochniak, E., Sumner, L. W., Hammond, A. et al. (2009) LAP3, a novel plant protein required for pollen development, is essential for proper exine formation. *Sexual plant reproduction* 22, 167.

Doğramacı, M., Horvath, D.P., Chao, W.S., Foley, M.E., Christoffers, M.J. and Anderson, J.V. (2010) Low temperatures impact dormancy status, flowering competence, and transcript profiles in crown buds of leafy spurge. *Plant Molecular Biology* 73, 207-226.

Doyle, J.J. and Doyle, M. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19, 11–15.

Du, D., Zhang, Q., Cheng, T., Pan, H., Yang, W. and Sun, L. (2013) Genome-wide identification and analysis of late embryogenesis abundant (LEA) genes in *Prunus mume*. *Molecular Biology Reports* 40, 1937-1946.

Ebine, K. and Ueda, T. (2015) Roles of membrane trafficking in plant cell wall dynamics. *Frontiers in Plant Science* 6, 878.

Egea, J., Berenguer, T., García, J. and Dicenta, F. (2000) 'Antoneta' and 'Marta' Almonds. *HortScience* 35, 1358-1359.

Egea, L., García, J., Egea, J., Berenguer, T., Gambín, M. and Patiño, J. (1985) Variedades de almendro de la Región de Murcia. CSIC-CEBAS Murcia.

Egea, L. and García, J.E. (1988) Lignes de recherche actuella sur l'amandier au CEBAS Murcie. Objectifs et premières observations. 2 Colloque du GREMPA. Montpellier (Nîmes). Septembre 1975,1-11.

Egea, G., Nortes, P.A., González-Real, M.M., Baille, A. and Domingo, R. (2010) Agronomic response and water productivity of almond trees under contrasted deficit irrigation regimes. *Agricultural and Water Management* 97, 171-181.

Egea, J., Ortega, E., Martínez-Gómez, P. and Dicenta, F. (2003) Chilling and heat requirements of almond cultivars for flowering. *Environmental and Experimental Botany* 50, 79-85.

El Yaacoubi, A., Malagi, G., Oukabli, A., Citadin, I., Hafidi, M., Bonhomme, M. and Legave, J.M. (2016) Differentiated dynamics of bud dormancy and growth in temperate fruit trees relating to bud phenology adaptation, the case of apple and almond trees. *International Journal of Biometeorology* 60, 1695-1710.

Erez, A. (1982) The rest in the apple and natural and artificial means to break it (in German). *Erwerbs-Obstbau* 24, 116-118.

Erez, A. (1995) Means to compensate for insufficient chilling to improve bloom and leafing. *Acta Horticulturae* 395, 81–95.

Erez A. (2000) Bud dormancy; phenomenon, problems and solutions in the tropics and subtropics. in temperate fruit crops in warm climates. In Erez A., ed. Springer, Dordrecht.

Erez, A., Couvillon, G.A. and Hendershott, C.H. (1979) The effect of cycle length on chilling negation by high temperatures in dormant peach leaf buds. *Journal of the American Society for Horticultural Science* 104, 573–576.

Erez, A. and Couvillon, G.A. (1987). Characterization of the influence of moderate temperatures on rest completion in peach. *Journal of the American Society for Horticultural Science* 112, 677-680.

Erez, A., Fishman, S., Linsley-Noakes, G.C. and Allan, P. (1990). The dynamic model for rest completion in peach buds. *Acta Horticulturae* 276, 165–174.

Fadón, E., Herrero, M. and Rodrigo, J. (2015) Flower bud dormancy in *Prunus* species. In Anderson, J.V., ed. Advances in Plant Dormancy. Springer, Cham. Pp. 123-135.

Fadón, E., Herrero, M. and Rodrigo, J. (2018) Dormant flower buds actively accumulate starch over winter in sweet cherry. *Frontiers in Plant Science* 9, 171.

Fadón, E. and Rodrigo, J. (2018) Unveiling winter dormancy through empirical experiments. *Environmental and Experimental Botany* 152, 28–36.

Falavigna, V.d.S., Guitton, B., Costes, E. and Andrés, F. (2019) I want to (bud) break free: The potential role of *DAM* and *SVP*-Like genes in regulating dormancy cycle in temperate fruit trees. *Frontiers in plant science* 9, 1990-1990.

Fan, S., Bielenberg, D.G., Zhebentyayeva, T.N., Reighard, G.L., Okie, W.R., Holland, D. and Abbott, A.G. (2010) Mapping quantitative trait loci associated with chilling requirement, heat requirement and bloom date in peach (*Prunus persica*). *New Phytologist* 185, 917-930.

FAO (2017). FAOSTAT. Food and Agriculture Organization of the United Nations, Rome, Italy.

Farrar, K., Evans, I.M., Topping, J.F., Souter, M.A., Nielsen, J.E. and Lindsey, K. (2003) *EXORDIUM*–a gene expressed in proliferating cells and with a role in meristem function, identified by promoter trapping in Arabidopsis. *The Plant Journal* 33, 61-73.

Felipe, A.J. (1977) Phenological states of almond. Proceedings of the Third GREMPA Colloquium, Bari, Italy, 101-103.

Felipe, A.J. (2000) El almendro: el material vegetal. I. Lérida, Spain.

Felipe, A. and Socias i Company, R. (1985) L'amélioration génétique de l'amandier à Saragosse. *Options Méditerranéennes* 85, 9-14.

Felipe, A. and Socias i Company, R. (1987) 'Ayles','Guara', and'Moncayo'almonds. *HortScience* 22, 961-962.

Felker, F.C. and Robitaille, H.A. (1985) Chilling accumulation and rest of sour cherry flower buds. *Journal of American Society of Horticultural Sciences* 110, 227-232.

Feng, S. and Jacobsen, S.E. (2011) Epigenetic modifications in plants: an evolutionary perspective. *Current Opinion in Plant Biology* 14, 179–186.

Fernández i Martí, A., Gradziel, T.M. and Socías i Company, R. (2014) Methylation of the S*f* locus in almond is associated with *S*-RNase loss of function. *Plant Molecular Biology* 86, 681–689.

Fischer-Kilbienski, I., Miao, Y., Roitsch, T., Zschiesche, W., Humbeck, K. and Krupinska, K. (2010) Nuclear targeted AtS40 modulates senescence associated gene expression in *Arabidopsis thaliana* during natural development and in darkness. *Plant Molecular Biology* 73, 379-390.

Fishman, S., Erez, A. and Couvillon, G. (1987a) The temperature dependence of dormancy breaking in plants: computer simulation of processes studied under controlled temperatures. *Journal of Theoretical Biology* 126, 309-321.

Fishman, S., Erez, A. and Couvillon, G.A. (1987b) The temperature dependence of dormancy breaking in plants: Mathematical analysis of a two-step model involving a cooperative transition. *Journal of Theoretical Biology* 124, 473-483.

Foyer, C.H., López-Delgado, H., Dat, J.F. and Scott, I.M. (1997) Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiologia Plantarum* 100, 241–254.

Fulgoni, V., Abbey, M., Davis, P., Jenkins, D., Lovejoy, J., Most, M., Sabate, J. and Spiller, G. (2002) Almonds lower blood cholesterol and LDL-cholesterol but not HDL-cholesterol in human subjects: results of a meta-analysis. *FASEB Journal* 16, 981-982.

Fresnedo-Ramírez, J., Chan, H.M., Parfitt, D.E., Crisosto, C.H. and Gradziel, T.M. (2017) Genome-wide DNA-(de)methylation is associated with noninfectious bud-failure exhibition in almond (*Prunus dulcis* [Mill.] D.A. Webb). *Scientific Reports* 7, 42686.

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemical Journal* 282, 821-828.

Fujiwara, S., Oda, A., Yoshida, R., Niinuma, K., Miyata, K., Tomozoe, Y., Tajima, T., Nakagawa, M., Hayashi, K., Coupland, G. et al. (2008) Circadian clock proteins LHY and CCA1 regulate SVP protein accumulation to control flowering in Arabidopsis. *The Plant Cell* 20, 2960-2971.

Gabay, G., Faigenboim, A., Dahan, Y., Izhaki, Y., Itkin, M., Malitsky, S., Elkind, Y. and Flaishman, M.A. (2019) Transcriptome analysis and metabolic profiling reveal the key role of α-linolenic acid in dormancy regulation of European pear. *Journal of Experimental Botany* 70, 1017-1031.

Gai, S., Zhang, Y., Liu, C., Zhang, Y. and Zheng, G. (2013) Transcript profiling of *Paoenia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. *PLoS ONE* 8, e55297.

Gannouni, T.A., Campoy, J.A., Quero-García, J., Barreneche, T., Arif, A., Albouchi, A. and Ammari, Y. (2017) Dormancy related traits and adaptation of sweet cherry in Northern Africa: A case of study in two Tunisian areas. *Scientia Horticulturae* 219, 272-279.

Gao, F., Jordan, M.C. and Ayele, B.T. (2012) Transcriptional programs regulating seed dormancy and its release by after-ripening in common wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal* 10, 465-476.

García, J.E. (1986) Cultivo del almendro en zonas semiáridas del sureste español. *Anales de Edafología y Agro-biología* 45, 827-841.

Garg, R., Narayana Chevala, V.V.S., Shankar, R. and Jain, M. (2015) Divergent DNA methylation patterns associated with gene expression in rice cultivars with contrasting drought and salinity stress response. *Scientific Reports* 5, 14922.

Gericke, O. (2015). Factors inducing the transition from dormancy to flowering in fruit trees: Meeting the challenges of climate change. Master Thesis., University of Copenhagen, Denmark.

Champagnat, P. (1983). Bud dormancy, correlation between organs, and morphogenesis in woody plants. *Soviet Plant Physiology* 30, 458-471.

Gholizadeh, J., Sadeghipour, H.R., Abdolzadeh, A., Hemmati, K., Hassani, D. and Vahdati, K. (2017) Redox rather than carbohydrate metabolism differentiates endodormant lateral buds in walnut cultivars with contrasting chilling requirements. *Scientia Horticulturae* 225, 29-37.

Ghrab, M., Ben Mimoun, M., Masmoudi, M.M. and Ben Mechlia, N. (2014) The behaviour of peach cultivars under warm climatic conditions in the Mediterranean area. *International Journal of Environmental Studies* 71, 3-14.

González-Rossia, D., Reig, C., Dovis, V., Gariglio, N. and Agustí, M. (2008) Changes on carbohydrates and nitrogen content in the bark tissues induced by artificial chilling and its relationship with dormancy bud break in *Prunus* sp. *Scientia Horticulturae* 118, 275-281.

Gradziel, T.M. (2009) Almond (*Prunus dulcis*) breeding. In Breeding plantation tree crops: temperate species. Springer, New York. Pp. 1-31.

Gradziel, T.M. (2011). 2 Origin and Dissemination of Almond. *Horticultural Reviews* 38, 23.

Gradziel, T. and Kester, D.E. (1994) Breeding for resistance to *Aspergillus flavus* in almond. *Acta Horticultare* 373, 111-117.

Gradziel, T.M. and Kester, D. (1996) Genetic improvements. In Almond production manual. University of California, 70-75.

Gradziel, T.M. and Kester, D.E. (1998) Breeding for self-fertility in California almond cultivars. *Acta Horticulturae*. 470, 109-117.

Gradziel, T. and Kester, D. (1999) The university of California almond breeding programme. II. breeding objectives and projects. *Nucis Newsletter* 8, 10-14.

Gradziel, T.M., Lampinen, B., Connell, J.H. and Viveros, M. (2007) 'Winters' almond: an early-blooming, productive, and high-quality pollenizer for 'Nonpareil'. *HortScience* 42, 1725-1727.

Gradziel, T., Martínez-Gómez, P., Dicenta, F. and Kester, D. (2001) The utilization of related *Prunus* species for almond variety improvement. *Journal of the American Pomological Society* 55, 100.

Gradziel, T.M. and Martínez-Gómez, P. (2013) Almond Breeding. In Janick, J., ed. Plant Breeding Reviews. John Wiley & Sons, Hoboken. Pp. 207-258.

Grasselly, C. (1972) L'amandier: caractères morphologiques et physiologiques des variétés, modalité de leurs transmissions chez les hybrides de première génération. PhD Thesis, Université de Bordeaux, Bordeaux, France.

Grasselly, C. (1976) Origine et évolution de l'amandier cultivé. *Options Méditerranéennes* 32, 45-49.

Grasselly, C. (1978) Observations sur l'utilisation d'un mutant d'amandier à floraison tardive dans un programme d'hybridation. *Annales de l'Amélioration des Plantes 28,* 685–695.

