Molecular aspects of dormancy in peach (*Prunus persica* [L.] Batsch)

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ABSTRACT

Dormancy is one of the most important adaptive mechanisms developed by perennial plants, in order to survive the low temperatures of autumn and winter in temperate climates. The study of the genes regulated during dormancy release is crucial to understand the process, with the final objective of the development of new varieties with a better adaptation to certain environments; and this is particularly important considering the increasing economical weight of fruit crops in low and medium chilling regions as the Mediterranean area. We focused on the molecular and physiological mechanisms underlying the maintenance and release of seasonal dormancy in peach. In order to achieve this we first used suppression subtractive hybridization (SSH) to identify genes expressed in dormant and dormancy-released buds in two cultivars with different chilling requirements, 'Zincal-5' and 'Springlady', and subsequently validated their differential expression utilizing a peach cDNA microarray platform containing transcripts enriched in flower buds. Additionally, we carried out a genome-wide search of peach genes related to dormancy release by hybridizing the previous cDNA microarray with mRNA samples from 10 cultivars showing different dormancy behaviour, followed by an expression correlation analysis.

Among the most relevant genes identified in these two first works, we found the *DORMANCY ASSOCIATED MADS-box* genes *DAM4*, *DAM5* and *DAM6*, described independently by other groups working in peach and other species. The central role of *DAM* genes in dormancy regulation has also been confirmed by additional functional approaches as the analysis of the non-dormant *evg* mutant, QTL analysis, and transgenic approaches.

In our second work we focused on the molecular mechanisms of *DAM6* down-regulation concomitant with dormancy release in flower buds. A ChIP analysis of *DAM6* promoter and structural gene revealed chromatin modification events similar to those observed in vernalization of *Arabidopsis* and cereals. We showed that *DAM6* is transcriptionally active in dormant buds collected in October, when a short chromatin region around its ATG was trimethylated in histone H3 at K4 (H3K4) and acetylated at the N-terminal tail of H3. Concomitantly with *DAM6* repression, H3K4 became demethylated and H3 deacetylated. Later H3K27 was found trimethylated along a genomic region larger than 4kb, including promoter, coding sequence and intron. Due to their relevance in dormancy regulation, *DAM* genes could be used as expression markers to assess the dormancy stage of an individual plant and to evaluate the chilling requirements of new cultivars. In fact, we have shown in this work that the expression pattern of

DAM5, together with other transcripts (BD396, DB247, SB280 and PpB63), correlates well with chilling requirements values of five different varieties (‘Big Top’, ‘Catherina’, ‘Fergold’, ‘Maruja’ and ‘Springlady’) measured following Utah and Dynamic models.

Some of the genes identified in transcriptomic experiments using flower buds, as *DAM1*, *DAM5* and *DAM6*, were also regulated during the cold stratification of peach seeds, suggesting the presence of common regulatory pathways in the dormancy process of buds and seeds. These similarities between bud and seed dormancy have possible important implications in the evaluation of bud chilling requirements of early and late flowering genotypes directly at seed level greatly reducing the time needed for evaluating plant material in breeding programs.

Among others, a significant number of genes identified in this work were homologous to ABA and drought related genes from other species. ABA, in fact, has been proposed to promote and maintain bud dormancy although few molecular data support this prediction. Our data contribute to highlight a prominent role of ABA in dormancy processes and also uncover elements of the ABA and drought regulatory response in peach, as an ABA-INSENSITIVE5 (ABI5) binding protein (AFP)-like, a dehydration-responsive element (DRE)-binding protein (DREB2C)-like, a calcium-binding annexin, and several genes regulated by stress signalling pathways.

RESUMEN

La latencia es uno de los mecanismos adaptativos más importantes desarrollados por las plantas perennes para sobrevivir a las bajas temperaturas estacionales en climas templados. El estudio de los genes regulados durante la salida de la latencia es crucial para entender este proceso y poder obtener nuevas variedades con una adecuada adaptación climática a la zona de cultivo. Esto es particularmente relevante en el área mediterránea, donde el peso económico del cultivo de frutales con bajos y medios requerimentos de frío se esta incrementando notablemente. En esta tesis se han estudiado aspectos moleculares y fisiológicos que rodean al mantenimiento y salida de latencia en melocotonero. Para ello, se ha utilizado la técnica de hibridación substractiva supresiva, que ha permitido identificar genes expresados en yemas latentes y no latentes en dos variedades con diferentes requerimientos en horas frío, 'Zincal 5' y 'Springlady'. Posteriormente, se ha validado su expresión diferencial mediante una micromatriz de cDNA que contenía transcritos enriquecidos en yema floral. Además, mediante hibridación de la micromatriz anterior con muestras de RNA procedentes de 10 cultivares que diferían en su comportamiento respecto a la latencia, se han identificado genes cuya expresión correlacionaba con el estado de latencia del material ensayado.

Dentro de los genes más relevantes identificados en estos dos trabajos se encuentran los genes *DORMANCY ASSOCIATED MADS-box (DAM) DAM4, DAM5* and *DAM6*, previamente descritos de forma independiente por otros autores que trabajan en melocotonero y otras especies leñosas Su papel en la latencia se ha confirmado por medio de pruebas funcionales como el análisis del mutante no latente *evg*, el mapeo de QTL, y mediante el uso de plantas transgénicas.

En un segundo trabajo se ha estudiadoel mecanismo molecular de la represión de *DAM6* durante la salida de latencia en yema floral. El análisis de inmunoprecipitación de cromatina en la zona del promotor y gen estructural de *DAM6* reveló modificaciones similares a las observadas en la vernalización de *Arabidopsis* y cereales. Los resultados han demostrado que *DAM6* se transcribe en yema latente recolectada en octubre, cuando una pequeña región de cromatina alrededor del ATG se encuentra trimetilada en la lisina 4 (K4) de la histona H3 y acetilada en la cola N-terminal de H3. En paralelo a la represión de *DAM6*, H3K4 se demetila y H3 se deacetila. Posteriormente, H3K27 se encuentra trimetilada a lo largo de una región genómica mayor de 4kb, que incluye promotor, secuencia codificante e intrón.

Debido a su relevancia en la regulación de la latencia, los genes *DAM* podrían ser utilizados como marcadores de expresión para evaluar el estado de latencia de una planta individual, y para

evaluar los requerimentos en frío de nuevas variedades. De hecho en este trabajo se ha demostrado que la expresión de *DAM5*, junto con otros transcritos (BD396, DB247, SB280 y PpB63), se correlaciona con las estimaciones de requerimentos de frío en cinco cultivares diferentes (‘Big Top’, ‘Catherina’, ‘Fergold’, ‘Maruja’ y ‘Springlady’), medidos mediante los modelos Utah y Dynamic.

Algunos de los genes identificados en los experimentos de transcriptómica con yema floral, como *DAM1*, *DAM5* y *DAM6*, son regulados durante la estratificación en frío de las semillas de melocotonero, sugiriendo la existencia de rutas de regulación comunes en los procesos de latencia de semilla y yema. Estas similitudes entre la latencia de yema y semilla podrían justificar el empleo de la semilla para la estimación de los requerimentos de frío de diferentes genotipos, reduciendo considerablemente el tiempo necesario para la evaluación del material vegetal en los programas de mejora.

Un numero significativo de genes identificados en este trabajo muestran homología a genes relacionados con el ABA y estrés hídrico en otras especies. El ABA, es considerado uno de los principales inductores de la latencia en la yema, sin embargo pocos datos moleculares apoyan esta idea. Nuestros datos contribuyen a resaltar el importante papel del ABA en el proceso de latencia y también a desentrañar los elementos de la respuesta reguladora del ABA y el estrés hídrico en melocotonero. Entre ellos se encuentran una proteína similar a ABA-INSENSITIVE5 (ABI5) BINDING PROTEIN (AFP), una proteína de regulación de la respuesta al estrés hídrico similar a DREB2C, y una anexina.

RESUM

La latència és un dels mecanismes adaptatius més importants desenrotllats per les plantes perennes per a sobreviure a les baixes temperatures estacionals en climes temperats. L'estudi dels gens regulats durant l'eixida de la latència és crucial per a entendre este procés i poder obtindre noves varietats amb una adequada adaptació climàtica a la zona de cultiu. Açò és particularment rellevant en l'àrea mediterrània, on el pes econòmic del cultiu de fruiters amb baixos i mitjans requeriments de fred s'està incrementant notablement. En esta tesi s'han estudiat aspectes moleculars i fisiològics que rodegen al manteniment i eixida de latència en bresquillera. Per a això, s'ha utilitzat la tècnica d'hibridació substractiva supressiva, que ha permés identificar gens expressats en gemmes latents i no latents en dos varietats amb diferents requeriments en hores fred, 'Zincal 5' i 'Springlady'. Posteriorment, s'ha validat la seua expressió diferencial per mitjà d'una micromatriu de cDNA que contenia transcrits enriquits en gemma floral. A més, per mitjà d'hibridació de la micromatriu anterior amb mostres de RNA procedents de 10 cultivars que diferien en el seu comportament respecte a la latència, s'han identificat gens l'expressió dels quals correlacionava amb l'estat de latència del material assajat.

Dins dels gens més rellevants identificats en estos dos treballs es troben els gens *DORMANCY ASSOCIATED MADS-box (DAM) DAM4*, *DAM5* i *DAM6*, prèviament descrits de forma independent per altres autors que treballen en bresquillera i altres espècies llenyoses El seu paper en la latència s'ha confirmat per mitjà de proves funcionals com l'anàlisi del mutant no latent *evg*, el mapeig de QTL, i per mitjà de l'ús de plantes transgèniques.

En un segon treball s'ha estudiat el mecanisme molecular de la repressió de *DAM6* durant l'eixida de latència en gemmes florals. L'anàlisi d'inmunoprecipitació de cromatina en la zona del promotor i gen estructural de *DAM6* va revelar modificacions semblants a les observades en la vernalització *d'Arabidopsis* i cereals. Els resultats han demostrat que *DAM6* es transcriu en gemma latent recol.lectada en octubre, quan una xicoteta regió de cromatina al voltant de l' ATG es troba trimetilada en la lisina 4 (K4) de la histona H3 i acetilada en la cua N-terminal d' H3. En paral.lel a la represió de *DAM6*, H3K4 es demetila i H3 es deacetila. Posteriorment, H3K27 es troba trimetilada en una regió genómica major de 4kb, que inclou promotor, seqüència codificant i intró.

Alguns dels gens identificats en els experiments de transcriptòmica amb gemma floral, com *DAM1*, *DAM5* i *DAM6*, són regulats durant l'estratificació en fred de les llavors de bresquillera, suggerint l'existència de rutes de regulació comuna en els processos de latència de llavor i gemma.

Estes similituds entre la latència de gemma i llavor podrien justificar l'ús de la llavor per a l'estimació dels requeriments de fred de diferents genotips, reduint considerablement el temps necessari per a l'avaluació del material vegetal en els programes de millora.

Un nombre significatiu de gens identificats en este treball mostren homologia a gens relacionats amb l'ABA i estrés hídric en altres espècies. L'ABA, és considerat un dels principals inductors de la latència en la gemma, no obstant això poques dades moleculars recolzen esta idea. Les nostres dades contribuïxen a ressaltar l'important paper de l'ABA en el procés de latència i també a desentranyar els elements de la resposta reguladora de l'ABA i l'estrés hídric en bresquillera. Entre ells es troben una proteïna semblant a ABA-INSENSITIVE5 (ABI5) BINDING PROTEUET (AFP), una proteïna de regulació de la resposta a l'estrés hídric semblant a DREB2C, i una anexina.

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ABBREVIATION LIST

ABA	Abscisic Acid
BD	Blooming Date
BLASTN/P	Basic Local Alignment Tool Nucleotide/Protein
ChIP	Chromatin Immunoprecipitation
CR	Chilling Requirement
CU	Chilling Units
DAM	Dormancy Associated MADS-box
DR	Dormant bud cDNA library
EST	Expressed Sequence Tag
evg	*evergrowing*
GA	Gibberellins, Gibberellic Acid
H3K27	Histone H3 Lysine-27
H3K4	Histone H3 Lysine-4
HC	Hydrogen Cyanamide
HR	Heat Requirement
IAA	Indoleacetic Acid
JA	Jasmonic Acid
LD	Long Day
LG	Linkage Group
MADS	**M**CM1/**A**GAMOUS/**D**EFICIENS/**S**ERUM RESPONSE FACTOR
MTB	Mean Time to Budbreak
QTL	Quantitative Trait Loci
RD	Dormancy release bud cDNA library
SCAR	Sequences Characterized Amplified Region
SD	Short Day
SSH	Subtractive Suppressive Hybridization
SSR	Simple Sequence Repeated
SZ	‘Springlady’ cDNA library
ZS	‘Zincal 5’ cDNA library

1. GENERAL INTRODUCTION

1.1. Dormancy as an adaptation to the environment

Perennial plants are able to survive under unfavourable climatic conditions through a seasonal stage of latent growth named dormancy. The study of dormancy has been a matter of interest for scientists covering almost a century of work, including genetics, physiology, biochemistry, agronomy, and molecular biology. Lang et al. (1987) defined dormancy for the first time as the "temporary suspension of visible growth of any plant structure containing a meristem". He further distinguished between paradormancy, when growth is inhibited by distal organs, endodormancy when growth is inhibited by internal signals, and ecodormancy, which is provoked by environmental conditions. Despite some limitations in this definition, Lang's description had been widely utilized by the scientific community for years. A more recent definition of dormancy has been proposed by Rohde & Balherao (2007), as "the inability to resume growth from meristems (and other organs and cells) under favourable conditions". Dormancy is a physiological mechanism that involves different metabolic pathways: light perception, hormones, cell cycle and abiotic stress resistance.