Grasselly, C. and Crossa-Raynaud, P. (1980) L'amandier. Techniques agricoles et productions méditerranéenes. G.P. Maisonneuve- Larose. Paris, 446 pp.

Gregersen, P.L. and Holm, P.B. (2007) Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal* 5, 192-206.

Habu, T., Yamane, H., Sasaki, R., Yano, K., Fujii, H., Shimizu, T., Yamamoto, T. and Tao, R. (2014) Custom microarray analysis for transcript profiling of dormant vegetative buds of Japanese apricot during prolonged chilling exposure. *Journal of the Japanese Society for Horticultural Science* 83, 1-16.

Halaly, T., Pang, X., Batikoff, T., Crane, O., Keren, A., Venkateswari, J., Ogrodovitch, A., Sadka, A., Lavee, S. and Or, E. (2008) Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. *Planta* 228, 79-88.

Hamilton, J.A., El Kayal, W., Hart, A.T., Runcie, D.E., Arango-Velez, A. and Cooke, J.E.K. (2016) The joint influence of photoperiod and temperature during growth cessation and development of dormancy in white spruce (*Picea glauca*). *Tree Physiology* 36, 1432-1448.

Hänninen, H. (2016) Boreal and temperate trees in a changing climate. Biometeorology. Springer, Dordrecht.

Hao, X., Chao, W., Yang, Y. and Horvath, D. (2015) Coordinated Expression of *FLOWERING LOCUS T* and *DORMANCY ASSOCIATED MADS-BOX*-like genes in leafy spurge. *PLoS ONE* 10, e0126030-e0126030.

Harrington, C.A., Gould, P.J. and Clair, J.B.S. (2010) Modeling the effects of winter environment on dormancy release of Douglas-fir. *Forest Ecology and Management* 259, 798-808.

Hatch, A. H. and Walker, D. R. (1969) Rest intensity of dormant peach and apricot leaf buds as influenced by temperature, cold hardines and respiration. *Journal of American Society of Horticultural Sciences* 94, 304–307.

Hedley, P.E., Russell, J.R., Jorgensen, L., Gordon, S., Morris, J.A., Hackett, C.A., Cardle, L. and Brennan, R. (2010) Candidate genes associated with bud dormancy release in blackcurrant (*Ribes nigrum* L.). *BMC Plant Biology* 10, 202.

Heide, O.M. (2003) High autumn temperature delays spring bud burst in boreal trees, counterbalancing the effect of climatic warming. *Tree Physiology* 23, 931-936.

Heide, O.M. (2008) Interaction of photoperiod and temperature in the control of growth and dormancy of *Prunus* species. *Scientia Horticulturae* 115, 309-314.

Heide, O.M. and Prestud, A.K. (2005) Low temperatures, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *Tree Physiology* 25, 109-114.

Hernández Mora, J.R., Micheletti, D., Bink, M., Van de Weg, E., Cantín, C., Nazzicari, N., Caprera, A., Dettori, M.T., Micali, S., Banchi, E. et al. (2017) Integrated QTL detection for key breeding traits in multiple peach progenies. *BMC Genomics* 18, 404.

Hillman, J. R. (1984). Apical Dominance. In Wilkins, M.B., ed. Advanced Plant Physiology. Pitman, London. Pp 127-148.

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y. and Matsui, H. (2001) A large family of class III plant peroxidases. *Plant and Cell Physiology* 42, 462-468.

Hoenicka, H., Nowitzki, O., Hanelt, D. and Fladung, M. (2008) Heterologous overexpression of the birch *FRUITFULL*-like MADS-box gene *BpMADS4* prevents normal senescence and winter dormancy in *Populus tremula* L. *Planta* 227, 1001-1011.

Horvath, D. (2009) Common mechanisms regulate flowering and dormancy. *Plant Science* 177, 523-531.

Horvath, D.P., Anderson, J.V., Chao, W.S. and Foley, M.E. (2003) Knowing when to grow: signals regulating bud dormancy. *Trends in Plant Science* 8, 534-540.

Horvath, D.P., Chao, W.S., Suttle, J.C., Thimmapuram, J. and Anderson, J.V. (2008) Transcriptome analysis identifies novel responses and potential regulatory genes involved in seasonal dormancy transitions of leafy spurge (*Euphorbia esula* L.). *BMC Genomics* 9, 536.

Howe, G.T., Horvath, D.P., Dharmawardhana, P., Priest, H.D., Mockler, T.C. and Strauss, S.H. (2015) Extensive transcriptome changes during natural onset and release of vegetative bud dormancy in *Populus*. *Frontiers in Plant Science* 6, 989.

Huang, J., Xia, T., Li, G., Li, X., Li, Y., Wang, Y., Wang, Y., Chen, Y., Xie, G., Bai, F.-W., et al. (2019) Overproduction of native endo-β-1,4-glucanases leads to largely enhanced biomass saccharification and bioethanol production by specific modification of cellulose features in transgenic rice. *Biotechnology for Biofuels* 12, 11.

Ibáñez, C., Collada, C., Casado, R., González-Melendi, P., Aragoncillo, C. and Allona, I. (2013) Winter induction of the galactinol synthase gene is associated with endodormancy in chestnut trees. *Trees* 27, 1309-1316.

Ibáñez, C., Kozarewa, I., Johansson, M., Ögren, E., Rohde, A. and Eriksson, M.E. (2010) Circadian clock components regulate entry and affect exit of seasonal dormancy as well as winter hardiness in *Populus* trees. *Plant Physiology* 153, 1823-1833.

Imani, A. and Mehr-Abadi, S. M. (2012) Floral differentiation and development in early, middle and late blooming almond cultivars. *African Journal of Microbiology Research*, 6, 5301-5305.

Ionescu, I.A., Moller, B.L. and Sánchez-Pérez, R. (2017a) Chemical control of flowering time. *Journal of Experimental Botany* 68, 369–382.

Ionescu, I.A., López-Ortega, G., Burow, M., Bayo-Canha, A., Junge, A., Gericke, O., Møller, B.L. and Sánchez-Pérez, R. (2017b) Transcriptome and metabolite changes during hydrogen cyanamide-induced floral bud break in sweet cherry. *Frontiers in Plant Science* 8, 1233-1233.

IPCC (2018) Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levelsand related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. In Masson-Delmotte, V., Zhai, P., Pörtner, H.O., Roberts, D., Skea, J., Shukla, P.R., Pirani, A., Moufouma-Okia, W., Péan, Pidcock, C.R., Connors, S., Matthews, J.B.R., Chen, Y., Zhou, X., Gomis, M.I., Lonnoy, E., Maycock, T., Tignor, M. and Waterfield, T. eds. (In Press).

Ito, A., Saito, T., Sakamoto, D., Sugiura, T., Bai, S. and Moriguchi, T. (2015) Physiological differences between bud breaking and flowering after dormancy completion revealed by *DAM* and *FT/TFL1* expression in Japanese pear (*Pyrus pyrifolia*). *Tree Physiology* 36, 109-120.

Jehanzeb, M., Zheng, X. and Miao, Y. (2017) The role of the *S40* gene family in leaf senescence. *International Journal of Molecular Sciences* 18, 2152.

Jiang, D., Wang, Y., Wang, Y. and He, Y. (2008) Repression of *FLOWERING LOCUS C* and *FLOWERING LOCUS T* by the Arabidopsis Polycomb repressive complex 2 components. *PLoS ONE* 3, e3404.

Jiang, D., Yang, W., He, Y. and Amasino, R.M. (2007) Arabidopsis relatives of the human lysine-specific demethylase1 repress the expression of *FWA* and *FLOWERING LOCUS C* and thus promote the floral transition. *The Plant Cell* 19, 2975-2987.

Jonkers, H. (1979) Bud dormancy of apple and pear in relation to the temperature during the growth period. *Scientia Horticulturae* 10, 149-154.

Julian, C., Rodrigo, J. and Herrero, M. (2011) Stamen development and winter dormancy in apricot (*Prunus armeniaca*). *Annals of Botany* 108, 617-625.

Jung, C. and Müller, A.E. (2009) Flowering time control and applications in plant breeding. *Trends in Plant Science* 14, 563-573.

Kalberer, S.R., Wisniewski, M. and Arora, R. (2006) Deacclimation and reacclimation of cold-hardy plants: current understanding and emerging concepts. *Plant Science* 171, 3-16.

Kaufmann, K., Pajoro, A., and Angenent, G.C. (2010) Regulation of transcription in plants: Mechanisms controlling developmental switches. *Nature Reviews Genetics* 11, 830.

Kester, D.E. (1965) Inheritance of time of bloom in certain progenies of almond. *Proceedings of American Society of Horticultural Science* 87, 214-221.

Kester, D.E. and Asay, R.N. (1973) Correlation among chilling requirements for germination, blooming and leafing in almond. *Genetics* 74, 5135–5135.

Kester, D., Asay, R. and Micke, W. (1984) Solano, Sonora and Padre almonds. *HortScience* 19, 138-139.

Kester, D.E. and Gradziel, T.M. (1996) Almonds. In Janick, J. and Moore, J.N., eds. Fruit Breeding, Volume III: Nuts. Wiley: New York. Pp: 1-97.

Kester, D.E., Gradziel, T.M., and Grasselly, C. (1991) Almonds (*Prunus*). *Genetic Resources of Temperate Fruit and Nut Crops* 290, 701-760.

Kester, D.E., Micke, W., Gradziel, T. and Weinbaum, S. (1990) Almond cultivar research in California. *Programme de recherche Agrimed Amélioration génétique de deux espèces de fruits secs méditerranéens: l'amandier et le pistachier*, 67.

Kester, D.E., Raddi, P. and Asay, R.N. (1977) Correlations of chilling requirements for germination blooming and leafing within and among seedling population of almond. *Journal of the American Society for Horticultural Science* 102, 145-148.

Kim, S.A., Ahn, S.Y. and Yun, H.K. (2016) Transcriptome analysis of grapevine shoots exposed to chilling temperature for four weeks. *Horticulture, Environment and Biotechnology* 57, 161-172.

Kim, S.J. and Brandizzi, F. (2014) The plant secretory pathway: an essential factory for building the plant cell wall. *Plant Cell Physiology* 55, 687–693.

Kitamura, Y., Takeuchi, T., Yamane, H., and Tao, R. (2016) Simultaneous down-regulation of *DORMANCY-ASSOCIATED MADS-box6* and *SOC1* during dormancy release in Japanese apricot (*Prunus mume*) flower buds. *The Journal of Horticultural Science and Biotechnology* 91, 476-482.

Kobayashi, A., Takahashi, A., Kakimoto, Y., Miyazawa, Y., Fujii, N., Higashitani, A. and Takahashi, H. (2007) A gene essential for hydrotropism in roots. *Proceedings of the National Academy of Sciences* 104, 4724-4729.

Koike, M., Takezawa, D., Arakawa, K. and Yoshida, S. (1997) Accumulation of 19-kDa plasma membrane polypeptide during induction of freezing tolerance in wheat suspension-cultured cells by abscisic acid. *Plant and Cell Physiology* 38, 707-716.

Koonin, E.V. and Aravind, L. (2002) Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell Death And Differentiation* 9, 394.

Krueger, F. and Andrews, S.R. (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* 27, 1571-1572.

Krupinska, K., Haussühl, K., Anke, S., van der Kooij, T.A.W., Leckband, G., Horst, L. and Falk, J. (2002) A novel nucleus-targeted protein is expressed in barley leaves during senescence and pathogen infection. *Plant Physiology* 130, 1172-1180.

Kumar, G., Arya, P., Gupta, K., Randhawa, V., Acharya, V. and Singh, A.K. (2016a) Comparative phylogenetic analysis and transcriptional profiling of MADS-box gene family identified *DAM* and *FLC*-like genes in apple (*Malusx domestica*). *Scientific Reports* 6, 20695.

Kumar, G., Rattan, U. K., and Singh, A. K. (2016b) Chilling-mediated DNA methylation changes during dormancy and its release reveal the importance of epigenetic regulation during winter dormancy in apple (*Malus x domestica* Borkh.). *PloS ONE*, 11, e0149934.

Kuroda, H., Sugiura, T., and Ito, D. (2002) Changes in hydrogen peroxide content in flower buds of Japanese pear (*Pyrus pyrifolia* Nakai) in relation to breaking of endodormancy. *Journal of the Japanese Society for Horticultural Science* 71, 610-616.

Ladizinsky, G. (1999) On the origin of almond. *Genetics Resources and Crop Evolution* 46, 143-147.

Lämke, J. and Bäurle, I. (2017) Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biology* 18, 124.

Lamp, B., Connell, J., Duncan, R., Viveros, M. and S. Polito, V. (2001) Almond flower development: Floral initiation and organogenesis. *Journal of the American Society for Horticultural Science* 126, 689-696.

Lang, G.A., Early, J.D., Martin, G.C. and Darnell, R.L. (1987) Endo-, para- and ecodormancy: Physiological terminology and classification for dormancy research. *HortScience* 22, 371-377.

Lata, C. and Prasad, M. (2011) Role of DREBs in regulation of abiotic stress responses in plants. *Journal of Experimental Botany* 62, 4731-4748.

Le Provost, G., Herrera, R., Paiva, J.A., Chaumeil, P., Salin, F. and Plomion, C. (2007) A micromethod for high throughput RNA extraction in forest trees. *Biological Research* 40, 291-297.

Lee, S.J., Kang, J.Y., Park, H.J., Kim, M.D., Bae, M.S., Choi, H.I. and Kim, S.Y. (2010) DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. *Plant Physiology* 153, 716-727.

Legave, J.M., Blanke, M., Christen, D., Giovannini, D., Mathieu, V. and Oger, R. (2013) A comprehensive overview of the spatial and temporal variability of apple bud dormancy release and blooming phenology in Western Europe. *International Journal of Biometeorology* 57, 317-331.

Leida, C., Conesa, A., Llácer, G., Badenes, M.L. and Ríos, G. (2012a) Histone modifications and expression of *DAM6* gene in peach are modulated during bud dormancy release in a cultivar-dependent manner. *New Phytologist* 193, 67-80.

Leida, C., Romeu, J.F., García-Brunton, J., Ríos, G. and Badenes, M.L. (2012b) Gene expression analysis of chilling requirements for flower bud break in peach. *Plant Breeding* 131, 329-334.

Leida, C., Conejero, A., Arbona, V., Gómez-Cadenas, A., Llácer, G., Badenes, M.L. and Ríos, G. (2012c) Chilling-dependent release of seed and bud dormancy in peach associates to common changes in gene expression. *PLoS ONE* 7, e35777.

Leida, C., Terol, J., Martí, G., Agustí, M., Llácer, G., Badenes, M.L. and Ríos, G. (2010) Identification of genes associated with bud dormancy release in *Prunus persica* by suppression subtractive hybridization. *Tree Physiology* 30, 655-666.

Lenz, A., Hoch, G. and Vitasse, Y. (2016) Fast acclimation of freezing resistance suggests no influence of winter minimum temperature on the range limit of European beech. *Tree Physiology* 36, 490-501.

Li, L., Stoeckert, C.J. and Roos, D.S. (2003) OrthoMCL: Identification of Ortholog Groups for Eukaryotic Genomes. *Genome Research* 13, 2178-2189.

Li, F., Wu, X., Lam, P., Bird, D., Zheng, H., Samuels, L., Jetter, R. and Kunst, L. (2008) Identification of the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in Arabidopsis. *Plant Physiology* 148, 97-107.

Li, J., Xu, Y., Niu, Q., He, L., Teng, Y. and Bai, S. (2018) Abscisic Acid (ABA) promotes the induction and maintenance of pear (*Pyrus pyrifolia* white pear group) flower bud endodormancy. *International Journal of Molecular Sciences* 19, 310.

Liu, Y., Ye, N., Liu, R., Chen, M. and Zhang, J. (2010) H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in Arabidopsis seed dormancy and germination. *Journal of Experimental Botany* 61, 2979-2990.

Lloret, A., Martínez-Fuentes, A., Agustí, M., Badenes, M.L. and Ríos, G. (2017) Chromatin-associated regulation of sorbitol synthesis in flower buds of peach. *Plant Molecular Biology* 95, 507–517.

López-Molina, L., Mongrand, S., Kinoshita, N. and Chua, N.H. (2003) AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes and Development* 17, 410-418.

Luedeling, E. (2012) Climate change impacts on winter chill for temperate fruit and nut production: A review. *Scientia Horticulturae* 144, 218-229.

Luedeling, E. and Brown, P.H. (2011) A global analysis of the comparability of winter chill models for fruit and nut trees. *International Journal of Biometeorology* 55, 411-421.

Luedeling, E., Girvetz, E.H., Semenov, M.A. and Brown, P.H. (2011) Climate change affects winter chill for temperate fruit and nut trees. *PLoS ONE* 6, e20155.

Luedeling, E., Zhang, M. and Girvetz, E.H. (2009) Climatic changes lead to declining winter chill for fruit and nut trees in California during 1950–2099. *PLoS ONE* 4, e6166.

Marengo, J.A. and Camargo, C.C. (2008) Surface air temperature trends in Southern Brazil for 1960–2002. *International Journal of Climatology: A Journal of the Royal Meteorological Society* 28, 893-904.

Marchese, A., Bošković, R., Martínez-García, P. and Tobutt, K. (2008) The origin of the self-compatible almond 'Supernova'. *Plant breeding* 127, 105-107.

Martínez, M., Serra, C., Burgueño, A. and Lana, X. (2010) Time trends of daily maximum and minimum temperatures in Catalonia (NE Spain) for the period 1975–2004. *International Journal of Climatology: A Journal of the Royal Meteorological Society* 30, 267-290.

Martínez-García, P.J., Mañas, F., López, P., Dicenta, F. and Ortega, E. (2011) Molecular and phenotypic characterization of the *S*-locus and determination of flowering time in new 'Marcona'and 'Desmayo Largueta'-type almond (*Prunus dulcis*) selections. *Euphytica* 177, 67-78.

Martínez-Gómez, P., Prudencio, A.S., Gradziel, T.M. and Dicenta, F. (2017) The delay of flowering time in almond: a review of the combined effect of adaptation, mutation and breeding. *Euphytica* 213, 197.

Maulión, E., Valentini, G.H., Kovalevski, L., Prunello, M., Monti, L.L., Daorden, M.E., Quaglino, M. and Cervigni, G.D.L. (2014) Comparison of methods for estimation of chilling and heat requirements of nectarine and peach genotypes for flowering. *Scientia Horticulturae* 177, 112-117.

Mazzitelli, L., Hancock, R.D., Haupt, S., Walker, P.G., Pont, S.D.A., McNicol, J., Cardle, L., Morris, J., Viola, R., Brennan, R. et al. (2007) Co-ordinated gene expression during phases of dormancy release in raspberry (*Rubus idaeus* L.) buds. *Journal of Experimental Botany* 58, 1035-1045.

Measham, P.F., Darbyshire, R., Turpin, S.R. and Murphy-White, S. (2017) Complexity in chill calculations: A case study in cherries. *Scientia Horticulturae* 216, 134-140.

Mielke, E.A. and Dennis, F.G. (1978) Hormonal control of flower bud dormancy in sour cherry (*Prunus cerasus* L.) III. Effects of leaves, defoliation and temperature on levels of abscisic acid in flower primordia. *Journal of American Society of Horticultural Sciences* 103, 446–449.

Miyazawa, Y., Moriwaki, T., Uchida, M., Kobayashi, A., Fujii, N. and Takahashi, H. (2012) Overexpression of *MIZU-KUSSEI1* enhances the root hydrotropic response by retaining cell viability under hydrostimulated conditions in *Arabidopsis thaliana*. *Plant and Cell Physiology* 53, 1926-1933.

Moriwaki, T., Miyazawa, Y., Fujii, N. and Takahashi, H. (2012) Light and abscisic acid signalling are integrated by *MIZ1* gene expression and regulate hydrotropic response in roots of *Arabidopsis thaliana*. *Plant, Cell and Environment* 35, 1359-1368.

Muncharaz, M. (2003). El almendro: Manual técnico. Aedos, Barcelona.173.

Musa-Veloso, K., Paulionis, L., Poon, T. and Lee, H.Y. (2016) The effects of almond consumption on fasting blood lipid levels: a systematic review and meta-analysis of randomised controlled trials. *Journal of Nutritional Science* 5, e34-e34.

Nakamura, S., Pourkheirandish, M., Morishige, H., Kubo, Y., Nakamura, M., Ichimura, K., Seo, S., Kanamori, H., Wu, J., Ando, T. et al. (2016) Mitogen-Activated Protein Kinase 3 regulates seed dormancy in barley. *Current Biology* 26, 775-781.

Naor, A., Flaishman, M., Stern, R., Moshe, A. and Erez, A. (2003) Temperature effects on dormancy completion of vegetative buds in apple. *Journal of the American Society for Horticultural Science*, 128, 636-641.

Nielsen, K.L., Indiani, C., Henriksen, A., Feis, A., Becucci, M., Gajhede, M., Smulevich, G. and Welinder, K.G. (2001) Differential Activity and structure of highly similar peroxidases. Spectroscopic, crystallographic, and enzymatic analyses of lignifying *Arabidopsis thaliana* Peroxidase A2 and Horseradish Peroxidase A2. *Biochemistry* 40, 11013–11021.

Nishizawa, A., Yabuta, Y. and Shigeoka, S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiology* 147, 1251-1263.

Noh, Y.S. and Amasino, RM. (2003) *PIE1*, an ISWI family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell 15, 1671-1682.

Okie, W.R. and Blackburn, B. (2011) Increasing chilling reduces heat requirement for floral budbreak in peach. *HortScience* 46, 245-252.

Olukolu, B. A., Trainin, T., Fan, S., Kole, C., Bielenberg, D. G., Reighard, G. L. et al. (2009). Genetic linkage mapping for molecular dissection of chilling requirement and budbreak in apricot (*Prunus armeniaca* L.). *Genome*, 52(10), 819-828.

Or, E., Vilozny, I., Eyal, Y. and Ogrodovitch, A. (2000) The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. *Plant Molecular Biology* 43, 483-494.

Ortega, E., and Dicenta, F. (2004) Suitability of four different methods to identify self-compatible seedlings in an almond breeding programme. *The Journal of Horticultural Science and Biotechnology*, 79(5), 747-753.

Oukabli, A. and Mahhou, A. (2007) Dormancy in sweet cherry (*Prunus avium* L.) under Mediterranean climatic conditions. *Biotechnology, Agronomy, Society and Environment* 11, 122-128.

Oukabli, A., Bartolini, S. and Viti, R. (2003) Anatomical and morphological study of apple (*Malus× domestica* Borkh.) flower buds growing under inadequate winter chilling. *The Journal of Horticultural Science and Biotechnology* 78, 580-585.

Park, B.S., Eo, H.J., Jang, I.C., Kang, H.G., Song, J.T. and Seo, H.S. (2010) Ubiquitination of LHY by SINAT5 regulates flowering time and is inhibited by DET1. *Biochemical and Biophysical Research Communications* 398, 242-246.

Park, M.J., Kwon, Y.J., Gil, K.E., and Park, C.M. (2016). LATE ELONGATED HYPOCOTYL regulates photoperiodic flowering via the circadian clock in Arabidopsis. *BMC Plant Biology* 16, 114.

Pascual, J., Cañal, M.J., Correia, B., Escandon, M., Hasbún, R., Meijón, M., Pinto, G. and Valledor, L. (2014) Can epigenetics help forest plants to adapt to climate change? In Álvarez-Venegas, R., De la Peña, C. and Casas-Mollano, J.A., eds. Epigenetics in plants of agronomic importance: Fundamentals and applications. Springer, Cham. Pp. 125-146.

Patwari, P., Salewski, V., Gutbrod, K., Kreszies, T., Dresen-Scholz, B., Peisker, H., Steiner, U., Meyer, A.J., Schreiber, L. and Dörmann, P. (2019) Surface wax esters contribute to drought tolerance in Arabidopsis. *The Plant Journal* 98, 727-744.

Pérez, F.J. and Lira, W. (2005) Possible role of catalase in post-dormancy bud break in grapevines. *Journal of Plant Physiology* 162, 301-308.

Pérez, F.J., Vergara, R. and Rubio, S. (2008) H_2O_2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds. *Plant Growth Regulation* 55, 149-155.

Petersen, T.N., Brunak, S., von Heijne, G. Nielsen, J. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods* 8, 785–786.

Petri, J.L. and Leite, G.B. (2003). Consequences of insufficient winter chilling on apple tree bud-break. Paper presented at: VII International Symposium on Temperate Zone Fruits in the Tropics and Subtropics, 662.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29, e45-e45.

Pilati, S., Brazzale, D., Guella, G., Milli, A., Ruberti, C., Biasioli, F., Zottini, M. and Moser, C. (2014) The onset of grapevine berry ripening is characterized by ROS accumulation and lipoxygenase-mediated membrane peroxidation in the skin. *BMC Plant Biology* 14, 87.