Dormancy is the result of plant adaptation to the environment and the variation in intensity is due to the different pressure exercised at different latitudes. Among the same species, this climatic variations generated different genotypes with a distinct dormancy intensity (Heide et al., 1977). In cases where this pressure was released, some annual plants at the tropical climates have been observed to reconstitute their ancestral woody or evergrowing habit (Okubo et al., 2000).

1.1.1 Seasonal cycle of dormancy

During autumn, just before growth cessation in winter, paradormancy marks the first step to a deeper dormant state. When the terminal bud is formed, auxin production and transport imposes a basitonic gradient of bud growth ability in axillary buds (Champagnat et al., 1975; Wareing, 1956). From the beginning of March to the end of August, removal of the apical bud allows the growth of the axillary buds. The same effect is obtained with defoliation from August to September while is totally ineffective later, being an isolated axillary bud unable to resume growth under favourable conditions. Between September and November, depending on climatic conditions, the bud break ability is null, due to endodormancy induction.

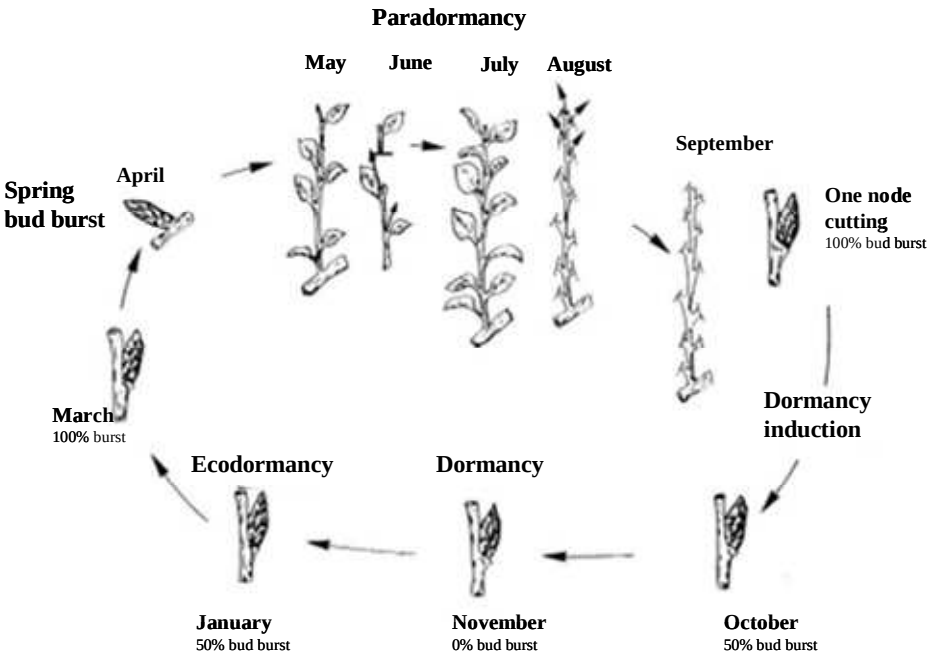

Figure 1.1 Development cycle of axillary buds on a tree shoot. From April to August, the bud is inhibited by paradormancy: a simple decapitation allows immediate growth resumption. But progressively, decapitation and defoliation are both needed to obtain the same result, and finally in September, both treatments have no result. True dormancy appears on October., as shown by the dramatic decrease in bursting ability of buds on 'one-node cutting' but it is released in January. (Based on Champagnat 1989).

The plant, in a first step, develops a moderate resistance to cold that increases considerably after leaf senescence and abscission as a response to lower temperatures (Perry, 1971). From this moment the tree is considered to be endodormant. Once chilling requirements are fulfilled and climatic conditions are favourable (25ºC), buds need only 2 weeks for bud break (Figure 1.1).

1.1.2 Environmental and physiological factors

The first studies devoted to bud dormancy were held during the 50's and 60's, with a relevant dedication to hormones that were thought to be the cause of dormancy establishment. Hemberg's (1949) studies showed that an endogenous substance called "dormin" was involved in bud dormancy induction and its synthesis was a plant response to environmental cues (Eagles & Wareing, 1963). This "dormin" was later named abscisic acid (ABA).

Evidences in favour and against ABA as dormancy inductor opened the question whether ABA was more involved in increase freezing tolerance than dormancy regulation (Welling et al., 1997; Rinne et al., 1998; Mielke & Dennis, 1978; Trewavas & Jones, 1991; Tanino, 2004). A relationship between ABA and water content under SD or dormancy induced by water stress was

observed in *Betula pubescens, Prunus persica, Vitis vinifera* and *Vitis riparia* in buds and cortex tissue (Erez et al., 1998; Fennel & Line, 2001; Rinne et al., 1994; Welling et al., 1997; Koussa et al., 1998), suggesting a link between ABA content and water status with bud dormancy depth (Tamura et al., 1993). After dormancy establishment, a reduction of free water and an accumulation of dehydrins were observed, improving plant tolerance to freezing.

A first connection between day-length shortening and dormancy induction was observed by Garner and Allard (1923), and later confirmed by others (Kramer et al., 1936; Downs & Bothwick, 1956; Nithsch, 1957; Weieser, 1970; Allona et al., 2008). In photoperiod-insensitive varieties and *Euphorbia esula* low temperatures replaces the effect of SD (Heide & Pestrud, 2005; Horvath 2009), as it was also described in strawberry (Ito & Saito, 1962; Guttridge, 1985). Went (1948, 1953) proposedthe term thermoperiodism to indicate the importance of the variation of temperature between day and night. A more recent review by Tanino (2010) raises the hypothesis of the existence of two separated processes involved in dormancy induction in northern ecotypes: one driven by low temperatures while another induced by warm temperature-photoperiod ensuring dormancy development and cold adaptation under both favourable and unfavourable conditions. This hypothesis could explain the different and controversial responses of dormancy to warm and low temperatures described in the literature until now.

Concomitantly with dormancy establishment, water mobility decreases and hydrophilic molecules accumulate (Faust et al., 1995; Erez et al., 1998). Simultaneously, plasmodesmata are blocked by 1,3 β-glucan (Rinne et al., 2001; Rinne & Van der Schoot, 2003) and calcium depositions (Jian et al., 1997), and a change in aquaporin gene expression is reported in peach bud (Yooyongwech et al., 2008). Chilling, on the other side, is responsible for restoring symplastic connections by enhancing production of 1,3 β-glucanase. This process is supposed to be due to gibberellins (GA) production that induce 1,3 β-glucanases genes transcription as shown in tobacco (Leubner-Metzger et al., 1996).

After growth cessation and under low temperature conditions starch is converted to maltose and simple hexoses, with accumulation in stems and buds (Rinne et al, 1994; Kuroda & Sagisaka, 1993). Some evidences suggest a role of sucrose and other sugars as signalling molecules in euphorbia, poplar and aspen (Horvath et al., 2002; Olsen et al., 1997; Erikkson et al., 2000). Sugars have been described to interact with ABA and GA during the formation of potato tuber, an organ that also undergoes dormancy processes (Xu et al., 1998).

Other hormones different from ABA have been related to dormancy maintenance and release. Ethylene has been shown to interact with ABA during bud set and dormancy, as observed in an ethylene-insensitive transgenic birch under SD conditions (Ruonala et al., 2006). Ethylene is considered as an intermediate between light and ABA signals, hypothetically activated by transient low hexose pools (Ruttink et al., 2007).

GA has been proposed as ABA antagonist in growth processes. Under long day-length photoperiod (LD) GA accumulates, accelerating growth. On the contrary, SD has been shown to block certain steps in biosynthesis of GA, leading to growth cessation in poplar trees (Olsen et al., 1997; Eriksson, 2000).

Auxins and cytokinins are involved in bud apical dominance (Rohde et al., 2000). Auxin is also required to maintain the cambium in a meristematic state whereas its sensitivity is reduced during dormancy (Schrader et al., 2003).

Coville was the first, in 1920, to observe chilling requirements effects. He reported that certain native bushes from Washington D.C. remained completely dormant under warm controlled conditions, thus concluding that plants wouldn't resume normal growth in the warm weather without a period of chilling (Coville, 1920). An incomplete fulfilment of chilling requirements causes bud break delay, low bud break rate, lack of uniformity of leafing and blooming, and higher flower-bud drop, that directly influence yield and plant architecture, including a decrease in fruit commercial quality (Erez, 2000; Legave et al., 1982; Viti & Monteleone, 1991, 1995; Topp et al., 2008). On the other hand, an early fulfilment of chilling requirements in cold regions may increase the risk of spring frosts.

1.1.3 Economical importance of dormancy

The incomplete accumulation of chilling still represents a challenge for fruit crop production in temperate and warm climates, because most of the species and cultivars of temperate areas were originated and cultivated between the parallels 34 and 48 of the northern hemisphere (Faust. 2000).

Mediterranean climate area includes Mediterranean Sea borderlands, central and coastal southern California, central Chile, the southern tip of Africa, and part of the southernmost of Australia (Figure 1.2). These locations concentrates the early fruit production that initiates the commercial season, where a high interest to obtain new varieties adapted to this climate exists (Topp et al.,

2008). The expected changes in World climate conditions confer to dormancy and stress processes an increasing relevance, and represents a great challenge for plant breeders worldwide (Luedeling et al., 2011).

Several strategies have been developed to break bud dormancy such as over tree sprinkling of water, shading and defoliation. But the effectiveness of these treatments depends on the time of application. Defoliation, for example, triggers bud break only when performed before endodormancy (Saure, 1973; Janick, 1974; Edwards, 1987). In tropical climates, another agronomical practice is the cultivation at high altitudes that may help to fulfil chilling requirements (Arora et al., 2003).

Chemicals products as hydrogen cyanamide (HC) have also been used to overcome dormancy entrance, with some disadvantages as phytotoxicity and certain environmental impact. With such perspective, the development of new low chilling varieties represents an interesting solution that would minimize the negative ecological impact of chemical dormancy breaking agents.

Maybe for these reasons, in the last 20-30 years the interest on plant bud dormancy has increased, as indicated by the different international symposia emphasizing the complexity and relevance of this process (Corvallis, Oregon USA 1995; Angers, France in 2000; Wageningen, The Netherlands 2004 and Fargo, North Dakota USA 2009). Definitely, a better basic knowledge of dormancy will contribute in a fundamental way to the development of new varieties.

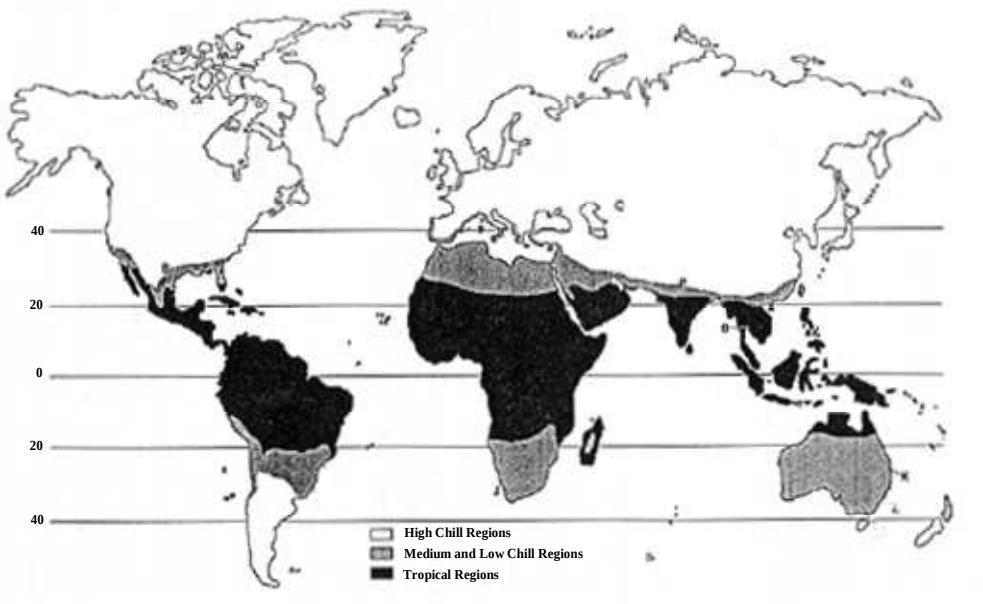

Figure 1.2 Worldwide distribution of medium and low chill region of major stone fruit breeding programs. (adapted from Byrne et al., 2000).

1.2 Climatic models for dormancy release

Once the relationship between dormancy release and the accumulation of low temperatures was established (Coville, 1920), bud dormancy release models started to be developed to determine in which way climate influences the fulfilment of chilling requirements. The effective temperature affecting dormancy breaking was estimated to be 7.2ºC, approximated in certain cases to 7ºC (Samish et al., 1954; Vegis et al., 1964; Weldon et al., 1934). Initially, temperature was considered the only major influence on dormancy release, and chilling requirement was consolidated as a parameter with a practical use to establish the success of a cultivar in a certain environment (Samish & Lavee, 1962). The first models based on the linear accumulation of chilling hours (Weinberger, 1950) soon demonstrated their limitations, as chilling requirement measurements were highly dependent on the year and location. A first improvement was provided by Richardson (1974) with the Utah Model that attributed different chill unit values to temperature ranges. Saure (1985) proposed a dual model considering a dual temperature action on dormancy; in a first phase low temperatures support the establishment of dormancy while in a second phase it contributes to dormancy release. This duality was also considered by Cesaraccio et al. (2004), who distinguished chilling days (from harvest to bloom) from antichilling days (during quiescence). Gradually, different models were developed taking into account bud sensitivity to temperature following its dormancy-stage (Fuchigami et al., 1987), the interaction between photoperiod and temperature (Hanninen et al., 1995), as well as diurnal and nocturnal temperature differences (Sugiura et al., 2002).