Polito, V.S., Pinney, K., Heerema, R. and Weinbaum, S. (2002) Flower differentiation and spur leaf area in almond. *Journal of Horticultural Science and Biotechnology* 77, 474-478.

Pomar, F., Caballero, N., Pedreño, M.A. and Ros-Barceló, A. (2002) H_2O_2 generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. *FEBS Letters* 529, 198–202.

Ponti, L., Gutierrez, A.P., Ruti, P.M. and Dell'Aquila, A. (2014) Fine-scale ecological and economic assessment of climate change on olive in the Mediterranean Basin reveals winners and losers. *Proceedings of the National Academy of Sciences* 111, 5598-5603.

Poonam, V., Kumar, G., S Reddy L, C., Jain, R., K Sharma, S., K Prasad, A. and S Parmar, V. (2011) Chemical constituents of the genus *Prunus* and their medicinal properties. *Current Medicinal Chemistry* 18, 3758-3824.

Punt, W. (1964) Names found to be incorrect for which no legitimate alternative name is available. *Regne Vegetabile* 36, 9.

Rabot, A., Mortreau, E., Ben Baaziz, K., Hamama, L., Boummaza, R., Sakr, S., Henry, C., Lothier, J., Le Gourrierec, J., Leduc, N. et al. (2012) Insight into the role of sugars in bud burst under light in the rose. *Plant and Cell Physiology* 53, 1068-1082.

Ramos, A., Pérez-Solís, E., Ibáñez, C., Casado, R., Collada, C., Gómez, L., Aragoncillo, C. and Allona, I. (2005) Winter disruption of the circadian clock in chestnut. *Proceedings of the National Academy of Sciences* 102, 7037-7042.

Ramsay, J. and Martin, G.C. (1970) Seasonal changes in growth promoters and inhibitors in buds of apricot. *Journal of American Society of Horticultural Sciences* 95, 569-574.

Ranocha, P., Dima, O., Nagy, R., Felten, J., Corratgé-Faillie, C., Novák, O., Morreel, K., Lacombe, B., Martinez, Y., Pfrunder, S. et al. (2013) Arabidopsis WAT1 is a vacuolar auxin transport facilitator required for auxin homoeostasis. *Nature Communications* 4, 2625.

Reinoso, H., Luna, V., Pharis, R. P. and Bottini, R. (2002) Dormancy in peach (*Prunus persica*) flower buds. V. Anatomy of bud development in relation to phenological stage. *Canadian Journal of Botany* 80, 656-663.

Richardson, E. (1974) A model for estimating the completion of rest for'Redhaven'and'Elberta'peach trees. *HortScience* 9, 331-332.

Richardson, E., Seeley, S., Walker, D., Anderson, J. and Ashcroft, G. (1975) Pheno-climatography of spring peach bud development [Cold damage]. *HortScience* 10, 236–237.

Rinne, P.L.H., Welling, A., Vahala, J., Ripel, L., Ruonala, R., Kangasjärvi, J. and van der Schoot, C. (2011) Chilling of dormant buds hyperinduces *FLOWERING LOCUS T* and recruits GA-inducible 1,3-β-glucanases to reopen signal conduits and release dormancy in *Populus*. *The Plant Cell* 23, 130-146.

Ríos, G., Leida, C., Conejero, A. and Badenes, M.L. (2014) Epigenetic regulation of bud dormancy events in perennial plants. *Frontiers in Plant Science* 5, 247.

Ríos, G., Tadeo, F.R., Leida, C. and Badenes, M.L. (2013) Prediction of components of the sporopollenin synthesis pathway in peach by genomic and expression analyses. *BMC Genomics* 14, 12.

Rodrigo, M.J., Alquezar, B. and Zacarías, L. (2006) Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *Journal of Experimental Botany* 57, 633-643.

Rodríguez, A. and Sánchez-Tamés, R. (1986) Dormancy and seasonal changes of plant growth regulators in hazel buds. *Physiologia Plantarum* 66, 288-292.

Rodríguez, A., Pérez-López, D., Sánchez, E., Centeno, A., Gómara, I., Dosio, A. and Ruiz-Ramos, M. (2019) Chilling accumulation in fruit trees in Spain under climate change. *Natural Hazards and Earth System Sciences* 19, 1087-1103.

Rodríguez, J.A., Sherman, W.B., Scorza, R., Wisniewski, M. and Okie, W.R. (1994) Evergreen'peach, its inheritance and dormant behavior. *Journal of the American Society for Horticultural Science* 119, 789-792.

Rodríguez-Falcón, M., Bou, J. and Prat, S. (2006) Seasonal control of tuberization in potato: Conserved elements with the flowering response. *Annual Review of Plant Biology* 57, 151-180.

Rohde, A. and Bhalerao, R.P. (2007) Plant dormancy in the perennial context. *Trends in Plant Science* 12, 217-223.

Romeu, J.F., Monforte, A.J., Sánchez, G., Granell, A., García-Brunton, J., Badenes, M.L. and Ríos, G. (2014) Quantitative trait loci affecting reproductive phenology in peach. *BMC Plant Biology* 14, 52-52.

Ros-Barceló, A. (1998) The generation of H_2O_2 in the xylem of *Zinnia elegans* is mediated by an NADPH-oxidase-like enzyme. *Planta* 207, 207–216

Rothkegel, K., Greve, M., Tapia, S., Bravo, S., Almeida, A.M., Montes, C., Sánchez, E. and Prieto, H. (2017) DNA methylation and small interference RNAs participate in the regulation of MADS-box genes involved in dormancy in sweet cherry (*Prunus avium* L.). *Tree Physiology* 37, 1739-1751.

Routray, P., Li, T., Yamasaki, A., Yoshinari, A., Takano, J., Choi, W.G., Sams, C.E. and Roberts, D.M. (2018) Nodulin Intrinsic Protein 7;1 is a tapetal boric acid channel involved in pollen cell wall formation. *Plant Physiology* 178, 1269-1283.

Rubio, S., Noriega, X. and Pérez, F.J. (2018) Abscisic acid (ABA) and low temperatures synergistically increase the expression of *CBF/DREB1* transcription factors and cold-hardiness in grapevine dormant buds. *Annals of Botany* 123, 681-689.

Ruiz, D., Campoy, J.A. and Egea, J. (2007) Chilling and heat requirements of apricot cultivars for flowering. *Environmental and Experimental Botany* 61, 254-263.

Ruttink, T., Arend, M., Morreel, K., Storme, V., Rombauts, S., Fromm, J., Bhalerao, R.P., Boerjan, W. and Rohde, A. (2007) A molecular timetable for apical bud formation and dormancy induction in poplar. *Plant Cell* 8, 2370-2390.

Sabate, J. and Haddad, E. (2001) Almond-rich diets simultaneously improve plasma lipoproteins and alpha-tocopherol levels in men and women. *Annals of Nutrition and Metabolism* 45, 596.

Saeed, A., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T. and Thiagarajan, M. (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34, 374-378.

Salazar, J.A., Ruiz, D., Campoy, J.A., Tartarini, S., Dondini, L. and Martínez-Gómez, P. (2016) Inheritance of reproductive phenology traits and related QTL identification in apricot. *Tree Genetics and Genomes* 12, 71.

Sánchez-Pérez, R., Del Cueto, J., Dicenta, F. and Martínez-Gómez, P. (2014) Recent advancements to study flowering time in almond and other *Prunus* species. *Frontiers in Plant Science* 5, 334-334.

Sánchez-Pérez, R., Dicenta, F. and Martínez-Gómez, P. (2004) Identification of *S*-alleles in almond using multiplex PCR. *Euphytica* 138, 263-269.

Sánchez-Pérez, R., Dicenta, F. and Martínez-Gómez, P. (2012) Inheritance of chilling and heat requirements for flowering in almond and QTL analysis. *Tree Genetics and Genomes* 8, 379-389.

Sánchez-Pérez, R., Jørgensen, K., Olsen, C.E., Dicenta, F. and Møller, B.L. (2008) Bitterness in almonds. *Plant Physiology* 146, 1040-1052.

Sánchez-Pérez, R., Ortega, E., Duval, H., Martínez-Gómez, P. and Dicenta, F. (2007) Inheritance and correlation of important agronomic traits in almond. *Euphytica* 155, 381–391.

Sánchez-Pérez, R., Pavan, S., Mazzeo, R., Moldovan, C., Cigliano, R.A., Del Cueto, J., Ricciardi, F., Lotti, C., Ricciardi, L. and Dicenta, F. (2019) Mutation of a bHLH transcription factor allowed almond domestication. *Science* 364, 1095-1098.

Santamaría, M., Hasbún, R., Valera, M., Meijón, M., Valledor, L., Rodríguez, J.L. and Rodríguez, R. (2009) Acetylated H4 histone and genomic DNA methylation patterns during bud set and bud burst in *Castanea sativa*. *Journal of Plant Physiology* 166, 1360–1369.

Sathe, S.K., Teuber, S.S., Gradziel, T.M. and Roux, K.H. (2001) Electrophoretic and immunological analyses of almond (*Prunus dulcis* L.) genotypes and hybrids. *Journal of Agriculture and Food Chemistry* 49, 2043-2052.

Saura-Calixto, F., Bauza, M., Martinez de Toda, F. and Argamenteria, A. (1981) Amino acids, sugars, and inorganic elements in the sweet almond (*Prunus amygdalus*). *Journal of Agricultural and Food Chemistry* 29, 509-511.

Sawa, M. and Kay, S.A. (2011) *GIGANTEA* directly activates *Flowering Locus T* in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 108, 11698-11703.

Saze, H. (2008) Epigenetic memory transmission through mitosis and meiosis in plants. *Seminars in Cell and Developmental Biology* 19, 527-536.

Schirra, M. (1997) Postharvest technology and utilization of almonds. *Horticultural reviews* 20, 267-311.

Schröder, F., Lisso, J., Lange, P. and Müssig, C. (2009) The extracellular EXO protein mediates cell expansion in Arabidopsis leaves. *BMC Plant Biology* 9, 20-20.

Schwartz, M.D., Ahas, R. and Aasa, A. (2006) Onset of spring starting earlier across the northern hemisphere. *Global Change Biology* 12, 343–351.

Sedgley, M. and Collins, G. (2002) Almond improvement in Australia. *Fruits* 57, 129-134.

Seeley, S. (1990). Hormonal transduction of environmental stresses. HortScience 25, 1369–1376.

Segura, J.M.A., Kodad, O. and Ansón, J. (2016) 'Vialfas', una nueva variedad de almendro del CITA de Aragón de floración extra–tardía. *Revista de fruticultura* 49, 60-67.

Shirasawa, K., Isuzugawa, K., Ikenaga, M., Saito, Y., Yamamoto, T., Hirakawa, H. and Isobe, S. (2017) The genome sequence of sweet cherry (*Prunus avium*) for use in genomics-assisted breeding. *DNA Research* 24, 499-508.

Shkolnik, D., Nuriel, R., Bonza, M.C., Costa, A. and Fromm, H. (2018) MIZ1 regulates ECA1 to generate a slow, long-distance phloem-transmitted Ca^{2+} signal essential for root water tracking in Arabidopsis. *Proceedings of the National Academy of Sciences* 115, 8031-8036.

Silva, C., Garcia-Mas, J., Sánchez, A.M., Arús, P. and Oliveira, M. (2005) Looking into flowering time in almond (*Prunus dulcis* (Mill) D. A. Webb): the candidate gene approach. *Theoretical and Applied Genetics* 110, 959-968.

Singh, R.K., Maurya, J.P., Azeez, A., Miskolczi, P., Tylewicz, S., Stojkovič, K., Delhomme, N., Busov, V. and Bhalerao, R.P. (2018) A genetic network mediating the control of bud break in hybrid aspen. *Nature Communications* 9, 4173.

Singh, R.K., Svystun, T., AlDahmash, B., Jönsson, A.M. and Bhalerao, R.P. (2017) Photoperiod- and temperature-mediated control of phenology in trees – a molecular perspective. *New Phytologist* 213, 511-524.

Socias i Company, R. (2002). The relationship of *Prunus webbii* and almond revisited. *Nucis Newsletter* 11, 17-19.

Socias i Company, R. and Felipe, A.J. (1999) Blanquerna, Cambra y Felisia: Tres nuevos cultivares autógamos de almendro. *Información Técnica Económica Agraria Producción vegetal* 95, 111-117.