An adaptation of the Utah Model to warm climates (Dynamic model) was developed in Israel (Fishman et al., 1987a; 1987b). In this model chilling time is accumulated in two steps. A first accumulation of an intermediate product is promoted by chilling temperatures while warm temperatures have an opposite effect on this process. Once a sufficient amount of intermediate product is accumulated, Chill Portions are continuously accumulated. Then, after a period of cold, moderate temperatures have a positive effect on dormancy release (Erez & Couvillon, 1987). Several modifications to the Utah Model including temperature effects not previously considered were successively proposed. Thus, a more complex and accurate model arose taking into account different climatic conditions and crops: peach in Israel (Erez, 2000; Erez & Lerner, 1990; Fishman et al., 1987 a, 1987b), ornamental peach in Japan (Pawasut et al., 2004), kiwifruit (Allan et al., 1997), *Eucalyptus nitens* (Gardner & Bertling, 2005), peach in South Africa (Allan et al., 1995), peach in Chile (Perez et al., 2008) and in France (Balandier et al., 1993a), cherry

(Alburquerque et al., 2008), almond (Egea et al., 2003), apricot in Spain (Ruiz et al., 2007) and Italy (Viti et al., 2010), walnut in California (Luedeling et al., 2009e) and apple in northern Italy (Valentini et al., 2001). In particular the Dynamic model along with the Chill Hours and the Utah Models failed in tropical climatic conditions of Reunion Island (Balandier et al., 1993). This could mainly be due to the lack of biological and physiological basis: the majority of the models are developed with detached shoots or potted trees in growth chamber trying to simulate their behaviour in open field (Campoy et al., 2011a).

As a conclusion, bud dormancy release models are as accurate as our understanding of the cellular mechanisms underlying dormancy release (Arora et al., 2003). A deeper knowledge on the molecular and physiological mechanisms controlling dormancy would contribute to develop more robust models for dormancy evaluation; as an example by considering the interaction between photoperiod and temperature into chill model (Campoy et al., 2011a).

1.3 Common feature between bud and seed dormancy

During the growing season also seeds and tubers undergo dormancy. The growth of bud and embryo meristems is strictly regulated by dormancy mechanisms until environmental conditions are optimal for long-term survival. Dormancy is then released after a period of chill in both seeds and buds suggesting a common mechanism of control. This chilling treatment in seeds is called stratification. Insufficient cold stratification of seeds causes deformations in the growth habit as physiological dwarfing (Pollock, 1962).

In most popular model organisms, physiological dormancy integrates contributions from the embryo and the seed coat, being the coat component at least partially due to the mechanical resistance to breakage of endosperm and testa layers. In fact some species require scarification or fire, since smoke contains some dormancy breaking compounds as nitrogen oxides and a butenolide that stimulates germination.

Several recent findings support the hypothesis that germination control by seed covering layers is promoted by the action of several cell-wall modifying proteins, as 1,3 β-glucanases. Similar enzymes are involved in bud dormancy, producing degradation of callose deposition in the neck region of plasmodesmata (Rinne et al., 2001; 2011). In the cases of seeds, they could facilitate endosperm rupture of seeds, promoting cell separation (Finch-Savage et al., 2006).

The ratio of the hormones ABA and GA is considered a relevant factor regulating seed dormancy processes (Kucera et al., 2005). Dormancy maintenance depends on high ABA:GA ratios while its

release occurs with low ABA:GA (Cadman et al., 2006). In addition to hormone content and synthesis, the transition from a dormant to a non-dormant state is characterized by an altered sensitivity to ABA and GA. Other hormones as ethylene, brassinosteroids, auxin and cytokinins have been also proposed to affect dormancy and germination (Finkelstein et al., 2008).

The molecular factors and pathways conditioning seed dormancy status have been enumerated in several recent reviews (Bentsink et al., 2007; Finkelstein et al., 2008; Holdsworth et al., 2008; North et al., 2010; Finch-Savage et al., 2006).

Early studies showed that the B3 class transcription factors encoded by *VIVIPAROUS 1* (*VP1*) in maize and *ABA-INSENSITIVE 3* (*ABI3*) in *Arabidopsis* are involved in seed development and dormancy (McCarty et al., 1991; 1995; Giraudat et al., 1992).

At hormonal level, bud dormancy resembles seed dormancy (Powell 1987). Multiple physiological and transcriptomic studies (Rohde et al., 2002; Arora et al., 2003; Horvath et al., 2003; Rohde & Balherao, 2007) support the role of ABA in bud dormancy events. Transgenic poplars overexpressing and downregulating *PtABI3* suggested a role of *ABI3* in bud development influencing cellular differentiation of vegetative tissues; in addition to its role on seed dormancy (Rohde et al., 2002).

The modification of the chromatin structure contributes to the transcriptional regulation of dormancy in seeds. Single and double mutants in the *HISTONE MONOUBIQUITINATION* genes (*HUB1* and *HUB2*) coding for C3HC4 RING finger proteins with histone modifying activity showed a decreased dormancy phenotype (Liu et al., 2007). The identification of two histone deacetylases further suggested that chromatin structure is involved in the control of seed development (Tanaka et al., 2008).

1.4 Genetic and molecular aspects of dormancy process

1.4.1 Genetic studies and mapping of dormancy associated loci

Classical genetic approaches for the description of traits related to dormancy contributed to the understanding of the dormancy process. In blueberry, for example, cold hardiness trait best fits in a simple additive dominance model, while chilling requirement is controlled by two genes with equal effect (Rowland et al., 1999). Moreover, two genetic studies suggested that one major gene controlled the low chilling requirement trait in apple and apricot (Hauagge & Cummins, 1991; Tzonev & Erez, 2003).

A Quantitative Trait Loci (QTL) mapping of bud set and bud flush in *Populus* suggested the existence of respectively three and six QTLs (Frewen et al., 2000), which was more recently confirmed in four different pedigrees (Rohde et al., 2011). *PHYB2* and *ABI1B* genes, involved respectively in the perception of photoperiod and ABA response signal, mapped to two of these QTLs affecting bud set and bud flush (Frewen et al., 2000).

Two QTLs for blooming date were detected on LG2 and LG7 (Linkage Group) by Dirlewanger et al. (1999) in a peach F_2 population. One major gene (*Late blooming or Lb*) was mapped on LG4 by Ballester et al. (2001) in an almond F_1 population; and one QTL for blooming time was mapped on LG4 by Verde et al. (2002) in a peach backcross (BC_1) population. Fan et al. (2010) identified 20 QTLs associated to chilling requirement (CR), heat requirement (HR) and blooming date traits (BD) after evaluating a F_2 population of peach for 2 consecutive years. The strongest QTL found in this work mapped on linkage group 1, in a region containing the locus responsible for the non-dormant phenotype of the *evergrowing (evg)* mutant of peach (Fan et al., 2010).

In apricot, QTLs analysis of a cross between "Perfection" (high chilling variety) and "A1740" (low chilling variety) showed the most significant QTLs on LG1, LG5 and LG7 (Olukolu et al., 2009); while Campoy et al. (2011b) identified one major QTL for flowering time on LG5, linked to UDAp423r and AMPA-105 SSRs loci.

Additionally, a candidate gene approach associated two genes homologous to *Arabidopsis LEAFY* and MADS-box genes to two QTLs in almond (Silva et al., 2005), indicating that our knowledge on the genetic control of flowering time in annual plants may be applied to perennial tree species.

Few molecular markers have been associated to dormancy yet. A SCAR identified in red osier dogwood (*Cornus sericea* L.) was reported to be effective (>92%) to distinguish northern from southern dogwood ecotypes, differing in temperature-induced dormancy (Svedsen et al., 2007).

1.4.2. Molecular aspects of dormancy process.

1.4.2.1 The Vernalization model in *Arabidopsis* and cereals

The reproductive success of a plant depends on the synchronization of flowering time with the optimal environmental conditions. To do so certain Brassicaceae and cereals have developed a signal perception and transduction pathway that senses prolonged periods of cold during winter or vernalization.

A key gene in the regulation of vernalization in *Arabidopsis* is *FLOWERING LOCUS C (FLC). FLC* is a *MADS*-box transcription factor that acts as a repressor of floral transition by repressing

the floral integrator *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF CONSTANS 1 (SOC1)* (Searle et al., 2006; Figure 1.3). The cold-dependent induction of *VERNALIZATION INSENSITIVE 3 (VIN3)* during vernalization results in stable repression of *FLC*. When *FLC* is repressed, *FT* expression is induced under long days condition, and the FT protein is transported from the leaf to the shoot apex where induces flowering (Corbesier et al., 2007). Repression of *FLC* ends with meiosis, allowing the vernalization response in the next generation (Sheldon et al., 2000).

In cereals, *VERNALIZATION1 (VRN1)* gene coding for a MADS-box transcription factor related to the *Arabidopsis* genes *APETALA1 (AP1)*, *FRUITFULL (FUL)* and *CAULIFLOWER (CAL)* regulates meristem identity favouring the vegetative to reproductive transition (Yan et al., 2003). *VRN1* expression increases quantitatively as a response to cold, just in an opposite manner to *FLC* (Oliver et al., 2009). Resetting of *VRN1* expression occurs in autumn and in the progeny (Yan et al., 2003; Sasani et al., 2009).

VERNALIZATION2 (VRN2) encodes for a zinc-finger protein without clear homologues in *Arabidopsis* that is downregulated during prolonged periods of cold. *VRN1* and *VRN2* act respectively as positive and negative regulators of the floral induction (Figure 1.3). Loss-of-function mutations in *VRN2* confer a spring growth habit in wheat (that responds to LD photoperiod) (Trevaskis et al., 2003; Yan et al., 2004). However, *vrn2* mutants still respond to cold and induce *VRN1* after vernalization (Karsai et al., 2005).

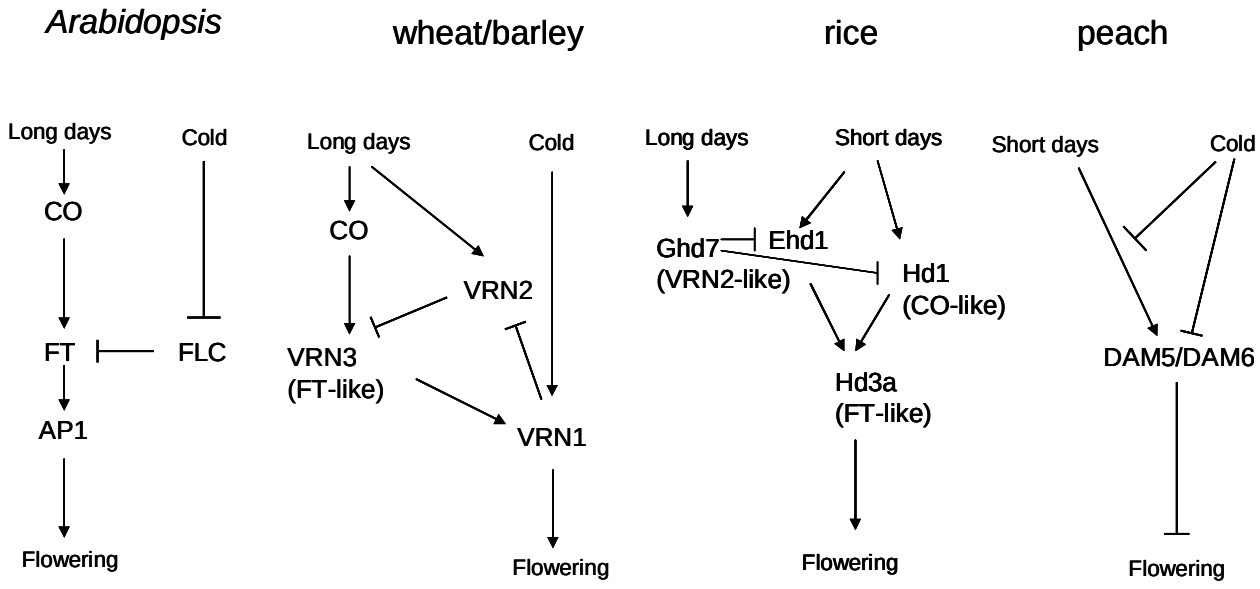

Figure 1.3 Genetic pathway controlling flowering in *Arabidopsis*, wheat/barley, rice and peach. Arrows show promoting effects, T-bars show repressing effects (Trevaskis et al., 2007; Alonso-Blanco et al., 2009; Jiménez et al., 2010b).

The expression of *VERNALIZATION3 (VRN3)* gene, considered as the homolog of *FT* in cereals, is up-regulated under LD conditions in wheat and barley as well as *VRN2*, whereas FT in rice is up-regulated under SD (Izawa et al., 2002).

While the day-length response is conserved between *Arabidopsis* and temperate cereals (wheat and barley), with *CONSTANS* (*CO*) inducing *FT* expression in specific day-lengths to promote flowering, the mechanism that integrates vernalization into this photoperiodic response is slightly different. In *Arabidopsis* a single gene, *FLC*, is responsible for regulating flowering by means of *FT* repression, while in cereals *VRN1* and *VRN2* are performing this role. The first is induced by prolonged cold while the second one responds to photoperiod. (Trevaskis et al., 2007b).

Rice differs from *Arabidopsis* and temperate cereals in that the flowering pathway only depends on the photoperiod and no vernalization response has been reported. Under short-day conditions, expression of the CO orthologue *Hd1(Heading date 1)* and *Ehd1(Early heading date 1)*, with no homologues in the *Arabidopsis* flowering pathway, promotes flowering in rice (Figure 1.3). Both genes induce the expression of *Hd3a (Heading date 3a)*, a rice orthologue of *Arabidopsis FT* (Izawa et al., 2007). Under long days conditions, expression of the VRN2-like gene *Ghd7 (Grain number, plant height and heading date 7)* represses flowering (Itoh et al., 2010).

1.4.2.2 Chromatin modification in vernalization

In both, *Arabidopsis* and cereals, the transcription of key vernalization genes is regulated by similar epigenetic mechanisms. In *Arabidopsis*, the stable down-regulation of *FLC* by vernalization is associated to specific histone modifications involving di- and tri-methylation of histone H3 lysine-27 (H3K27me2, H3K27me3), and methylation of H3 lysine-9 and H4 arginine-3. On the opposite, marks associated to active transcription such as H3 acetylation and H3 lysine-4 di- and trimethylation (H3K4me2, H3K4me3) are removed (Bastow et al., 2004; Sung et al., 2004; Finnegan et al., 2005; Sung et al., 2006; Finnegan et al., 2007; Schmitz et al., 2008; Doyle & Amasino, 2009).

In temperate cereals, *VRN1* is up-regulated during vernalization, coinciding with a reduction in H3K27me3 and an increase in H3K4me3 (Oliver et al., 2009; Hemming & Trevaskis, 2011).

The Polycomb Repressive Complex 2 (PRC2) is involved in H3K27 trimethylation and stable chromatin repression of *Arabidopsis FLC* during winter and wheat *VRN1* during summer (Hemming et al., 2009; Oliver et al., 2009). Chromatin state is restored at spring to ensure the plant is able to respond to vernalization again the next year. Different Polycomb subunits similar

to proteins from animals are combined in different ways to constitute specific PRC2 complexes involved in many aspects of plant development, as vernalization, seed germination and the transition from juvenile to adult phase (Hennig & Derkacheva, 2009).

1.4.2.3 The dormancy pathway in peach

In perennial plants *SHORT VEGETATIVE PHASE (SVP)-like/AGAMOUS-LIKE24 (AGL24)-like MADS*-box genes named *DORMANCY ASSOCIATED MADS*-box (*DAM*) have been found related to dormancy processes in poplar (Ruttink et al., 2007), raspberry (Mazzitelli et al., 2007), japanese apricot (Yamane et al., 2008), leafy spurge (Horvath et al., 2008) and blackcurrant (Hedley et al., 2010), suggesting similar mechanisms of dormancy control in perennial plants.

In peach (*Prunus persica* (L.) Batsch.), six tandemly repeated *DAM* genes were identified in studies employing the *evg* natural mutant (Bielenberg et al., 2008). The *evg* mutant, first identified in Mexico (Rodriguez et al., 1994), maintains apical growth and persistent leaves during the period of low temperatures and short days, presenting a relative low frost resistance (Arora et al., 1996; Arora & Wisniewski, 1994). On the contrary, lateral buds show a wild-type behaviour.

The *evg* locus has been mapped and identified in a genomic region of 132 kb in the wild-type, that was demonstrated to be partially deleted in *evg* in four of the six clustered *MADS*-box genes (Bielenberg et al., 2004; 2008).

The first report on QTL mapping of dormancy-related variables in peach identified twenty QTLs for chilling requirements (CR), bloom date (BD) and heat requirement (HR). The main QTL associated to the three traits localizes in the *evg* region, indicating that *DAM* genes are strong candidates for these traits (Fan et al., 2010). The publication of the peach genome (IPGI http://www.rosaceae.org/peach/genome) allowed the identification of the exact position of this region, although *DAM1*, *DAM2* and *DAM3* transcript models are not correctly annotated in the public repository (Figure 1.4)

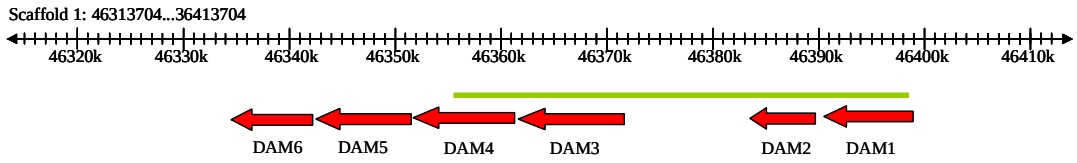

Figure 1.4 Physical map representation of DAM genes on the peach genome. Arrows indicates the sense orientation and green bar indicates the deletion in the *evg* mutant (adapted from Bielenberg et al., 2008).

The deletion in the *evg* mutant affected a region containing four genes and caused the lack of transcription of six *DAM* genes (Bielenberg et al., 2008). This fact suggests a possible cross-regulation between the four deleted genes and the other two.

Four different seasonal expression patterns have been observed indicating specific roles of these genes in growth and development (Li et al., 2009). The expression of *DAM1*, *DAM2* and *DAM4* was more closely associated to terminal bud formation, although all six genes showed seasonal expression changes. Gene expression of *DAM3*, *DAM5* and *DAM6* was strongly up-regulated by SD and successively reduced by chilling temperatures (Li et al., 2009). *DAM5* and *DAM6* levels directly correlated with the time required for bud break so they have been proposed as quantitative repressors of bud dormancy release (Jiménez et al., 2010b). The down-regulation of these genes is supposed to be necessary to reset dormancy mechanism for the next year (Hemming & Trevaskis, 2011). These observations were further confirmed by Yamane et al. (2011) in *Prunus persica* treated with cyanamide, but also in other woody plants as Japanese apricot (*Prunus mume*) (Yamane et al., 2008), poplar (Druart et al., 2007), raspberry (Mazzitelli et al., 2007) and leafy spurge (Horvath et al., 2008). In a recent work, Sasaki et al. (2011) focused on *PmDAM6* as a candidate gene to control dormancy in Japanese apricot. Transgenic poplar constitutively expressing *PmDAM6* showed growth cessation and terminal bud set in environmental conditions favorable to growth.

Since an epigenetic mechanism regulates the expression of *FLC* in *Arabidopsis* and *VRN1* in cereals, it is expected a similar control of bud dormancy in perennial trees. Interestingly the *DAM1* gene of leafy spurge shows altered levels of H3K27me3 and H3K4me3 at two different dormancy stages (Horvath et al., 2010).

1.4.3 Transcriptomic approaches

Genomics offers useful tools for the study of biological issues in species showing difficulties for genetic approaches (Rowland et al., 1997; Wisniewsky et al., 2004). Several studies have been dedicated to dormancy entrance, release or maintenance in different species as blueberry (Dhanaraj et al., 2004), *Rhododendron* (Wei et al., 2005), *Populus* (Schrader et al., 2004), raspberry (Mazzitelli et al., 2007) and grapevine (Mathiason et al., 2008), as summarized in Table 1.1.

The most relevant transcriptomic studies on dormancy release are described and the genes classified by ontological categories: membrane alterations, sugar metabolism and carbohydrate

catabolism, stress response and detoxification, biosynthesis of hormones and hormone receptors, light perception, cell cycle, division and growth, transport genes, regulation of gene expression and vernalization pathway.

Table 1.1 Genomic studies in perennial plants related to dormancy

Method	N of ESTs/gene/part of the plant	Treatments	Species	Reference
Microarray	1400 unigenes differentially expressed	crown buds collected monthly 2002-2006	Leafy spurge (*Euphorbia esula*)	Horvath et al., 2002
Microarray	4270 ESTs 12 randomly selected differential expressed genes	bud releasing from dormancy	Grape (*Vitis vinifera*)	Pacey Miller et al., 2003
Microarray	7 cDNA libraries 12376 clones sequenced, differential expression of SVP/AGL24-type MADS-box gene	Induction of dormancy (leaf senescence)	Populus genus	Andersson et al., 2004
EST sequencing	430 and 483 EST clones from 2 libraries	Non-acclimated and after 600 chilling units floral buds	Blueberry (*Vaccinium corymbosum*)	Dhanaraj et al., 2004
EST sequencing	4500 full lenght EST clones	dehydratation, high salinity, chilling, heat, ABA, H2O2 in leaves	Poplar (*Populus nigra v. Italica*)	Nanjo et al., 2004
Microarray (POP1 array)	33000 ESTs; 1598 contigs and 1478 singletons from dormant; 950 contigs and 746 singletons	Summer/autumn cambium (active growth/dormancy)	Poplar (*Popolus tremula*)	Schrader et al., 2004
EST sequencing	15 574 contigs + 6804 singlet	16 different libraries	Poplar (*Popolus tremula*)	Sterky et al., 2004
Microarray	2171 ESTs	seasonal changes in earlywood-latewood from xylem and shoot tip library	Loblolly pine (*Pinus teada*)	Yang & Loopstra 2005
cDNA from SSH	10 SSH libraries, 108 unigenes, 8 randomly selected differential expression genes	bark tissue at 5ºC and 25ºC under SD or LD	Peach (*Prunus persica*)	Bassett et al., 2006
SSH / nylon membrane hybridization	801 ESTs 233 unigenes	buds during budbreak (6 stages) and shoot outgrowth	Sessile oak (*Quercus petraea*)	Derory et al., 2006
SSH	516 clones differentially expressed from 2 SSH libraries	dormant and growing crown buds	Leafy spurge (*Euphorbia esula*)	Jia et al., 2006
Microarray	Idem from Schrader et al., 2004	Cambial meristematic cells during dormancy-activity cycle	Aspen (*Populus tremula*)	Druart et al., 2007
Microarray and Northern blot Hybridization	5516 ESTs Differential expression of 9 genes	HC treatment/ control in budbreak	Grape (*Vitis vinifera*)	Keilin et al., 2007
Microarray	1469 ESTs from buds from nodes 3 to 12	0, 500, 1000, 1500 and 2000 hours of chilling	Grapevine (*Vitis riparia*)	Mathiason et al., 2009
Microarray	5.300 screened clones from 2 cDNAs libraries, 88 differentially expressed unigenes	endodormant vs paradormant bud	Raspberry (*Rubus idaeus*)	Mazzitelli et al., 2007
cDNA-AFLP + RT-PCR	592 TDF (Transcript-Derived Fragments)	seasonal changes throught 115 d covering all dormant periodin axillary and apical buds	Poplar (*Populus tremula x Popolus alba*)	Rohde et al., 2007

Microarray (TIGR potato microarray vers. 3 and 4)	TIGR potato microarray, 47 upregulated genes in natural dormancy release, 589 cDNA after 1 day BE treatment, 319 cDNA after 8 days, 26 down-, 12 up-regulated genes.	meristem from tubers collected in natural and bromoethane (BE) dormancy release	Potato (*Solanum tuberosum*)	Campbell et al., 2008
Northern blot Hybridization	Differential expression of 7 genes involved in oxidative stress mechanism	Heat Shock and HC treatment on budbreak	Grape (*Vitis vinifera*)	Halaly et al., 2008
Microarray	one SSH library 185 clones differentially expressed	buds recollected during dormancy release	Tree peony (*Paeonia suffruticosa*)	Xin et al., 2008
SSH	2 SSH libraries, 26 unigenes	buds endodormant vs paradormant and endodormant vs paradormant	Japanese apricot (*Prunus mume*)	Yamane et al., 2008
Microarray	1582 genes upregulated and 1116 downregulated	buds treated with HC	Kiwifruit (*Actinidia deliciosa*)	Walton et al., 2009
SSH	11.520 screened clones, 106 sequenced contigs, 23 up-regulated genes during SD exposure	Growth cessation and bud dormancy entrance using the *evg* mutant	Peach (*Prunus persica*)	Jiménez et al., 2010a

1.4.3.1 Genes involved in membrane alterations: cell wall modification and cell expansion

The composition of plasma membrane proteins varies with the season: in autumn the increase in fatty acid desaturation of membrane lipids and the change in glycoproteins composition contributes to maintain fluidity at low temperatures (Martz et al., 2006; Yoshida et al., 1984).

Cell wall modification enzymes are expressed during the time course of bud dormancy; as extensin-like hydroxyproline glycoproteins are up-regulated at the end of dormancy. In the same period, the transcription of polygacturonase-inhibiting proteins is down-regulated. In fact, dormancy process includes a phase of cell wall restructuration that could be involved in the reestablishment of cell-to-cell communication through plasmodesmata during dormancy release, as observed in birch (Rinne et al., 2001).

1.4.3.2 Genes involved in sugar metabolism and carbohydrate catabolism

In autumn, accumulation of sucrose and starch occurs in response to low and freezing temperatures and, under SD condition, starch is mobilized mostly during the night.

During chilling fulfilment, genes involved in carbohydrate metabolism (enzymes as sucrose synthase, hexose transporter and several genes involved in the processing of glucose-6-phosphate) are inhibited. Mathiason et al. (2009) identified grapevine ESTs with similarity to sucrose synthase, hexose transporter, glyceraldehyde-3-phosphate dehydrogenase and chalcone synthase; as also found in raspberry buds (Mazzitelli et al., 2007).