Socias i Company, R. and Felipe, A. J. (2007) 'Belona'and 'Soleta'Almonds. *HortScience* 42, 704-706.

Socias i Company, R., Felipe, A. and Aparisi, J.G. (1999) A major gene for flowering time in almond. *Plant breeding* 118, 443-448.

Socias i Company, R., Kodad, O., Alonso, J. M. and Felipe, A. J. (2008) 'Mardía'almond. *HortScience* 43, 2240-2242.

Spach, É. (1843) Monographia generis *Amygdalus.* P. Renouard.

Srinivasan, C., Dardick, C., Callahan, A., and Scorza, R. (2012) Plum (*Prunus domestica*) trees transformed with poplar *FT1* result in altered architecture, dormancy requirement, and continuous flowering. *PLoS ONE*, 7, e40715.

Stöckigt, J., Barleben, L., Panjikar, S. and Loris, E.A. (2008) 3D-Structure and function of strictosidine synthase – the key enzyme of monoterpenoid indole alkaloid biosynthesis. *Plant Physiology and Biochemistry* 46, 340-355.

Sudawan, B., Chang, C.S., Chao, H.F., Ku, M.S.B. and Yen, Y.F. (2016) Hydrogen cyanamide breaks grapevine bud dormancy in the summer through transient activation of gene expression and accumulation of reactive oxygen and nitrogen species. *BMC Plant Biology* 16, 202.

Szálay, L., Timon, B., Németh, S., Papp, J. and Tóth, M. (2010) Hardening and dehardening of peach flower buds. *HortScience* 45, 761-765.

Tabuenca, M.C. (1964) Necesidades de frío invernal de variedades de albaricoquero, melocotonero y peral. *Anales de Estación Experimental de Aula Dei* 7, 113-131.

Tabuenca, M.C. (1972) Chilling requirements in almond. *Anales de la Estación Experimental de Aula Dei* 11, 325–329.

Takemura, Y., Kuroki, K., Jiang, M., Matsumoto, K. and Tamura, F. (2015) Identification of the expressed protein and the impact of change in ascorbate peroxidase activity related to endodormancy breaking in *Pyrus pyrifolia*. *Plant Physiology and Biochemistry* 86, 121–129.

Tamura, F., Tanabe, K. and Banno, K. (1992) Effect of chilling treatment on intensity of bud dormancy, respiration and endogenous growth regulators in Japanese pear 'Nijisseiki'. *Journal of Japanese Society of Horticultural Sciences* 60, 763–769.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., et al. (2017) agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic acids research* 45, W122-W129.

Tong, Z., Gao Z., Wang F., Zhou J. and Zang, Z. (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. *BMC Molecular Biology* 10, 71.

Trainin, T., Bar-Ya'akov, I. and Holland, D. (2013) *ParSOC1*, a MADS-box gene closely related to Arabidopsis *AGL20/SOC1*, is expressed in apricot leaves in a diurnal manner and is linked with chilling requirements for dormancy break. *Tree Genetics and Genomes* 9, 753-766.

Tromp, J. (1976) Flower-bud formation and shoot growth in apple as affected by temperature. *Scientia Horticulturae* 5, 331–338.

Unterholzner, S.J., Rozhon, W., Papacek, M., Ciomas, J., Lange, T., Kugler, K.G., Mayer, K.F., Sieberer, T. and Poppenberger, B. (2015) Brassinosteroids are master regulators of gibberellin biosynthesis in Arabidopsis. *The Plant Cell* 27, 2261-2272.

Valdés, A.E., Övernäs, E., Johansson, H., Rada-Iglesias, A. and Engström, P. (2012) The homeodomain-leucine zipper (HD-Zip) class I transcription factors ATHB7 and ATHB12 modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic acid receptor gene activities. *Plant Molecular Biology* 80, 405-418.

van Bel, A.J.E., Furch, A.C.U., Buxa, S.V., Will, T., Musetti, R. and Hafke, J.B. (2014) Spread the news: systemic dissemination and local impact of Ca^{2+} signals along the phloem pathway. *Journal of Experimental Botany* 65, 1761-1787.

van der Schoot, C., Paul, L.K., Paul, S.B. and Rinne, P.L.H. (2011) Plant lipid bodies and cell-cell signaling: a new role for an old organelle? *Plant Signaling and Behavior* 6, 1732-1738.

van Gurp, T.P., Wagemaker, N.V., Wouters, B., Vergeer, P., Ouborg, J.N. and Werhoeben, K.J.V. (2016) epiGBS: reference-free reduced representation bisulfite sequencing. *Nature Methods* 13, 322–329.

Vargas Garcia, F. J. and Romero Romero, M. A. (1993) 'Masbovera','Glorieta'and 'Francolí', three new almond varieties from IRTA. I International Congress on Almond 373, 75-82.

Vargas, F. J., Romero, M. A., Clavé, J., Vergés, J., Santos, J. and Batlle, I. (2006) Four new almond varieties released by IRTA: 'Vayro', 'Marinada', 'Constantí' and 'Tarraco'. *Nucis-Newsletter*, 13, 9-12.

Vargas, F. J., Romero, M. A., Rovira, M. and Girona, J. (1984) Amélioration de l'amandier par croisement de variétés. Résultats préliminaires à Tarragone (Espagne). *Options Méditerranéennes* 1, 101-122.

Varriale, A. (2017) DNA Methylation in plants and its implications in development, hybrid vigour, and evolution. In Rajewsky N., Jurga S., Barciszewski J., eds. Plant Epigenetics. Springer, Cham.

Vavilov, N. I. (1930) Wild progenitors of the fruit trees of Turkistan and the Caucasus and the problem of the origin of fruit trees. *Report and Proceedings of the 9th International Horticultural Congress* 27-86.

Verde, I., Jenkins, J., Dondini, L., Micali, S., Pagliarani, G., Vendramin, E., Paris, R., Aramini, V., Gazza, L., Rossini, L. et al. (2017) The Peach v2.0 release: high-resolution linkage mapping and deep resequencing improve chromosome-scale assembly and contiguity. *BMC Genomics* 18, 225-225.

Viggiano, L. and de Pinto, M.C. (2017) Dynamic DNA methylation patterns in stress response. In Rajewsky, N., Jurga, S. and Barciszewski, J., eds. Plant Epigenetics. Springer, Berlin. Pp: 281–302.

Vining, K.J., Pomraning, K.R., Wilhelm, L.J., Priest, H.D., Pellegrini, M. and Mockler, T. C. (2012) Dynamic DNA cytosine methylation in the *Populus trichocarpa* genome: tissue-level variation and relationship to gene expression. *BMC Genomics* 13, 27.

Viola, R., Pelloux, J., Van der Ploeg, A., Gillespie, T., Marquis, N., Roberts, A.G. and Hancock, R.D. (2007) Symplastic connection is required for bud outgrowth following dormancy in potato (*Solanum tuberosum* L.) tubers. *Plant, Cell and Environment* 30, 973-983.

Vitasse, Y., Lenz, A. and Körner, C. (2014) The interaction between freezing tolerance and phenology in temperate deciduous trees. *Frontiers in Plant Science* 5.

Viti, R., Andreini, L., Ruiz, D., Egea, J., Bartolini, S., Iacona, C. and Campoy, J.A. (2010) Effect of climatic conditions on the overcoming of dormancy in apricot flower buds in two Mediterranean areas: Murcia (Spain) and Tuscany (Italy). *Scientia Horticulturae* 124, 217-224.

Viti, R., Bartolini, S. and Andreini, L. (2013) Apricot flower bud dormancy: main morphological, anatomical and physiological features related to winter climate influence. *Advances in Horticultural Science* 27, 5–17.

Vitra, A., Lenz, A. and Vitasse, Y. (2017) Frost hardening and dehardening potential in temperate trees from winter to budburst. *New Phytologist* 216, 113-123.

Voogd, C., Wang, T. and Varkonyi-Gasic, E. (2015) Functional and expression analyses of kiwifruit *SOC1*-like genes suggest that they may not have a role in the transition to flowering but may affect the duration of dormancy. *Journal of Experimental Botany* 66, 4699-4710.

Wang, S.Y. and Faust, M. (1994) Changes in the antioxidant system associated with budbreak in anna apple (*Malus domestica Borkh*) buds. *Journal of American Society of Horticultural Sciences* 119, 735–741.

Wang, D., Gao, Z., Du, P., Xiao, W., Tan, Q., Chen, X., Li, L. and Gao, D. (2015) Expression of ABA metabolism-related genes guggests similarities and differences between seed dormancy and bud dormancy of peach (*Prunus persica*). *Frontiers in Plant Science* 6, 1248.

Wang, J., Liu, J., Jiu, S., Li, Y., Whiting, M., She, W., Wang, L., Ma, C., Xu, W., Wang, S. et al. (2019) The MADS-box genes *PaMADS3/4/5* co-regulate multi-pistil formation induced by high temperature in *Prunus avium* L. *Scientia Horticulturae* 256, 108593.

Watkins, R. (1979) Cherry, plum, peach, apricot and almond. In Simmonds, N.W. ed. Evolution of crop plants. Longman, London. Pp:242–247.

Weinberger, J.H. (1950) Chilling requirements of peach varieties. *Proceedings American Society for Horticultural Science* 56, 122-8.

Welinder, K.G. (1992) Superfamily of plant, fungal and bacterial peroxidases. *Current Opinion in Structural Biology* 2, 388-393.

Wirthensohn, M. G. and Sedgley, M. (2001) Almond breeding in Australia. III International Symposium on Pistachios and Almonds 591, 245-248.

Wisniewski, M. E., Bassett, C. L., Renaut, J., Farrell Jr, R., Tworkoski, T. and Artlip, T. S. (2006) Differential regulation of two dehydrin genes from peach (*Prunus persica*) by photoperiod, low temperature and water deficit. *Tree physiology* 26, 575-584.

Wisniewski, M., Nassuth, A. and Arora, R. (2018) Cold hardiness in trees: A mini-review. *Frontiers in Plant Science* 9.

Wisniewski, M., Norelli, J., Bassett, C., Artlip, T. and Macarisin, D. (2011) Ectopic expression of a novel peach (*Prunus persica*) *CBF* transcription factor in apple (*Malus*× *domestica*) results in short-day induced dormancy and increased cold hardiness. *Planta* 233, 971-983.

Wu, T. D. and Watanabe, C. K. (2005) GMAP: a genomic mapping and alignment program for mRNA and EST sequences. *Bioinformatics* 21, 1859-1875.

Wu, R., Tomes, S., Karunairetnam, S., Tustin, S.D., Hellens, R.P., Allan, A.C., Macknight, R.C. and Varkonyi-Gasic, E. (2017) *SVP*-like MADS box genes control dormancy and budbreak in apple. *Frontiers in Plant Science* 8, 477.

Xiao, W., Custard, K.D., Brown, R.C., Lemmon, B.E., Harada, J.J., Goldberg, R.B. and Fischer, R.L. (2006) DNA methylation is critical for Arabidopsis embryogenesis and seed viability. *Plant Cell* 18, 805–814.

Xu, Z., Zhang, Q., Sun, L., Du, D., Cheng, T., Pan, H., Yang, W. and Wang, J. (2014) Genome-wide identification, characterisation and expression analysis of the MADS-box gene family in *Prunus mume*. *Molecular Genetics and Genomics* 289, 903-920.

Yaish, M.W., Colasanti, J. and Rothstein, S.J. (2011) The role of epigenetics processes in controlling flowering time exposed to stress. *Journal of Experimental Botany* 62, 3727–3735.

Yamane, H., Kashiwa, Y., Kakehi, E., Yonemori, K., Mori, H., Hayashi, K., Iwamoto, K., Tao, R. and Kataoka, I. (2006) Differential expression of dehydrin in flower buds of two Japanese apricot cultivars requiring different chilling requirements for bud break. *Tree Physiology* 26, 1559-1563.

Yamane, H., Ooka, T., Jotatsu, H., Hosaka, Y., Sasaki, R., & Tao, R. (2011). Expressional regulation of *PpDAM5* and *PpDAM6*, peach (*Prunus persica*) dormancy-associated MADS-box genes, by low temperature and dormancy-breaking reagent treatment. *Journal of experimental botany*, 62, 3481–3488. doi:10.1093/jxb/err028

Yamazaki, T., Miyazawa, Y., Kobayashi, A., Moriwaki, T., Fujii, N. and Takahashi, H. (2012) MIZ1, an essential protein for root hydrotropism, is associated with the cytoplasmic face of the endoplasmic reticulum membrane in Arabidopsis root cells. *FEBS Letters* 586, 398-402.

Yarur, A., Soto, E., León, G., and Almeida, A. M. (2016) The sweet cherry (*Prunus avium*) *FLOWERING LOCUS T* gene is expressed during floral bud determination and can promote flowering in a winter-annual Arabidopsis accession. *Plant reproduction*, 29, 311-322.

Yong, L.I., Fang, W.C., Zhu, G.R., Cao, K., Chen, C.W., Wang, X.W. and Wang, L.R. (2016) Accumulated chilling hours during endodormancy impact blooming and fruit shape development in peach (*Prunus persica*). *Journal of Integrative Agriculture* 15, 1267–1274.

Yoshida, R., Fekih, R., Fujiwara, S., Oda, A., Miyata, K., Tomozoe, Y., Nakagawa, M., Niinuma, K., Hayashi, K., Ezura, H. et al. (2009) Possible role of EARLY FLOWERING 3 (ELF3) in clock-dependent floral regulation by SHORT VEGETATIVE PHASE (SVP) in *Arabidopsis thaliana*. *New Phytologist* 182, 838-850.

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R. and Takahashi, S. (2001) EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. *The Plant Cell* 13, 2471-2481.

Zeinalabedini, M., Khayam-Nekoui, M., Grigorian, V., Gradziel, T.M. and Martínez-Gómez, P. (2010) The origin and dissemination of the cultivated almond as determined by nuclear and chloroplast SSR marker analysis. *Scientia Horticulturae* 125, 593-601.

Zhang, X., Garreton, V. and Chua, N.-H. (2005) The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes and Development* 19, 1532-1543.

Zhang, G.Z., Jin, S.H., Li, P., Jiang, X.Y., Li, Y.J. and Hou, B.K. (2017) Ectopic expression of *UGT84A2* delayed flowering by indole-3-butyric acid-mediated transcriptional repression of *ARF6* and *ARF8* genes in Arabidopsis. *Plant Cell Reports* 36, 1995-2006.

Zhao, K., Zhou, Y., Li, Y., Zhuo, X., Ahmad, S., Han, Y., Yong, X. and Zhang, Q. (2018) Crosstalk of *PmCBFs* and *PmDAMs* based on the changes of phytohormones under seasonal cold stress in the stem of *Prunus mume*. *International Journal of Molecular Sciences* 19, 15.

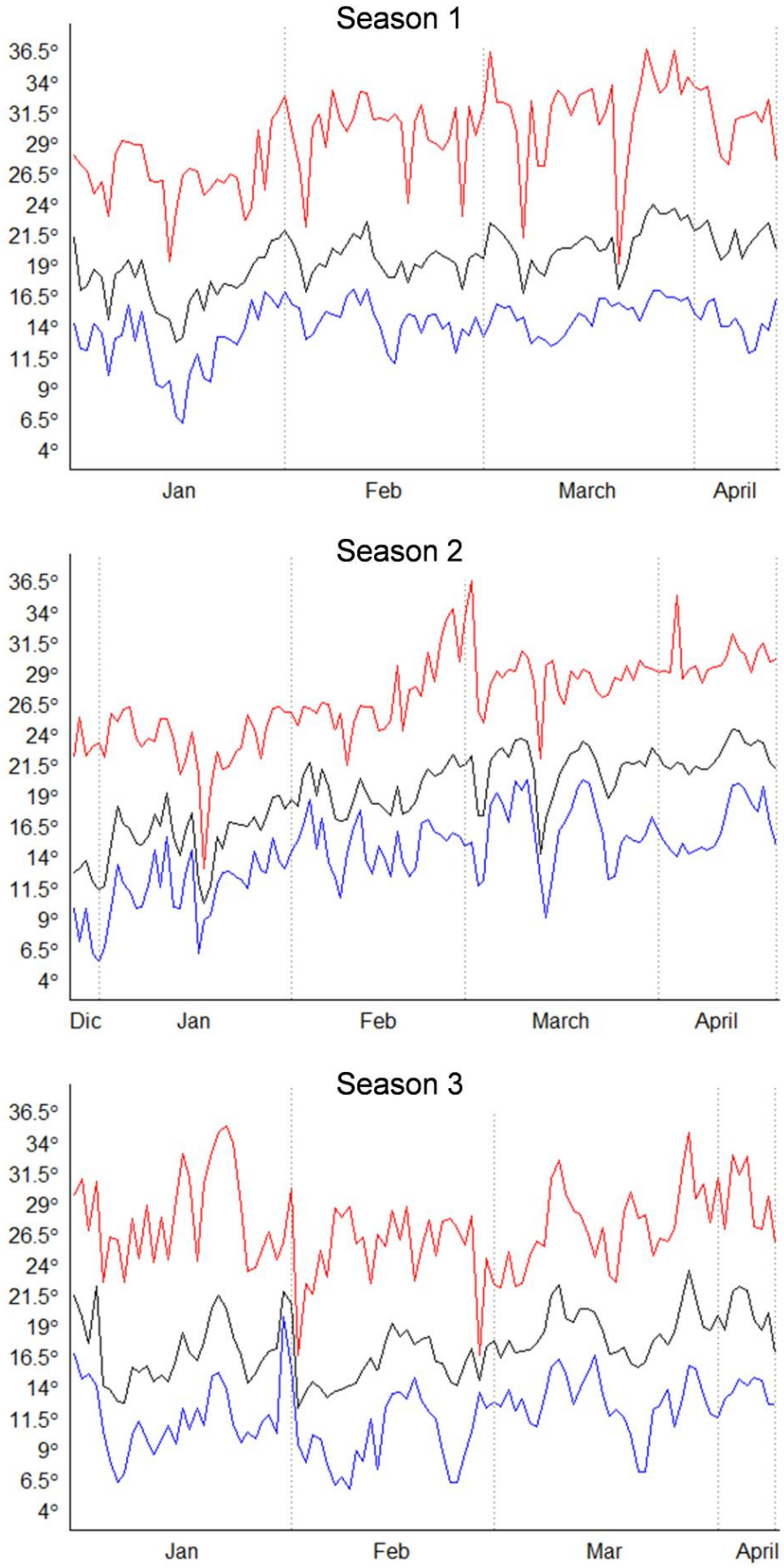
Zhang, Z., Zhuo, X., Zhao, K., Zheng, T., Han, Y., Yuan, C. and Zhang, Q. (2018) Transcriptome profiles reveal the crucial roles of hormone and sugar in the bud dormancy of *Prunus mume*. *Scientific Reports* 8, 5090-5090.

Zhebentyayeva, T.N., Fan, S., Chandra, A., Bielenberg, D.G., Reighard, G.L., Okie, W.R. and Abbott, A.G. (2014) Dissection of chilling requirement and bloom date QTLs in peach using a whole genome sequencing of sibling trees from an F2 mapping population. *Tree Genetics and Genomes* 10, 35-51.

Zhu, Y., Li, Y., Xin, D., Chen, W., Shao, X., Wang, Y. and Guo, W. (2015) RNA-Seq-based transcriptome analysis of dormant flower buds of Chinese cherry (*Prunus pseudocerasus*). *Gene* 555, 362-376.

10. ANNEXES

Annex 1. Maximum (red) minimum (blue) and medium (black) temperature recorded in the Spring chamber during the three seasons of study.



Annex 2. Candidate genes of screening for expression markers of endodormancy release in almond. Linkage Group (LG) location, genomic position in *Prunus dulcis* 'Lauranne' genome v.1 (https://www.rosaceae.org/node/9388636), cDNA sequences of candidate genes accessions and primers used in qRT-PCR reactions. Asterisks indicate that primer sequence was designed based on almond sequences. In other case, primers of the works referenced were used. RPII was used as endogenous control.

LG	Candidate gene	Genomic location	NCBI accession	Forward primer	Reverse primer	Reference
1	*DAM6*	Prudu.01G4818-19	MK578669-70	AGGAAATACTGGACCTGCGT*	GGTGGAGGTGGCAATTATGG*	
	AWPM-19	Prudu.01G037000	MN307431-33	CCCAGCCAATATGGCGAATATCAGAA	CATAGTGAGCAGTAAGTTTGTGCT	Leida et al. (2012b)
	AFP	Prudu.01G272600	MN307429-30	TTCCGTTGGTGGTGGAGTGGATGCA	TTACTAGCAGGGCTTCTTGCTTCAC	Leida et al. (2012b)
	P40	Prudu.01G098800	MH121042-44	TCTCCCTTCGTCCCAGTAAATGGTC	TTTCTGGGGAGGGTTTGCTTCCATC	Leida et al. (2012b)
2	*DREB2c*	Prudu.02G248400	MN271482	ATTTTGGAGCGGACCCCATG*	CCACCACCCTGTTGAACACA*	
4	*EMF2*	Prudu.04G136600	MN309827	CAGAAGCGAAAACCCCAAAC*	CATATCACCGCTGCTTCTCG*	
	GA2ox	Prudu.04G134200	MN2585447-49	TTTTGGACAGAACCCAGAAGA	TTGCTGAACACATTCCTTGG*	
	ARP4	Prudu.04G139500	MN258544-46	GCATCCAATGCTACTTGCAG	GCCTGATGCAAAAGATGTGA	
	LDL2	Prudu.04G172500	MN307434-36	AGTATGCCAGAGGAGGCAAC	CCACCTTGAAACCCAATGAC*	
	PIE1	Prudu.04G12100	MN307437	GGGGGTGTTGGAATAAACCT	ATACGGTGGCATCGATCTTG*	
	RPII			TGAAGCATACACCTATGATGATGAAG	CTTTGACAGCACCAGTAGATTCC	Tong et al. (2008)

Annex 3. Primers sequence, melting temperature (Tm) and amplicon size of DEGs analyzed by qRT-PCR for RNA-seq validation. *OEP16* and *60S* were used as endogenous controls.

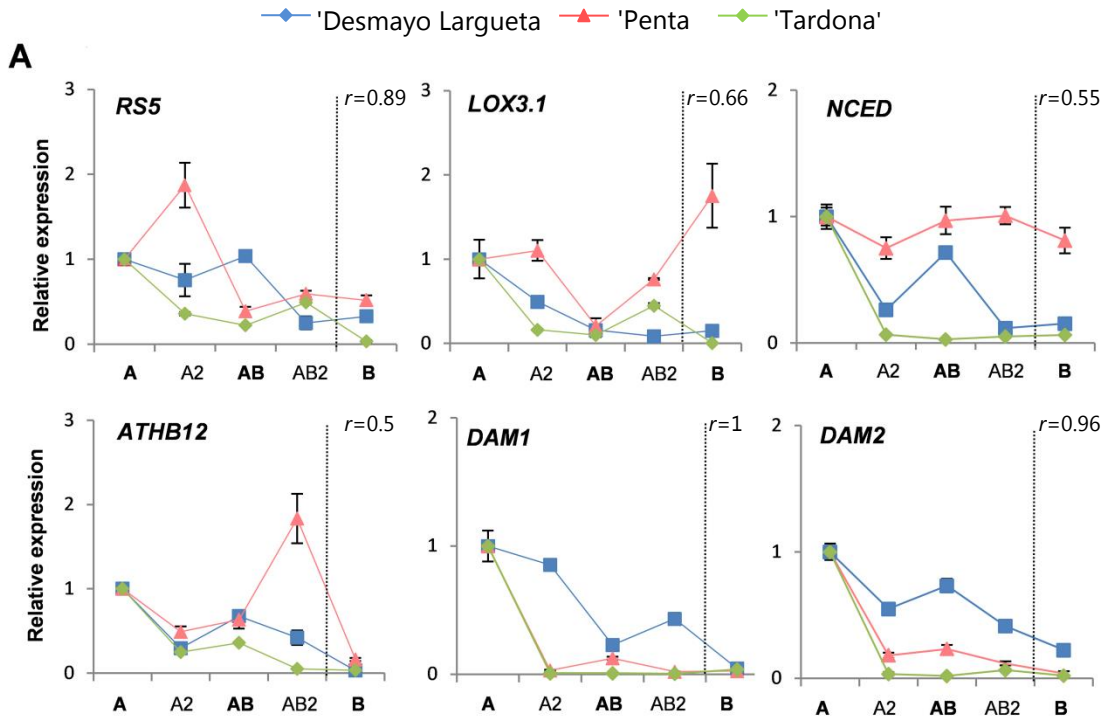
DEG name	Forward primer	Tm (°C)	Reverse primer	Tm (°C)	Product size (bp)
XET	GGTTTGGTTCTGGGTTCT	56.16	GCTTTGTCCTTTACTCGGT	56.8	115
4CL	GTTCAGCAGGAGCCACTT	59.1	ACAAGAATGGATGGCTTCA	56.00	123
NIP7	TTGAAGTTTGAGGAGATGGAACAG	59.73	GGCCAATAAAGACTCGGTTCAA	60.02	111
STR13	TTCATTAGCCTGCAGTTG	54.41	TTCCAAATGGGTTGCAGC	58.06	76
β-1.3-GLUCOSIDASE	AACTCCCTCCCTCTCCATC	59.09	CGGTGAGGTGGTTCTGGA	60.46	94
β-1.4-GLUCANASE	CTCCAGGGGTATATTGGG	54.82	AAGAGACCTGGAACAAAAG	53.99	148
SWEET10	GTGATGAGAAAAGTGGCAT	54.88	CCAGTGCCAACATTTTATAC	53.95	152
AIP2	ATGCAAGCGTCGTGGTAA	59.25	TGGGGTTTGTTGTTCCGTTT	59.23	93
DAM1	CCTCCACTTCTTAACCAGA	55.02	TGCTCCCAGTCTCTCTCT	58.00	93
DAM2	AGGAACTTGAGGAGAAGAG	54.72	TCAGTTCTTCCTCAGTTTCA	55.31	141
LOX3.1	TACGTAGCCAATGGTGTA	54.33	CGAGATCAAAGCGTGTTT	55.26	126
RS5	CTAGAAGGGGGAGAACGA	55.28	CCTAGAGAACGAGACCCA	54.94	80
MIZ1	GAGAGAAGGGGGTTGGAGA	59.32	GGCTTTGTCTCCTCTGTT	55.96	121
PBL15	GAAAAAGGACATGGGGGC	57.82	ACCCAAGCTAACCCCACA	57.71	131
ATHB12	CTGCAACTGAATAACCAAGG	56.01	TACAGCATACTCAGAGCC	54.86	90
60S	ATACCAGTTGAAGGATCGT	55.45	GGAATATGAGTTGCTAAGAAGG	55.44	105
S40	AATGTTTGGGCAAATTGGG	56.36	GACGTGGTAGTGGAGATT	55.75	115
NCED5	CTTTGGGGTGTGTTTTTCT	55.64	CTCTTCCTCCTCTTCTTCT	56.00	80
EXO7	TGTTGTTGCCGCCATTTG	59.12	GGTTGGTAATAGTGGCAC	54.51	104
OEP16	CCACGGACCCTTTCTAAA	55.63	TGAGCACACTTTGAAGAAG	54.77	99