These findings support the idea that during the fulfilment of chilling requirement buds reduce their metabolic activity.

Also the induction of a H^+ ATPase has been described concomitantly with bud break (Mazzitelli et al., 2007). This could be due to the increasing import of sugars needed to restore growth. On the contrary, a decrease in the activity of NAD-SDH (NAD-dependent sorbitol dehydrogenase), responsible for the conversion of unloaded sorbitol to fructose or other metabolites in sink tissue, is observed in raspberry (Mazzitelli et al., 2007) and Japanese pear (Ito et al., 2002).

Sucrose has also been proposed to act as a signalling molecule (Horvath et al., 2002). Dijkwel et al. (1997) and Short (1999) reported that sucrose inhibits phytocrome A (PHYA) signalling in *Arabidopsis* when combined with overexpression of phytochrome B (PHYB). Moreover this sugar inhibits the growth of leafy spurge crown buds through negative interaction with GA (Horvath 2002).

Rohde et al. (2007), working with poplar, identified 40 genes coding for proteins involved in catabolism and energy generation that were highly expressed during dormancy induction and dormancy maintenance. This observation suggests that lipid, sugar and fatty acid breakdown occurs during the induction of dormancy; not just during dormancy as proposed by previous studies (Sagisaka 1991). The expression of genes involved in polysaccharide cleavage and oligosaccharide production is high during the first phase of dormancy induction, having an osmoprotector function.

1.4.3.3 Proteins related to stress or protection responses and detoxification processes

Renaut et al. (2004) found that deactivation of reactive oxygen species and accumulation of dehydrins were some of the most prominent changes in the transcriptomic profile of poplar trees during seasonal chilling. Bud break is associated to free radical removal through the activation of peroxide scavenging systems such as catalase, ascorbate peroxidase, superoxide dismutase and glutathione reductase (Pacey Miller et al., 2003; Mazzitelli et al., 2007), when oxidative damages are more probable.

H_2O_2 is considered an occasional signalling molecule in plants that accumulates in grapevine buds treated with HC, suggesting a function in triggering the expression of genes related to dormancy release.

Dehydrins are the most studied proteins in relation to cold acclimation in woody plants. Although their function has not well been elucidated, their abundance during dehydration in plant suggests a

role in cellular protection (Ingram & Barteles, 1996). Their activity could be linked to calcium binding (Alsheikh et al., 2005), as well as to osmoregulation (Nylander et al, 2001; Wisniewsky et al., 1999), and radical scavenging (Hara et al., 2004). The simultaneous overexpression of two dehydrin genes in *Arabidopsis* (Puhakainen et al., 2004), and a citrus dehydrin in transgenic tobacco resulted in increased freezing tolerance and lipid peroxidation (Hara et al., 2003). The majority of these proteins are induced in response to low temperatures while few are induced under SD conditions, as also reported by Welling et al. (2004) in birch. In peach, a 60 kDa dehydrin was found at a lower concentration in the *evg* mutant when compared with cold-acclimated standard deciduous trees (Arora & Wisniewski; 1994). Numerous late embryogenesis abundant (LEA) proteins have been identified in different studies in a wide range of tissues (Horvath et al., 2003; Mazzitelli et al., 2007; Schrader et al., 2004; Basset et al., 2006; Jiménez et al., 2010a). Although the function of LEA proteins is not well known at the molecular level, they are supposed to have a role in protecting cells from desiccation and temperature stress.

1.4.3.4 Biosynthesis of hormones and hormones receptors

There is a set of overlapping hormonal signals in response to the environmental and physiological cues that are important in coordinating plant response during dormancy release.

In winter, auxin sensitivity and transport are reduced in poplar and birch (Schrader et al., 2003; Li et al., 2009). The opposite situation is found in potato, where auxin levels increase during dormancy and quickly drop off with dormancy release, suggesting opposite mechanisms involved in auxin production, transport and perception during dormancy (Horvath et al., 2003).

Short days preceding the cessation of apical growth cause a decrease in GA levels (Olsen et al., 1997). Coherently, transcript levels of the poplar gene *REPRESSOR of GA1(PtRGA1)*, highly similar to the repressor of gibberellin response *RGA1*, are up-regulated in dormant cambium (Schrader et al., 2004; Ruttink et al., 2007). Furthermore, a gene coding for a GA2 oxidase, an enzyme that inhibits the bioactive GAs via hydroxylation, was found increased in winter buds of *Prunus mume* (Yamane et al., 2008).

The involvement of ABA in dormancy establishment has been reported in poplar buds where genes encoding for ABA biosynthesis are up-regulated after 3 or 4 weeks of SD, coinciding with a transient ABA peak registered 4 weeks after the transition to SD (Ruttink et al., 2007). Moreover transgenic poplar overexpressing *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) doesn't form a terminal bud, highlighting the relevant role of ABA in dormancy acquisition (Rohde & Balherao,

2007; Rohde et al., 2002). During winter rest no transcription in genes involved in ABA biosynthesis has been registered, as reported by Schrader et al. (2004) in dormant cambium.

Mazzitelli et al. (2007) observed the presence of ABA-regulated transcripts during dormancy release in raspberry; as also observed by Campbell et al. (2008) in potato tubers, and by Horvath et al. (2008) in leafy spurge. These observations could indicate that ABA is involved in dormancy release by regulating a certain group of genes.

Also ethylene concentration varies during dormancy, as example a peak was detected before the initiation of endodormancy, suggesting this hormone is possibly induced by ABA. Nearly 10 genes associated to ethylene production or ethylene responses are expressed during paradormancy but repressed in endodormancy and ecodormancy (Horvath et al., 2008). Ruttink et al. (2007) also found a certain number of transcripts similar to *Arabidopsis* genes involved in ethylene signal transduction as *ETHYLENE RESPONSIVE FACTOR 4* (*AtERF4), AtERF5*, *AtERF5-LIKE*, perception as *ETR2* and *ERS1 (ETHYLENE RESPONSE SENSOR1),* and downstream signalling elements like *CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), ETHYLENE INSENSITIVE3 (EIN3), EIN3 BINDING F BOX1 (EBF1)* and *ETHYLENE-REGULATED NUCLEAR-LOCALIZED PROTEIN1 (ERN1).* Ethylene biosynthesis and signal transduction are simultaneously activated after 2 weeks of SD, just one week before the formation of the terminal bud.

The identification of a high number of genes regulated by jasmonic acid (JA) suggests a possible role of this hormone in dormancy progression. JA is usually associated to wounding and defence mechanisms. During the release of tuber dormancy in potato, the expression of *LOX* gene coding for a lipoxygenase involved in JA biosynthesis decreases, in addition to other genes involved in tuber development, protein storage and metabolism (Campbell et al. 2008).

1.4.3.5 Light perception

Phytochromes are proteins involved in red/far red light perception and regulate numerous physiological aspects of plant growth mediated by light quality and day length (Fankhauser & Chory, 1997), as well as circadian clock (Toth et al., 2001).

PHYTOCHROME A (PHYA) is supposed to regulate bud dormancy induction in poplar (Olsen et al., 1997) and aspen (Eriksson et al., 2000). Kim et al. (2002) showed that PHYTOCHROME B (PHYB) was the primary photoreceptor responsible for activation of cold-stress signalling mediated by light. *PHYTOCHROME C* (*PHYC)* is up-regulated during the fulfilment of chilling

requirements and mediates dormancy and germination responses to seasonal cues. *PHYC* also plays a role in cotyledon expansion in seedlings, and leaf area and stem length increase in adult plant (Mathiason et al., 2009).

The response to blue light involves a rhodopsin receptor whose gene expression decreases early and increases at the end of the chilling period, being interesting as an indicator of chilling requirements (Mathiason et al., 2009).

Horvath et al. (2008) observed that several circadian regulatory genes are surprisingly differentially expressed in crown buds of leafy spurge, that are situated below the soil surface. This is consistent with results in chestnut (*Castanea sativa* Mill.) showing that *CsTOC1* and *CsLHY* genes with daily cycling expression were constantly and highly expressed during dormancy. The oscillation was recovered when the plant returned to 22ºC. Notably, this phenomenon is not present in Arabidopsis (Ibañez et al., 2008; Ramos et al., 2005).

1.4.3.6 Cell cycle, division and growth

During the transition from para- to endodormancy the vast majority of genes involved in cell proliferation are down-regulated, including cell cycle regulators such as *CYCLIN A1 (CYCA1), CYCA2*, *CYCA3*, *CYCD3* and *CYCLIN-DEPENDENT KINASE A1 (CDKA1), CDKB1*, *CDKB2*, *CYCH1*, as well as *RETINOBLASTOMA PROTEIN*, *DP-E2F-LIKE PROTEIN3* and *CKS1* (Ruttink et al., 2007).

On the other side, genes involved in cell cycle, division and growth are induced during the chilling period. In this period cell cycle is re-established and cells start growing. Druart et al. (2007) reported that these genes were down-regulated upon dormancy establishment in aspen and then their expression remained low or slightly increased during ecodormancy. On the contrary, many of the genes involved in cell division are down-regulated during the transition from endodormancy to ecodormancy in leafy spurge (Horvath et al., 2008).

1.4.3.7 Transport genes

Some ATP binding cassette proteins (ABC) have been found down-regulated during the dormant period. Four of these proteins have been found in grape buds (Mathiason et al., 2009), two in leafy spurge crown buds (Horvath et al., 2008), and one in raspberry buds (Mazzitelli et al., 2007).

During dormancy establishment and release, plant is subjected to high variations in water content for the adjustment of osmotic pressure in cells. Aquaporins belong to a highly conserved group called major intrinsic proteins that control water movement between cells and possibly also modulate the transport of water through membranes to regulate osmotic pressure. This group of proteins is divided in four subfamilies, one of which is plasma membrane intrinsic proteins (PIP). Three of these PIPs were found induced during the period of accumulation in *Vitis* (Mathiason et al., 2009).

On the other side, water transport is reduced during dormancy induction by blockage of plasmodesmata through differential calcium (Jian et al., 1997) or 1,3 β-glucan deposition (Rinne et al., 2001; Rinne & Van der Schoot, 2003). During dormancy release a high expression of 1,3-β-glucanases, involved in plasmodesmata opening, was detected in peach bark (Bassett et al., 2006) as well as in poplar (Rinne et al., 2011).

Nitrate is a nitrogen source for ammonium and amino acids synthesis, which makes it essential for plant growth and development. During chilling accumulation two nitrate transporters were found up-regulated in *Vitis* (Mathiason et al., 2008), and one in orange (*Poncirus trifoliate*) (Zhang et al., 2005). This indicates that buds are preparing for protein production. A role of nitrate as a signal molecule favouring germination has been hypothesized (Alboresi et al., 2005); thus we cannot discard a related signalling function also in buds.

1.4.3.8 Regulation of gene expression

DAM-like transcription factors

DAM-like genes have been related to dormancy maintenance through sequencing and annotation of the *evg* mutant and QTLs analysis in peach. The relevance of these transcription factors is highlighted by the abundant literature showing dormancy-dependent regulation of *DAM-like* genes in other species as poplar (Ruttink et al., 2007; Druart et al., 2004), raspberry (Mazzitelli et al., 2007), Japanese apricot (Yamane et al., 2008), leafy spurge (Horvath et al., 2008), blackcurrant (Hedley et al., 2010) as well as in peach fruit tissue after cold storage (Ogundiwin et al., 2008). The seasonal expression pattern of *DAM* genes in peach indicates that they are induced at different stages: *DAM3*, *DAM5* and *DAM6* during dormancy entrance, and *DAM1*, *DAM2* and *DAM4* during terminal bud formation (Li et al., 2009). Recently, Jiménez et al. (2010b) proposed *DAM5* and *DAM6* as quantitative repressors of bud dormancy release. A transgenic poplar

expressing constitutively *DAM6* showed terminal bud formation and dormancy induction under normal growing conditions, thus confirming this supposed role (Sasaki et al., 2011).

Other transcription factors

The setting up of dormancy involves the transcriptional regulation of a large number of genes. Thus, the identification of transcription factors with dormancy-dependent gene expression represents an important step in understanding the process.

Schrader et al. (2004) identified in poplar cambium a gene with similarities to the CCAAT-binding factor *HAP2* from aspen, which is induced during dormancy. This protein is part of a complex that includes the seed dormancy regulator LEC1 (Kwong et al., 2003; Lee et al., 2003).

In poplar, Rohde et al. (2007) identified three regulatory genes *AP2*/*EREBP*, *ERF4* and *WRKY11* clearly expressed after 24 days under SD. Based of the function of their respective *Arabidopsis* homologues, these genes could be involved in ABA and ethylene signal transduction. The *ERF4* homologue in *Arabidopsis* is transcriptionally induced by ethylene, jasmonate, and ABA and acts as a transcriptional repressor capable of modulating ABA and ethylene responses. Overexpression of *ERF4* leads to ethylene insensitivity and increases ABA sensitivity. Together, the change in expression of *AP2/EREBP*, *ERF4* and *WRKY11* during dormancy induction supports a role in endodormancy set up in apical bud and paradormancy in axillary buds (Rohde et al., 2007).