Annex 4. Biological process-GO categories found in clusters 2, 3 and 4 ($P < 0,05$). No significantly enriched GO categories were found in cluster 1 (13 transcripts).

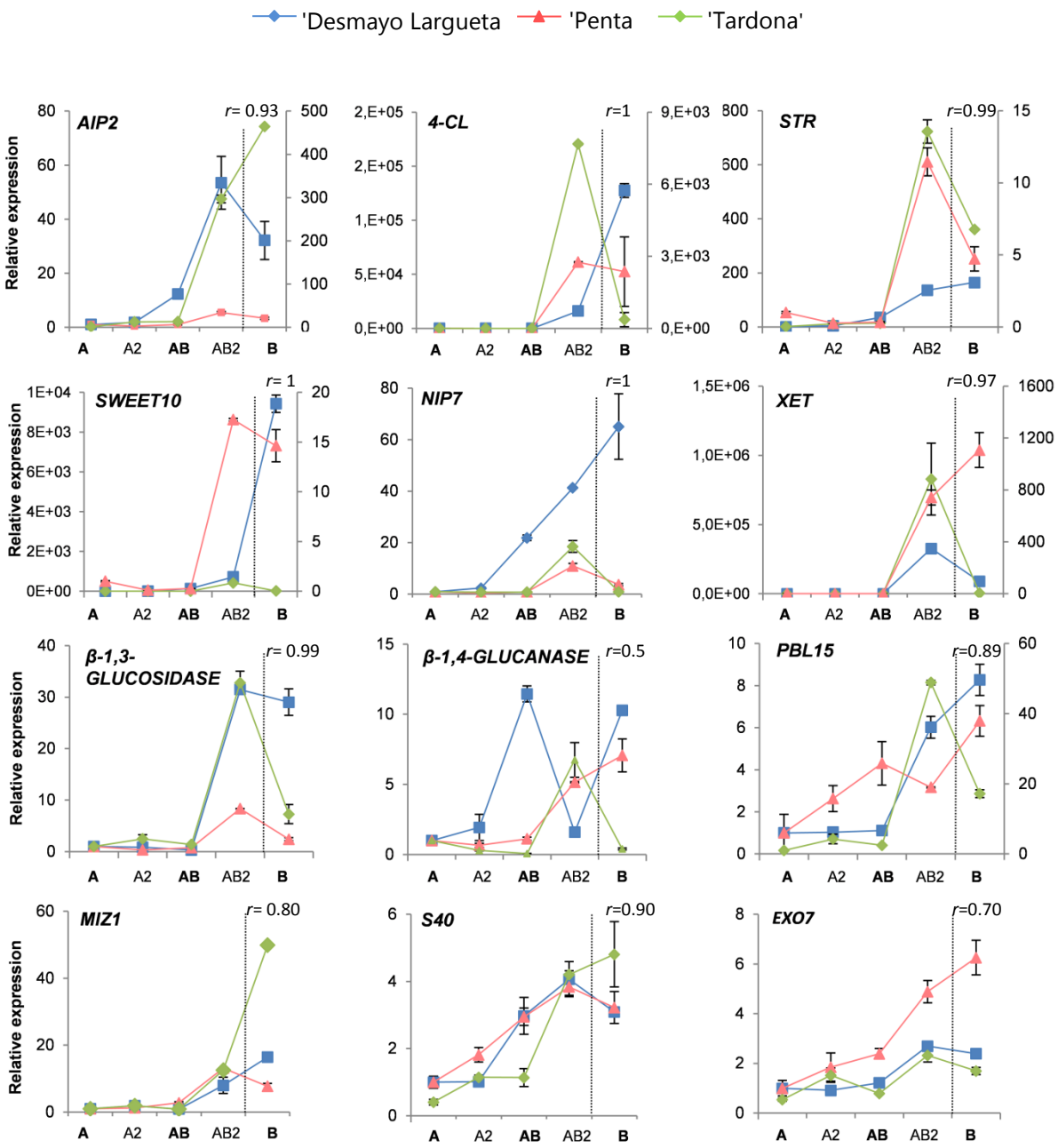
GO ID	GO Name	DEGs
CLUSTER 2 (385 transcripts)		
GO:0008152	metabolic process	136
GO:0009987	cellular process	83
GO:0071704	organic substance metabolic process	79
GO:0044238	primary metabolic process	74
GO:0055114	oxidation-reduction process	50
GO:0051179	localization	29
GO:0051234	establishment of localization	29
GO:0006810	transport	29
GO:0005975	carbohydrate metabolic process	24
GO:0055085	transmembrane transport	23
GO:0042221	response to chemical	16
GO:0006629	lipid metabolic process	14
GO:0070887	cellular response to chemical stimulus	13
GO:0032501	multicellular organismal process	11
GO:0044085	cellular component biogenesis	11
GO:0032502	developmental process	11
GO:0048856	anatomical structure development	11
GO:0007275	multicellular organism development	11
GO:0045229	external encapsulating structure organization	11
GO:0009636	response to toxic substance	11
GO:0098754	detoxification	10
GO:0097237	cellular response to toxic substance	10
GO:1990748	cellular detoxification	10
GO:0098869	cellular oxidant detoxification	10
GO:0051186	cofactor metabolic process	9
GO:0006091	generation of precursor metabolites and energy	9
GO:0022607	cellular component assembly	9
GO:0006979	response to oxidative stress	8
GO:0009653	anatomical structure morphogenesis	8
GO:0048869	cellular developmental process	8
GO:0032989	cellular component morphogenesis	8
GO:0048229	gametophyte development	8
GO:0009555	pollen development	8
GO:0048646	anatomical structure formation involved in morphogenesis	8
GO:0043062	extracellular structure organization	8
GO:0030198	extracellular matrix organization	8
GO:0085029	extracellular matrix assembly	8
GO:0010208	pollen wall assembly	8
GO:0010927	cellular component assembly involved in morphogenesis	8
GO:0010584	pollen exine formation	8
GO:0042737	drug catabolic process	7
GO:0022900	electron transport chain	7
GO:0072593	reactive oxygen species metabolic process	7
GO:0051187	cofactor catabolic process	7
GO:0042743	hydrogen peroxide metabolic process	7
GO:0042744	hydrogen peroxide catabolic process	7
GO:0008643	carbohydrate transport	5
GO:0031669	cellular response to nutrient levels	3
GO:0042594	response to starvation	3
GO:0009267	cellular response to starvation	3
GO:0016036	cellular response to phosphate starvation	3
GO:0080110	sporopollenin biosynthetic process	3
GO:1901568	fatty acid derivative metabolic process	2
GO:0048653	anther development	2
GO:0002933	lipid hydroxylation	2
CLUSTER 3 (170 transcripts)		
GO:0055085	transmembrane transport	13
GO:0051234	establishment of localization	16
GO:0051179	localization	16
GO:0006810	transport	16

GO ID	GO Name	DEGs
CLUSTER 4 (170)		
GO:0008152	metabolic process	94
GO:0044238	primary metabolic process	60
GO:0071704	organic substance metabolic process	60
GO:0044237	cellular metabolic process	56
GO:0055114	oxidation-reduction process	29
GO:0050789	regulation of biological process	27
GO:0065007	biological regulation	27
GO:0050794	regulation of cellular process	25
GO:0019219	regulation of nucleobase-containing compound metabolic process	21
GO:2000112	regulation of cellular macromolecule biosynthetic process	21
GO:0010556	regulation of macromolecule biosynthetic process	21
GO:0009889	regulation of biosynthetic process	21
GO:0031326	regulation of cellular biosynthetic process	21
GO:0051171	regulation of nitrogen compound metabolic process	21
GO:0080090	regulation of primary metabolic process	21
GO:0031323	regulation of cellular metabolic process	21
GO:0060255	regulation of macromolecule metabolic process	21
GO:0019222	regulation of metabolic process	21
GO:0019438	aromatic compound biosynthetic process	21
GO:1901362	organic cyclic compound biosynthetic process	21
GO:0006355	regulation of transcription, DNA-templated	20
GO:1903506	regulation of nucleic acid-templated transcription	20
GO:2001141	regulation of RNA biosynthetic process	20
GO:0051252	regulation of RNA metabolic process	20
GO:0010468	regulation of gene expression	20
GO:0006351	transcription, DNA-templated	20
GO:0097659	nucleic acid-templated transcription	20
GO:0032774	RNA biosynthetic process	20
GO:0034654	nucleobase-containing compound biosynthetic process	20
GO:0018130	heterocycle biosynthetic process	20
GO:0055085	transmembrane transport	15
GO:0042221	response to chemical	10
GO:0042737	drug catabolic process	8
GO:0017144	drug metabolic process	8
GO:0042744	hydrogen peroxide catabolic process	7
GO:0042743	hydrogen peroxide metabolic process	7
GO:0051187	cofactor catabolic process	7
GO:0072593	reactive oxygen species metabolic process	7
GO:0098869	cellular oxidant detoxification	7
GO:0097237	cellular response to toxic substance	7
GO:1990748	cellular detoxification	7
GO:0098754	detoxification	7
GO:0009636	response to toxic substance	7
GO:0006979	response to oxidative stress	7
GO:0070887	cellular response to chemical stimulus	7
GO:0006357	regulation of transcription by RNA polymerase II	5
GO:0006366	transcription by RNA polymerase II	5
GO:0022900	electron transport chain	5
GO:0044036	cell wall macromolecule metabolic process	4
GO:0010411	xyloglucan metabolic process	3

Annex 5. Expression dynamics of candidate DEGs by qRT-PCR. Endodormancy release (between AB2 and B state) is indicated by a dashed line. Relative expression of genes is represented by means of technical replicates of RNA pools from season 2. Endodormancy genes. Pearson correlation mean between relative expression to A and RPKM values of every cultivar is indicated on the right side at the top.



Annex 6. Expression dynamics of candidate DEGs by qRT-PCR. Endodormancy release (between AB2 and B state) is indicated by a dashed line. Relative expression of genes is represented by means of technical replicates of RNA pools from season 2. Ecodormancy genes. Pearson correlation mean between relative expression to A and RPKM values of every cultivar is indicated on the right side at the top. Relative expression of *4-CL*, *STR, SWEET10* and *XET* in 'Penta' samples is referred to the secondary Y axis. Relative expression of *AIP2*, *4-CL* and *PBL15* in 'Tardona' samples is referred to the secondary Y axis.



11. RESUMEN

11. RESUMEN

Los frutales de clima templado, como el almendro, han desarrollado un mecanismo para asegurar su supervivencia durante el invierno. Este mecanismo es conocido como letargo y se caracteriza por la ausencia de crecimiento activo visible en el árbol. En esta fase, las yemas vegetativas y florales se encuentran protegidas de posibles daños debidos a las bajas temperaturas.

La salida del letargo es un carácter dependiente del genotipo que, junto con la acumulación de calor, determina la época de floración de cada variedad. La salida del letargo requiere que se satisfagan unas determinadas necesidades de frío, que hacen que cuanto mayores sean, más tarde tenga lugar la época de floración. Retrasar la época de floración ha sido uno de los principales objetivos en los programas de mejora del almendro y las variedades tardías obtenidas han hecho posible la extensión del cultivo a zonas muy frías, nunca antes consideradas para la producción de almendra.

Sin embargo, se han observado problemas de producción asociados a las varieades de floración tardía con altos requerimientos de frío, como floración irregular o bajo índice de cuajado, que pueden deberse, por una parte, a las altas temperaturas (insuficiente acumulación de frío) durante el letargo y por otra, a las altas temperaturas durante la floración y el periodo de maduración del fruto. Así mismo, las bajas temperaturas una vez se ha superado el estado de letargo, pueden afectar negativamente a la producción del árbol ya que las yemas son especialmente sensibles en este estado a la caída de las temperaturas.

El letargo invernal se considera un estado cuantitativo cuya proceso de salida ocurre gradualmente. Diferentes procesos fisiológicos en especies del género *Prunus* se han observado durante la salida del letargo, como la formación de las tétradas durante el proceso de microsporogénesis, la diferenciación de los haces vasculares del xilema y la hidrólisis de carbohidratos, así como la toma de estos por el primordio de la yema floral.

El control genético de la salida del letargo es objeto de estudio de numerosos investigadores y se han hecho importantes progresos al respecto. Especialmente, se ha caracterizado la familia *DAM* de factores de transcripción (*DORMANCY ASSOCIATED MADS box*), cuya expresión está asociada al mantenimiento del estado de letargo. Así mismo, factores de transcripción de la familia *CBF* (C-REPEAT BINDING FACTORs) y *DREB (DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEINS)* juegan un papel en la respuesta a la acumulación de frío. Por otra parte, genes que regulan la época de floración en la planta herbácea modelo Arabidopsis se han estudiado en especies del género *Prunus,* así como genes relacionados con el fotoperiodo para el establecimiento del estado de letargo.

Además, se ha demostrado que los genes que codifican para enzimas relacionadas con la síntesis y metabolismo de las hormonas ABA (Acido Abscísico) y GAs (Giberelinas) están activamente implicadas, ya que el estado de letargo se relaciona directamente con el balance entre estas dos hormonas.

El objetivo de la presente Tesis Doctoral ha sido el análisis de las bases genéticas y moleculares de la salida del letargo y la época de floración en almendro, para identificar marcadores moleculares asociados a estos caracteres agronómicos.

Para ello, se han desarrollado los siguientes objetivos específicos: 1) La evaluación de la acumulación de frío para la salida del letargo usando diferentes modelos y la evaluación de la acumulación de calor para florecer en variedades de almendro con distintas época de floración; 2) La evaluación del efecto de diferentes temperaturas controladas en la época de floración, el índice de cuajado y las características del fruto y la época de maduración en variedades de almendro con distinta época de floración; 3) El análisis de expresión de genes candidatos asociados a la salida del letargo y a la época de floración en variedades de almendro y su evaluación como marcadores de expresión potenciales de la salida del letargo y la época de floración; 4) La identificación de nuevos marcadores de expresión mediante la secuenciación masiva del transcriptoma de yemas de flor en estado de letargo y fuera de letargo de variedades de almendro con distintos requerimientos de frío y 5) La identificación de marcas epigenéticas basadas en la metilación del ADN de yemas de flor en estado de letargo y fuera de letargo, en variedades de almendro con distinta época de floración.

El material vegetal utilizado en los ensayos consistió en la variedad tradicional de almendro 'Desmayo Largueta', de floración extra-temprana y maduración tardía; la variedad de floración extra-tardía y maduración temprana 'Penta' y la variedad de floración ultra-tardía y maduración tardía 'Tardona', ambas procedentes del Programa de Mejora del CEBAS-CSIC (Murcia). Los árboles de los que proviene el material vegetal se encuentran en la finca experimental "Tres Caminos" localizada en Santomera (Murcia). Para la evaluación de la salida del letargo se utilizó material vegetal consistente en varetas de almendro recogidas en la finca experimental de Santomera durante tres años (2014-2017). Para los análisis moleculares se recogieron yemas florales en distintos estados de letargo y fuera de letargo durante dos años (2015-2017).

Respecto al Objetivo 1) planteado en esta Tesis, la acumulación de frío se monitorizó para cada variedad ('Desmayo Largueta', 'Penta', 'Tardona') hasta que ésta había salido del letargo. Para llevar a cabo la evaluación de la salida del letargo, varetas recogidas en el campo fueron sometidas a condiciones de forzado en cámara controlada, y se evaluó la evolución fenológica a los diez días. La acumulación de calor se monitorizó desde el momento de salida de letargo hasta la época de floración de cada variedad en el campo.

La evaluación de la acumulación de frío para la salida del letargo mediante diferentes modelos y la evaluación de la acumulación de calor para florecer en variedades de almendro en condiciones naturales, mostró una mayor precisión del Modelo Dinámico, sobre el Modelo Richardson, sobre todo en los inviernos cálidos. La acumulación de calor para florecer aumentó en todas las variedades de estudio cuando la acumulación de frío fue menor. Así mismo, se observó una disminución en la productividad de las variedades tardías cuando la acumulación de frío durante el invierno fue insuficiente.

Respecto al Objetivo 2) planteado en esta Tesis, se utilizaron almendros cultivados en macetas que fueron transferidos en otoño a una cámara controlada (cámara de otoño, 20ºC) para prevenir la acumulación de frío. Dos macetas de almendros extra-tempranos (de la variedad 'Desmayo Largueta') y ultra-tardíos (de la variedad 'Tardona') respectivamente fueron sometidos a distintos tratamientos de temperatura en una cámara de invierno (7ºC), para hacer coincidir su salida del letargo. Una vez concluido el periodo de acumulación de frío, cada pareja de macetas fue llevada a un invernadero (cámara de primavera) donde pasaron el periodo de acumulación de calor para florecer, y esto se hizo semanalmente para cada tratamiento (pareja de almendros extra-tempranos y ultra-tardíos) durante diez semanas. Los almendros fueron polinizados en el invernadero y posteriormente transferidos a un abrigo sombreado donde tuvo lugar la maduración del fruto.

El control de la temperatura durante el letargo permitió retrasar la floración de la variedad extra-temprana, y de adelantar su época de maduración. El retraso en la floración de los diferentes tratamientos supuso un retraso gradual de la época de maduración. En el caso de la variedad ultra-tardía no se consiguió avanzar sustancialmente la floración. Las necesidades de calor para florecer fueron menores en la varieadad extra-temprana que en la ultra-tardía, y se observo una tendencia a disminuir el periodo de tiempo para florecer a lo largo de los tratamientos, relacionado probablemente con las temperaturas en el invernadero. Además, se observó que la floración en la variedad ultra-tardía fue deficiente debido a que las yemas florales no completaban su desarrollo y caían, lo que pudo estar relacionado con la brotación y de las yemas vegetativas y el crecimiento de las hojas. El escalonamiento de la floración no tuvo un efecto directo sobre el cuajado y las características del fruto estudiadas (peso del fruto, peso de la semilla y rendimiento). En concreto, estos valores fueron menores que aquéllos observados en condiciones naturales.

Respecto al Objetivo 3) planteado en esta Tesis, el estudio de genes candidatos asociados a la salida del letargo y la época de floración permitió la caracterización de un marcador potencial de la salida del letargo, correspondiente al gen de la peroxidasa 40 (*PdP40*). La actividad peroxidasa total de las yemas de flor, antes y tras la salida del letargo también fue analizada.

El marcador potencial *PdP40* procede de un escrutinio realizado mediante PCR cuantitativa entre genes candidatos asociados a la salida del letargo y la época de floración previamente descritos en especies del género *Prunus*. La secuencia codificante del gen *PdP40* reveló diferencias entre las secuencias de las variedades 'Desmayo Largueta' y 'Penta' y la variedad 'Tardona'.

Respecto al Objetivo 4) planteado en esta Tesis, se llevó a cabo un ensamblaje *de novo* del transcriptoma, teniendo en cuenta los datos de secuenciación de las variedades de almendro 'Desmayo Largueta', 'Penta' y 'Tardona' en estados de letargo comparables (letargo, fuera de letargo, estado intermedio). El estado intermedio fue seleccionado en base a la inducción de la expresión del gen *PdP40* analizado previamente. Un total de 1064 DEGs durante la salida del letargo fueron obtenidos por filtración en base a su coeficiente de variación entre muestras correspondientes a distintos estados de letargo en las tres variedades de almendro analizadas. A partir de esta primera selección se obtuvieron cuatro grupos de genes agrupados por su patrón de variación mediante clusterización jerárquica.

De esta manera se obtuvieron tres grupos de genes cuya expresión aumenta durante la salida del letargo y un grupo de genes cuya expresión disminuye durante la salida del letargo. Genes candidatos relacionados con la salida del letargo fueron seleccionados entre estos grupos, y su patrón de expresión fue analizado mediante PCR cuantitativa en muestras de dos años diferentes. Entre estos genes se encuentran aquellos relacionados con el transporte célula a célula y la degradación de los depósitos de callosa en los plasmodesmos, así como genes codificantes para enzimas biosintéticas de carbohidratos.

Además de estos genes, otros genes identificados mediante el análisis OrthoMCL a partir de la comparación con transcriptomas de cerezo y kiwi, fueron seleccionados para la validación del RNA-seq. En conclusión, los resultados de la secuenciación masiva del transcriptoma (RNA-seq), llevaron a la identificación de nuevos genes candidatos asociados a la fase de letargo y fuera de letargo. Además, fue posible identificar genes cuyo patrón de expresión durante la salida del letargo (basado en el coeficiente de variación entre muestras) está asociado a genotipos de almendro temprano y tardíos, respectivamente. Este es el caso de genes relacionados con resistencia a patógenos y proteínas de unión a ADN del tipo RICESLEEPER.

Respecto al Objetivo 5) planteado en esta Tesis, se llevó a cabo la técnica de epi-genotipado mediante secuenciación (epiGBS), que conlleva la digestión de ADN genómico y la ligación de adaptadores de secuenciación para la preparación de una librería. La detección de variantes en la metilación de citosinas (5mC) se basa en el

tratmiento del ADN con bisulfito. Los fragmentos secuenciados y que mostraron metilación diferencial fueron mapeados en el genoma del melocotonero para obtener la información de las regiones codificantes diferencialmente metiladas entre muestras.

Se observó que los fragmentos diferencialmente metilados se localizaron con más frecuencia en exones e intrones de los genes, seguido de las regiones aguas arriba y aguas abajo. Los resultados mostraron que en general, y a pesar de la limitada representación del genoma en la librería secuenciada, el estado de metilación depende más de la variedad (genotipo) que del estado de desarrollo (letargo/fuera de letargo). Los resultados del análisis de metilación diferencial de ADN de yemas de flor en distintos estados de letargo, permitió la identificación de genes (ortólogos de melocotonero) diferencialmente metilados en yemas de flor en estado de letargo, respecto a yemas activas, como es el caso del gen codificante para una proteína MAPK (MITOGEN-ACTIVATED PROTEIN KINASE) y una lipasa del tipo GDSL (motivo Glicina, Aspartato, Serina y Leucina) y genes diferencialmente metilados en yemas del almendro extra-temprano ('Desmayo Largueta') respecto a las del extra-tardío ('Penta'), como *LHY* (*LATE ELONGATED HYPOCOTYL*) *HSP* (*HYDROPHOBIC SEED PROTEIN*), *FAR1* (*FAR RED IMPAIRED RESPONSE1*) y genes involucrados en la regulación de la respuesta a auxina (*ARFs*, *AUXIN RESPONSE FACTORS*).