Chromatin regulation

FERTILIZATION INDEPENDENT ENDOSPERM (*FIE*)-like genes are members of the polycomb family of regulators of the chromatin structure that were observed to be strongly up-regulated in poplar cambium and buds during dormancy (Schrader et al., 2004; Ruttink et al., 2007). In *Arabidopsis*, *FIE* product acts as part of a complex that silences the transcription of genes necessary for proliferation, through modification of the chromatin structure. Also homologues of the chromatin-remodelling factor *PICKLE* (*PKL*), *CDC48-LIKE*, and *HISTONE1-3* are strongly up-regulated in poplar buds after shortening the day-length (Schrader et al., 2004). These findings also reinforce the idea that chromatin remodelling and modification processes are involved in dormancy (Ruttink et al., 2007).

CBFs

The best-known regulatory pathway in cold acclimation is the CBF/DREB1 cold response pathway that has been well characterized in *Arabidopsis* (Nakashima & Yamaguchi-Shinozaki, 2006). The *CBF* genes are induced within 15 min of cold exposure, followed by the induction of *CBF* target genes by its binding to LTRE/DRE/CRT elements in their promoters. Overexpression of *CBF* leads to increased freezing but also drought and salt tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999, Gilmour et al., 2000).

CBF orthologues of woody plants are similarly induced by low temperatures during normal growth, in dormant cambium in autumn (Schrader et al., 2004), as well as after SD exposure (Jaglo-Ottonsen et al., 1998), suggesting that they participate in both cold acclimation and freezing tolerance. In order to identify genes under *CBF* regulation, Benedict et al. (2006) compared the transcriptomes after cold exposure of wild-type *poplar* and a *CBF*-overexpressing line, concluding that many of these cold-regulated genes were in fact orthologues of genes from the *CBF* regulon in *Arabidopsis* (Welling et al., 2006).

1.5 Transgenic approaches towards the regulation of bud dormancy

Genes identified by transcriptomic approaches require a deeper functional study that can be achieved by gene introgression into a suitable plant. In classical breeding this is performed by means of repetitive backcrosses, being a long process greatly depending on the length of the reproductive cycle of the plant. A simpler and cleaner alternative is the production of transgenic plants. This technology has been used both for functional studies and introduction of agronomic traits in crops.

Olsen et al. (1997) reported that the overexpression of oat *PHYA* gene in aspen hybrid (*Populus tremula x P. tremuloides*) prevented this tree to enter dormancy even when critical day-length was changed from 15 to 6 h. These transgenic plants didn't present leaf abscission, growth cessation, and cold acclimation, as GA and indoleacetic acid (IAA) levels still remained high under SD conditions. A reduced expression of *PHYA*, on the other side, accelerated bud formation in response to SD (Erikkson et al., 2000).

Also in aspen, the overexpression of *PtFT* and *PtCO* induced a continuous growth habit under SD conditions (Böhlenius et al., 2006). Actually, plants engineered with the overexpression of *PtFT* are used to shorten the juvenility period in woody trees (Lewis & Kernodle, 2009).

Poplar overexpressing *ABI3*, as well as birch with a dominant negative version of *ETR1* failed to form buds and yet became dormant, suggesting that bud formation and dormancy set up are independent processes (Rohde et al., 2002; Ruonala et al., 2006).

Apple transformed with *CBF* transcription factor from peach showed higher sensitivity to short day-lenght and an increase to frost tolerance (Wisniewski et al., 2011).

The constitutive expression of the *BpMADS4* gene has been shown to induce early flowering in birch (*Betula pendula*) and apple (*Malus x domestica*), but not in poplar (Flachowsky et al., 2007). Transgenic trees maintain leaves, growth and the photosynthetic activity during autumn and winter (Hoenicka et al., 2008). This finding open new interesting questions about the differences in dormancy regulatory mechanisms among woody plants.

Plants transformed with *DAM6* from *Prunus mume* and *Prunus persica* have been obtained respectively in poplar and plum. Transgenic poplars showed growth cessation and terminal bud set under favourable conditions (Sasaki et al., 2011), while transgenic plums showed more branches and were dwarf (Fan, 2010).

The most interesting results obtained in controlling the time of flowering in woody trees, were achieved with the work by Weigel & Nilsson (1995). With the introgression of *PtFT* in *P.tremula* plants flowered within 4 weeks instead of 8-20 years in normal conditions. In citrus, early flowering and fruiting trees were obtained by transformation of *Poncirus trifoliata* with a citrus orthologue of *FT* (Endo et al., 2005) and with the *Arabidopsis AP1* gene (Peña et al., 2001).

MAIN OBJECTIVES

The general aim of this work is to understand the molecular and physiological mechanisms underlying the maintenance and release of seasonal dormancy in peach. To achieve this goal, successive specific objectives have been accomplished during this four-years work:

- Identification of peach genes related to dormancy release by suppression subtractive hybridization (SSH) and microarray hybridization.
- Evaluation of the identified genes to assess the chilling requirement of cultivars by analysis of expression.
- Study of chromatin modifications associated to dormancy release in the *DAM6* gene.
- Analysis of common physiological and molecular features shared by dormancy processes in buds and seeds.

2. Identification of genes associated with bud dormancy release in *Prunus persica* by suppression subtractive hybridization

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

Abstract

To better understand the molecular and physiological mechanisms underlying maintenance and release of seasonal bud dormancy in perennial trees, we identified differentially expressed genes during dormancy progression in reproductive buds from peach (*Prunus persica* [L.] Batsch) by suppression subtraction hybridization (SSH) and microarray hybridization. Four SSH libraries were constructed, which were respectively enriched in cDNA highly expressed in dormant buds (named DR), in dormancy released buds (RD), and in the cultivars with different chilling requirement 'Zincal 5' (ZS) and 'Springlady' (SZ), sampled after dormancy release. About 2,500 clones picked from the four libraries were loaded on a glass microarray. Hybridization of microarrays with the final products of SSH procedure was performed in order to validate the selected clones that were effectively enriched in their respective sample. Nearly 400 positive clones were sequenced, which corresponded to 101 different unigenes with diverse functional annotation. We obtained *DAM4*, *5*, and *6* genes coding for MADS-box transcription factors previously related to growth cessation and terminal bud formation in the *evergrowing* mutant of peach. Several other cDNAs are similar to dormancy factors described in other species and others have been related to bud dormancy for the first time in this study. Quantitative RT-PCR analysis confirmed differential expression of cDNAs coding for a Zn-finger transcription factor, a GRAS-like regulator, a DNA binding protein, and proteins similar to forisome subunits involved in the reversible occlusion of sieve elements in Fabaceae, among others.

Introduction

Perennial woody plants from temperate regions, such as peach species (*Prunus persica* [L.] Batsch), cease growth and become dormant during part of autumn and winter to elude the detrimental effect of the exposure to low temperatures. This state has been designated endodormancy because bud growth inhibition is due to signals internal to the bud itself, in contrast to bud growth inhibition by other distal organs (paradormancy), or by environmental factors (ecodormancy). For the purpose of this work, the term dormancy has been employed to refer to the endodormant state. The physiological and genetic control of bud dormancy has been reviewed by different authors (Arora et al., 2003; Horvath et al., 2003; Rohde & Bhalerao, 2007; Allona et al., 2008). In summary, these reviews emphasize the relevant role of day-length shortening, temperature, abscisic acid (ABA), ethylene and gibberellins (GA) as signals affecting bud set and dormancy onset, and discuss some molecular mechanisms related to the process, including cell cycle regulation, modification of the cell water status, and epigenetic regulation. Releasing of bud dormancy requires the completion of a

chilling period that leaves the bud in an ecodormant state, susceptible to initiate budbreak after a period of favourable temperatures. The length and intensity of this chilling requirement depend on the species and cultivar under study, suggesting genetic control of this process.

The identification of non-dormant mutants in hazelnut (Thompson et al., 1985) and the *evergrowing* mutant in peach showing defective terminal bud formation (Rodríguez et al., 1994) offered genetic tools to dissect the molecular control of bud dormancy. Recently, a genomic deletion has been identified in the mutant *evergrowing* that affects several members of a six tandemly repeated series of related MADS box genes (*DAM1-6*, for *DORMANCY-ASSOCIATED MADS-BOX*) in tight linkage to the observed phenotype (Bielenberg et al., 2008). Thus *DAM* genes are considered major candidates to control bud dormancy and meristem growth cessation through regulation of gene expression. However dormancy release is a complex trait that most likely involves numerous genes. In order to identify dormancy-related genes and to elucidate the molecular mechanisms underlying bud set and break in different species, several authors used distinct methodological approaches involving studies of gene transcription as cDNA microarray profiling (Schrader et al., 2004; Druart et al., 2007; Mazzitelli et al., 2007; Ruttink et al., 2007; Horvath et al., 2008; Mathiason et al., 2009), cDNA-AFLP (Rohde et al., 2007) and suppression subtraction hybridization (SSH, Jia et al., 2006; Xin et al., 2008; Yamane et al., 2008).

In this paper we describe the results of a transcriptomic approach for the isolation of cDNA fragments differentially transcribed during the fulfillment of the chilling requirement in peach flower buds. In order to achieve this goal, we first estimated the approximate dormancy-release date of the peach varieties ‘Zincal 5’ and ‘Springlady’ by measuring the mean time to budbreak (MTB). An RNA sample from ‘Springlady’ buds (medium chilling requirement) collected just after dormancy release was then compared in a first experiment against RNA from dormant buds of ‘Springlady’, and in a second experiment against RNA from dormancy released buds of ‘Zincal 5’ (low chilling). Thus we expected to identify genes regulated during dormancy progression and release in flower buds (first experiment) and other genes whose expression is due to or causing genotype-dependent differences in chilling requirement for dormancy release (second experiment). We employed the SSH procedure to perform both RNA comparisons (Diatchenko et al., 1996), which relies on the selective amplification and enrichment of abundant cDNAs in a sample (tester) when incubated and hybridized with an excess of a reference sample (driver). After hybridization of two sets of cDNA to be compared, an adaptor-based PCR approach allows subtraction of common cDNAs and amplification of rare and differentially expressed cDNAs. In addition to genes

previously associated with dormancy processes (ie. *DAM* genes), other genes not previously related to the dormancy process are described in this work, some of them identified for the first time in peach.

Materials and methods

Plant material

The peach cultivars 'Zincal 5' and 'Springlady' were employed in this study. The two orchards were located in the vicinity of Valencia (Spain). The samples required for the SSH procedure were obtained from flower buds of 'Springlady' collected on 14 November 2007 (dormant buds) and 8 January 2008 (dormancy released buds), and flower buds of 'Zincal 5' collected on 18 December 2007 (dormancy released buds). Daily average temperatures in the orchards under study were 11.4ºC on 14 November 2007, 8.7ºC on 8 January 2008 and 7.3ºC on 18 December 2007. No rainfall was recorded on these days. Budbreak measurements were performed on flower buds collected at different times from November 2007 until February 2008, whereas buds for RT-PCR analysis were collected from October 2008 until February 2009. Buds were routinely pooled from shoots obtained from different trees.

Budbreak measurements

CO_2 release was measured on excised flower buds introduced into a hermetic jar for 3-4 hours at 20ºC. Four 1-ml aliquots of the internal atmosphere were withdraw with a hypodermic syringe and injected into a gas chromatograph (Perkin Elmer Autosample). Carbon dioxide concentration was analysed by a thermal conductivity detector coupled to a Chromosorb column at 60 ºC. Single bud weight was calculated after weighing a pool of 100 reproductive buds and dividing the value by 100.

The mean time to budbreak (MTB) was calculated as described in Gariglio et al. (2006). Briefly, 20 shoots from the different cultivars at different times were placed with their basal tip in water and forced in a phytotron at 8-hour photoperiod (22.5 μmol/(m^2s)) at 20 °C during 6 weeks. The shoots were divided in four groups of five shoots each. The basal ends of the shoots were cut weekly and water was replaced daily. The number of opening buds was recorded three times per week. Time to budbreak of a group of shoots was the time in days required to open at least 50% of the flower buds. The results were expressed as the arithmetic mean of the time to budbreak for the four groups.

Isolation of RNA and mRNA purification

Total RNA was isolated from 0.5-2 g of flower buds (about 50-200 buds) by a guanidine thiocyanate-based protocol applied to high phenolic content tissues (Salzman et al., 1999). Poly A^+ RNA was subsequently purified by using the Oligotex® mRNA Purification System (Qiagen) according to the manufacturer's instructions. The mRNA concentration in the solution was measured with the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen). The poly A^+ RNA was concentrated by precipitation with two volumes of ethanol (overnight at –20 °C), in the presence of 33 mM NaCl and GlycoBlue™ Coprecipitant (Ambion), and then washed with ethanol 80%. The poly A^+ RNA was subsequently dissolved in RNAse free water at a concentration of 33 ng/µl.

SSH analysis

SSH was performed according to Diatchenko et al. (1996) with tester and driver cDNAs as shown in Table 1. Briefly, About 100 ng of poly A^+ RNA isolated from those samples as shown above was reverse transcribed to cDNA using the SMART™ PCR cDNA Synthesis Kit (Clontech). The resulting cDNA was digested with *Rsa*I and purified using the High Pure PCR Product Purification Kit (Roche). Subtraction was performed with the PCR-Select™ cDNA Subtraction Kit (Clontech) according to manufacturer's manual. The SSH products were purified using the High Pure PCR Product Purification Kit (Roche). Subtracted cDNAs were ligated into the pGEM®-T-Easy vector (Promega) and cloned into JM109 *Escherichia coli* cells. After growing on plates containing ampicillin, isopropyl-D-thiogalactopyranoside (IPTG) and 5-bromo-4-cloro-3-indolil-β-D-galactopiranósido (X-gal), white colonies were picked and incubated overnight at 37 ºC into 96-well plates containing ampicillin. Sterile glycerol was added to a final concentration of 20 % for stable storage of cultures at –80 ºC. The DR and RD libraries were respectively enriched in cDNA abundant in dormant and dormancy released buds from 'Springlady', whereas ZS and SZ libraries were enriched in transcripts from dormancy released buds of 'Zincal 5' and 'Springlady' respectively.

Microarray preparation and hybridization

The cloned cDNAs were directly amplified from *E coli* cultures. About 5 µl of stored cultures were used as template in 100 µl PCR reactions with Nested PCR primer 1 (5´-TCGAGCGGCCGCCCGGGCAGGT-3´) and Nested PCR primer 2R (5´-

AGCGTGGTCGCGGCCGAGGT-3´) by following these conditions: 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min, and a final extension of 72 °C for 5 min. Quality and success of the amplification was confirmed by agarose gel electrophoresis. PCR products were purified using the Multiscreen® PCR μ96 Filtration System (Millipore), and resuspended in water to a final concentration of 200–400 ng/μl. Before printing, purified PCR fragments were transferred to 384-well low volume, low profile plates (Corning) at a final concentration of 100–200 ng/μl in 50% dimethylsulphoxide. Each clone was spotted twice. Samples were spotted on Corning UltraGAPS glass slides, using a MicroGrid II spotting device from Biorobotics, in a 16-block format and 12 by 14 spots per block. Slides were crosslinked at 150 mJ and stored. Microarray hybridization was performed as previously described (Ríos et al., 2008). The SSH final products were labelled with the BioPrime® Plus Array CGH Genomic Labeling System (Invitrogen) according to manufacturer's instructions. About 500 ng of each sample was labelled with both, Alexa Fluor® 555 and Alexa Fluor® 647 fluorescent dyes. Data analysis was performed using the Limma package from the R statistical computing software (Gentleman et al., 2004). The *P*-value to control the false discovery rate was adjusted with the Benjamini and Hochberg's method. Each comparison of a pair of SSH products required four independent hybridizations with dye swap.

Sequence data analysis

Clones showing a *P*-value higher than 0.05 in the microarray analysis were not further considered. For sequencing purposes, we gave preference to clones that had at least a two-fold signal difference between SSH samples in both microarray comparisons or at least a four-fold signal difference in one of the comparisons. Only microarray-validated clones that consistently produced strong single bands following amplification and digestion with *RsaI* were sequenced. Base calling, trimming of low quality regions and vector masking were performed with phred and Crossmatch (Ewing & Green, 1998). Reading assembly was performed with the CAP3 (Huang & Madan, 1999). Similarity searches were performed with the standalone version of BLAST (Altschul et al., 1990), against the NCBI non-redundant protein database, using an e-value cutoff of 10^{-7}. Parsing of the BLAST results was performed with the Bio::SearchIO module from the Bioperl package (Stajich et al., 2002). Gene Ontology and functional annotations was performed with BLAST2GO (Conesa et al., 2005).

Real time RT-PCR

Extraction of RNA from reproductive buds at different developmental stages, poly A^+ RNA purification and quantification were performed as described above. About 80 ng of poly A^+ RNA were reverse transcribed with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) in a total volume of 20 µl. One µl of a ten times diluted first-strand cDNA was used for each amplification reaction in a final volume of 20 µl. Quantitative real-time PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems), using the Power SYBR® Green PCR Master Mix (Applied Biosystems). Reaction composition and conditions followed manufacturer's instructions. The primers employed are listed in Supplementary Figure 2. Cycling protocol consisted of 10 min at 95°C, then 40 cycles of 15 sec at 95°C for denaturation, and 60 sec at 60°C for annealing and extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve after the amplification and through size estimation of the amplified product. The comparative C_T ($\Delta\Delta C_T$) method was used to quantify those cDNAs with amplification efficiencies equivalent to the reference Actin gene. PpB20 amplicon, showing different amplification efficiency, was quantified by performing relative standard curves. Results were the average of 2 independent biological replicates repeated twice.

Results

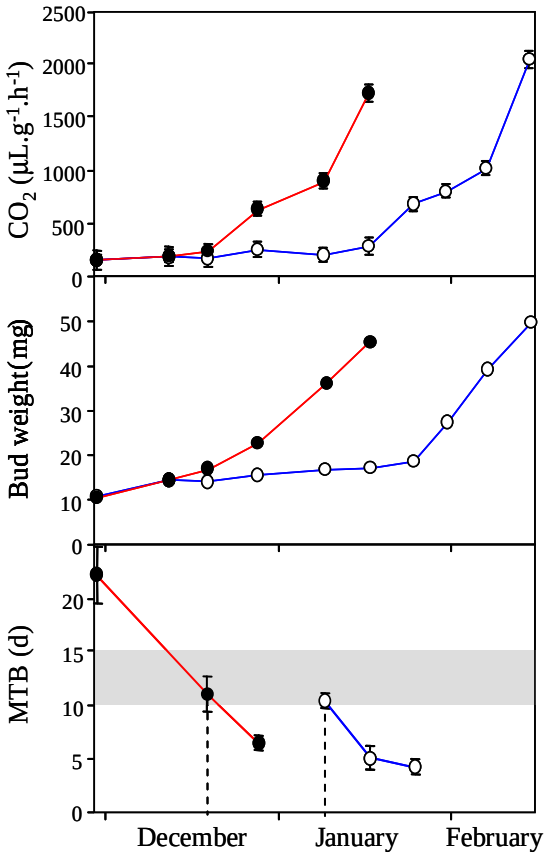

Figure 2.1. Flower bud parameters in 'Springlady' and 'Zincal 5' cultivars. The CO_2 release, weight and MTB of 'Springlady' (blue line-empty circles) and 'Zincal 5' (red line-filled circles) buds were recorded during autumn and winter seasons of years 2007-2008. The MTB interval from 10 to 15 days is shaded. Dashed lines label the genotype-specific dormancy release dates. Error bars represent standard deviations.

Two peach genotypes showing different chilling requirements

A genomic approach to the identification of genes and pathways involved in release of seasonal dormancy in peach requires a previous physiological assessment of the varieties under study. Dormancy release is conditioned by the fulfilment of the genotype-specific chilling requirement and other internal factors, which leave buds in an ecodormant state. Budbreak is subsequently happening after ecodormancy release under favourable environmental conditions. Whereas time to budbreak can be estimated by detecting the CO_2 produced by cell respiration in the bud and also by measuring the increase of bud weight due to the initiation of meristem growth, dormancy release time has to be measured by indirect means excluding environmental effects. This was accomplished by calculating the mean time to budbreak (MTB) of flower buds from shoots cut at different times and incubated under forcing conditions as shown in Materials and Methods. Those parameters were measured at different times during autumn and winter of years 2007-2008 in the cultivars under study 'Springlady' and 'Zincal 5'.

The burst in CO_2 release and increase in bud weight occurred in 'Zincal 5' about one month earlier than in 'Springlady' (Figure 2.1), indicating distinct requirements for bud metabolic activation and growth initiation in both cultivars. However their different chilling needs for dormancy release could only be estimated by MTB assessment. Since budbreak has a stochastic component, we decided to limit the MTB for dormancy release to an interval of 10-15 days, based on physiological works showing that MTB is usually stabilized in this time interval after dormancy releasing (Gariglio et al., 2006). Following this criterion 'Zincal 5' was three weeks earlier than 'Springlady', with release dates 18 December and 8 January respectively (Figure 2.1). Interestingly, 'Zincal 5' but not 'Springlady' was able to budbreak to some extent before its dormancy release date. As generally accepted, the length of the chilling period required for dormancy release is a more reliable and comparable measurement of the dormant behaviour of different peach cultivars than simple dates. Thus, by defining a chilling hour (CH) as one-hour interval at 7 °C or lower temperature (Weinberger 1950), we estimated a chilling requirement of 330 CH for 'Zincal 5' and 430 CH for 'Springlady' under our field conditions.

SSH analysis

As stated above, SSH is a widely used approach to isolate differentially expressed genes in two related samples. We designed a double SSH experiment to characterize the time and genotype dependent regulation of the peach bud transcriptome during seasonal dormancy. In the first experiment, cDNA samples from reciprocally subtracted libraries of dormant buds and dormancy-released buds of 'Springlady' were generated, which were respectively named DR and RD (Table 1). In the second experiment, reciprocal libraries from the cultivars 'Zincal 5' and 'Springlady' after bud dormancy release were produced (respectively ZS and SZ). A total of 2496 clones of putative differentially-expressed genes were isolated from the four libraries, with an insert size ranging from 200 to 1500 bp (not shown). Glass microarrays were constructed containing the amplified and purified inserts of these 2496 clones.

Table 2.1. SSH libraries elaborated in this work.

SSH library	Tester	Driver	Clones	Percentage of microarray-validated clones	
				M=1(*)	M=2(*)
DR	Dormant buds from 'Springlady'	Dormancy released buds from 'Springlady'	768	35 %	7 %
RD	Dormancy released buds from 'Springlady'	Dormant buds from 'Springlady'	768	48 %	24 %
ZS	Dormancy released buds from 'Zincal 5'	Dormancy released buds from 'Springlady'	480	15 %	3 %
SZ	Dormancy released buds from 'Springlady'	Dormancy released buds from 'Zincal 5'	480	21 %	9 %

(*) M value is log2-signal ratio.

In order to validate those clones effectively enriched in any of the four libraries, the final products of the four SSH experiments containing cDNAs accumulated under different conditions were labelled with fluorescent dyes, combined in pairs (DR against RD and ZS against SZ), and hybridized to the microarray as described in Material and Methods. With the exception of the RD library with 24%, the rest of the libraries had less than 10% of their respective clones differentially accumulated at levels greater than 4 fold (Log2 signal ratio >2; Table 2.1). A total of 364 clones showing 4 fold differences in cDNA accumulation for either the DR/RD or ZS/SZ comparisons, and those having 2 fold differences for both DR/RD and ZS/SZ comparisons were selected and produced suitable sequence data (Supplementary Figure 2.1). A search for coincident clones, as described in Materials and Methods, found 101 unigenes represented by 47 contigs and 54 singletons (Tables 2.2, 2.3 and 2.4). Forty-seven of the 101 unigenes were enriched in the RD sample, 46 in DR, 38 in ZS and 31 in SZ. It was expected to find many of the unigenes enriched in two independent SSH samples, as one of the operational criteria to sequence an EST was to have a threshold value of two fold differential cDNA accumulation in both DR/RD and ZS/SZ comparisons, but interestingly, an Euler diagram of overlapping groups showed a bias towards the coincidence of RD with ZS (29 unigenes) and DR with SZ groups (21 unigenes) (Figure 2.2).

About 50% of the unigenes were not found in a Blastn search against peach EST databases in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), arguing for a relevant number of novel ESTs. Of the 51 ESTs with significant similarity to previously described ESTs, 9 (18%) have been identified in a SSH approach to isolate low temperature and photoperiod regulated genes in bark (Bassett et al., 2006), whereas 24 (47%) have been recently described in an

article developing genomic tools for the identification of cold-responsive genes from peach fruit mesocarp (Ogundiwin et al., 2008; Tables 2.2, 2.3 and 2.4).

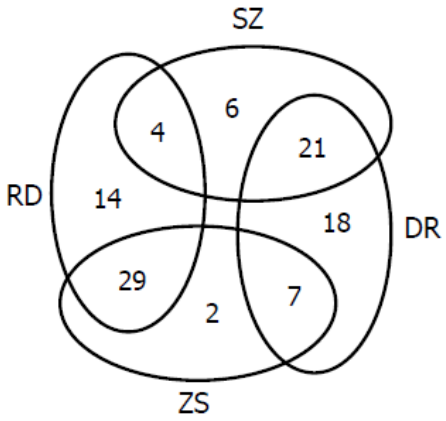

Figure 2.2. Euler diagram with overlapping unigenes. The diagram consists of four ellipses representing unigenes enriched in the dormancy released bud SSH sample (RD), unigenes enriched in the dormant bud sample (DR), unigenes enriched in the 'Zincal 5' sample (ZS), and unigenes enriched in the 'Springlady' sample (SZ). The number of shared unigenes is located in the overlapping sections.

Functional classification of unigenes

Tables 2.2, 2.3 and 2.4 contain the annotation and functional classification of unigenes based on Blastx best hits. These tables list separately the sequences enriched in the dormant sample DR (Table 2.2), in the dormancy-released sample RD (Table 2.3), and those that accumulated in ZS or SZ samples but not in DR or RD (Table 2.4). Unigenes representing very diverse biochemical, cellular and molecular functions were classified into six major categories: Metabolism, oxidation-reduction, stress and defence, signalling and transcription, transport and other. This last category (other) included also unigenes coding for proteins with unknown function or with no similarity in databases. From the 101 studied unigenes, only 13 corresponding to 54 clones did not show any Blastx hit at an E-value cut-off of 10^{-7}. The most remarkable case in this group is PpB94 containing 22 ESTs in the SZ group of 'Springlady' enriched transcripts. Some of the most striking observations arising from these lists were the high number of peroxidase ESTs found in the RD fraction (77) and the abundant number of transcription factors present in the DR and SZ groups (Tables 2.2 and 2.3).

Table 2.3 List of unigenes enriched in the dormacy-released sample (RD).

Unigenes	Expression group	EST	Blastx hit	Blastx E-value	Previous works
Metabolism					
PpB47	RD/ZS	13	acyl:coa ligase [*Populus trichocarpa*]	1×10^{-135}	
PpB48	RD	6	putative strictosidine synthase [*Arabidopsis thaliana*]	1×10^{-96}	
PpB49	RD/ZS	6	strictosidine synthase family protein [*Brassica napus*]	4×10^{-56}	
PpB50	RD	5	acyl:coa ligase [*Populus trichocarpa*]	3×10^{-48}	
PpB51	RD	5	chs-like protein [*Populus trichocarpa*]	4×10^{-79}	
PpB52	RD	3	Carboxyl-terminal peptidase, putative, expressed [*Oryza sativa*]	2×10^{-42}	
PpB53	RD/ZS	3	dihydroflavonol 4-reductase, putative [*Arabidopsis thaliana*]	3×10^{-102}	
PpB54	RD	2	carboxyl-terminal proteinase [*Zea mays*]	1×10^{-39}	
PpB55	RD/ZS	2	dihydroflavonol 4-reductase family [*Arabidopsis thaliana*]	2×10^{-37}	
PpB56	RD	1	dihydroflavonol 4-reductase family [*Arabidopsis thaliana*]	3×10^{-36}	
PpB57	RD	1	chalcone synthase family protein [*Arabidopsis halleri* subsp. *gemmifera*]	8×10^{-37}	
PpB58	RD	1	similar to putative polyubiquitin (UBQ10) [*Vitis vinifera*]	2×10^{-26}	
PpB59	RD/SZ	1	xyloglucan endotransglucosylase/hydrolase 5 [*Malus x domestica*]	2×10^{-127}	
PpB60	RD/ZS	1	glucose-methanol-choline (gmc) oxidoreductase, putative [*Ricinus communis*]	1×10^{-64}	
PpB61	RD/ZS	1	acyl:coa ligase [*Populus trichocarpa*]	6×10^{-22}	
PpB62	RD/ZS	1	flavonoid 3-hydroxylase, putative [*Ricinus communis*]	7×10^{-132}	
Oxidation-reduction					
PpB63	RD	41	Peroxidase 40 precursor, putative [*Ricinus communis*]	8×10^{-121}	
PpB64	RD/ZS	22	Peroxidase 9 precursor, putative [*Ricinus communis*]	1×10^{-47}	1,2
PpB65	RD	13	Peroxidase 9 precursor, putative [*Ricinus communis*]	2×10^{-48}	
PpB66	RD	2	alcohol dehydrogenase (ATA1) [*Arabidopsis thaliana*]	5×10^{-83}	
PpB67	RD	1	Peroxidase 9 precursor, putative [*Ricinus communis*]	1×10^{-15}	1
PpB68	RD/ZS	1	Cucumber peeling cupredoxin, putative [*Ricinus communis*]	8×10^{-16}	
Stress and defence					
PpB69	RD/ZS	4	dehydration-responsive protein RD22 [*Prunus persica*]	4×10^{-81}	
PpB70	RD/ZS	1	RD22-like protein [*Vitis vinifera*]	2×10^{-37}	
Signalling and transcription					
PpB71	RD/ZS	3	DNA binding protein, putative [*Ricinus communis*]	6×10^{-88}	
PpB72	RD	1	Transcription factor ICE1, putative [*Ricinus communis*]	2×10^{-10}	
PpB73	RD/ZS	1	DNA binding protein, putative [*Ricinus communis*]	1×10^{-51}	
Transport					
PpB74	RD/ZS	50	MEN-8 protein precursor, putative [*Ricinus communis*]	2×10^{-18}	
PpB75	RD/ZS	3	lipid binding protein, putative [*Ricinus communis*]	9×10^{-25}	
Other					
PpB76	RD/ZS	4	AAA ATPase containing von Willebrand factor type A [*Zea mays*]	1×10^{-10}	
PpB77	RD/SZ	3	tubulin alpha chain, putative [*Ricinus communis*]	1×10^{-158}	1
PpB78	RD/ZS	3	chlorophyll A/B binding protein, putative [*Ricinus communis*]	5×10^{-84}	
PpB79	RD/ZS	3	Rhicadhesin receptor precursor, putative [*Ricinus communis*]	4×10^{-70}	
PpB80	RD/ZS	2	conserved hypothetical protein [*Ricinus communis*]	2×10^{-33}	
PpB81	RD/SZ	1	early nodulin 93 protein [*Populus alba x Populus tremula* var. *glandulosa*]	2×10^{-27}	
PpB82	RD/ZS	1	predicted protein [*Populus trichocarpa*]	9×10^{-68}	
PpB83	RD/ZS	1	MtN3 [*Medicago truncatula*]	1×10^{-76}	1
PpB84	RD/ZS	1	conserved hypothetical protein [*Ricinus communis*]	3×10^{-24}	
PpB85	RD/ZS	1	Blue copper protein precursor, putative [*Ricinus communis*]	2×10^{-42}	
PpB86	RD/ZS	1	mazG nucleotide pyrophosphohydrolase domain protein [*Zea mays*]	5×10^{-39}	
PpB87	RD/ZS	9			
PpB88	RD/ZS	3			
PpB89	RD/ZS	2			
PpB90	RD	1			
PpB91	RD/SZ	1			
PpB92	RD/ZS	1			1,2
PpB93	RD/ZS	1			

The number of ESTs corresponding to each unigene and the Blastx best hit found in the non-redundant protein database from the National Center for Biotechnology Information (NCBI) are shown. ESTs described in previous works are labelled with 1 (Ogundiwin et al., 2008) or 2 (Bassett et al., 2006).

Table 2.2 List of unigenes enriched in the dormant bud sample (DR).

Unigenes	Expression group	EST	Blastx hit	Blastx E-value	Previous works
Metabolism					
PpB1	DR	4	UDP-galactose 4-epimerase [*Cyamopsis tetragonoloba*]	3×10^{-35}	1
PpB2	DR	2	UDP-glucosyltransferase, putative [*Ricinus communis*]	9×10^{-67}	
PpB3	DR	1	Glucan endo-1,3-beta-glucosidase precursor, putative [*Ricinus communis*]	2×10^{-146}	
PpB4	DR/SZ	1	(R)-limonene synthase, putative [*Ricinus communis*]	2×10^{-38}	
PpB5	DR/SZ	1	S-like ribonuclease [*Prunus dulcis*]	1×10^{-113}	
Oxidation-reduction					
PpB6	DR/SZ	1	cytochrome P450, putative [*Ricinus communis*]	2×10^{-102}	
PpB7	DR/SZ	1	cytochrome P450 [*Populus trichocarpa*]	4×10^{-55}	1
Stress and defence					
PpB8	DR	7	multidrug resistance pump, putative [*Ricinus communis*]	5×10^{-34}	
PpB9	DR	4	late embryogenesis abundant, putative [*Ricinus communis*]	8×10^{-08}	1
PpB10	DR/SZ	3	Indole-3-acetic acid-induced protein ARG2, putative [*Ricinus communis*]	1×10^{-15}	1,2
PpB11	DR/ZS	3	type II SK2 dehydrin [*Prunus persica*]	2×10^{-65}	1,2
PpB12	DR	2	multidrug resistance pump, putative [*Ricinus communis*]	5×10^{-104}	
PpB13	DR	1	allene oxide cyclase [*Pisum sativum*]	1×10^{-36}	
PpB14	DR	1	S-adenosylmethionine decarboxylase [*Malus x domestica*]	1×10^{-119}	1
PpB15	DR/ZS	1	heat shock protein, putative [*Ricinus communis*]	1×10^{-97}	
Signalling and transcription					
PpB16	DR/SZ	4	dam5 [*Prunus persica*]	3×10^{-36}	
PpB17	DR/SZ	3	dam6 [*Prunus persica*]	4×10^{-66}	
PpB18	DR	2	NAC domain protein, IPR003441 [*Populus trichocarpa*]	7×10^{-47}	1
PpB19	DR/SZ	2	zinc finger protein [*Camellia sinensis*]	4×10^{-27}	1
PpB20	DR/SZ	2	GRAS family transcription factor [*Populus trichocarpa*]	1×10^{-111}	
PpB21	DR	1	NAC domain protein NAC1 [*Gossypium hirsutum*]	3×10^{-35}	1
PpB22	DR	1	zinc finger protein, putative [*Ricinus communis*]	9×10^{-59}	
PpB23	DR	1	Mitogen-activated protein kinase kinase kinase, [*Ricinus communis*]	7×10^{-60}	1
PpB24	DR	1	transcription factor AP2-EREBP [*Lotus japonicus*]	2×10^{-29}	
PpB25	DR/SZ	1	sensory transduction histidine kinase, putative [*Ricinus communis*]	2×10^{-68}	
PpB26	DR/SZ	1	dam4 [*Prunus persica*]	6×10^{-41}	1
PpB27	DR/SZ	1	GRAS family transcription factor [*Populus trichocarpa*]	5×10^{-78}	
Transport					
PpB28	DR/SZ	8	plasma membrane intrinsic protein 2-2 [*Pyrus communis*]	2×10^{-82}	
PpB29	DR/SZ	2	forisome [*Canavalia gladiata*]	1×10^{-18}	
PpB30	DR/SZ	1	sorbitol transporter [*Prunus cerasus*]	1×10^{-64}	1
PpB31	DR/SZ	1	Mitochondrial carnitine/acylcarnitine carrier protein, [*Ricinus communis*]	3×10^{-26}	
PpB32	DR/SZ	1	sieve element-occluding protein 3 [*Medicago truncatula*]	2×10^{-33}	
Other					
PpB33	DR/ZS	3	pollen coat-like protein [*Arabidopsis thaliana*]	3×10^{-16}	1,2
PpB34	DR	1	peripheral-type benzodiazepine receptor, putative [*Ricinus communis*]	3×10^{-45}	
PpB35	DR	1	predicted protein [*Populus trichocarpa*]	4×10^{-14}	
PpB36	DR	1	indole-3-glycerol phosphate lyase IGL1 [*Lamium galeobdolon*]	9×10^{-55}	
PpB37	DR	1	poly(A)-binding protein C-terminal interacting protein 6 [*Cucumis sativus*]	2×10^{-23}	1
PpB38	DR	1	putative ripening-related protein [*Vitis vinifera*]	4×10^{-31}	
PpB39	DR/SZ	1	Anthranilate N-benzoyltransferase protein, putative [*Ricinus communis*]	4×10^{-60}	
PpB40	DR/SZ	1	3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase, [*Ricinus communis*]	6×10^{-26}	
PpB41	DR/ZS	1	conserved hypothetical protein [*Ricinus communis*]	1×10^{-75}	
PpB42	DR/ZS	1	light harvesting chlorophyll a /b binding protein [*Hedera helix*]	2×10^{-38}	
PpB43	DR/SZ	6			1
PpB44	DR/SZ	4			1,2
PpB45	DR/ZS	1			1,2
PpB46	DR/ZS	1			

The number of ESTs corresponding to each unigene and the Blastx best hit found in the non-redundant protein database from the National Center for Biotechnology Information (NCBI) are shown. ESTs described in previous works are labelled with 1 (Ogundiwin et al., 2008) or 2 (Bassett et al., 2006).

Table 2.4 List of unigenes exclusively enriched in the 'Zincal 5' (ZS) or 'Springlady' (SZ) samples.

Unigenes	Expression group	EST	Blastx hit	Blastx E-value	Previous works
PpB94	SZ	22			
PpB95	SZ	9	UVI4 (UV-B-INSENSITIVE 4) [*Arabidopsis thaliana*]	1×10^{-16}	
PpB96	SZ	2	putative RNA binding protein [*Nicotiana tabacum*]	1×10^{-130}	2
PpB97	SZ	1	sorbitol-6-phosphate dehydrogenase [*Prunus persica*]	4×10^{-50}	
PpB98	SZ	1	small basic intrinsic protein 1 [*Vitis vinifera*]	5×10^{-25}	
PpB99	SZ	1	hypothetical protein [*Vitis vinifera*]	5×10^{-13}	1
PpB100	ZS	2	hypothetical protein [*Vitis vinifera*]	1×10^{-14}	1
PpB101	ZS	2			2

The number of ESTs corresponding to each unigene and the Blastx best hit found in the non-redundant protein database from the National Center for Biotechnology Information (NCBI) are shown. ESTs described in previous works are labelled with 1 (Ogundiwin et al., 2008) or 2 (Bassett et al., 2006).

Such simple functional classification gave rise to interesting observations when relating to SSH groups: The RD unigenes were abundant in metabolism and oxidation-reduction categories, whereas DR group contained numerous unigenes related to stress and defence. On the other hand, the signalling and transport categories were rich in DR and SZ unigenes (Table 2.5).

Table 2.5. Functional classification of unigenes.

Functional classification	DR	RD	ZS	SZ
Metabolism	5	16	7	4
Oxidation-reduction	2	6	2	2
Stress and defence	8	2	4	1
Signalling and transcription	12	3	2	7
Transport	5	2	2	6
Other	14	18	21	11

Real time RT-PCR analysis of selected unigenes

In order to confirm the developmental and genotype-dependent regulation of genes obtained in this study, we selected nine unigenes for quantitative expression analysis on RNA collected from flower buds of 'Springlady' and 'Zincal 5' cultivars at different times during dormancy progression and release. An Actin gene was used as reference and *DAM1* as a control gene showing down-regulated expression during the time interval of the experiment (Figure 2.3). As formerly described by Li et al. (2009), *DAM1* expression level was higher in October sample and dropped to undetectable levels in the February and January samples of 'Springlady' and 'Zincal 5' respectively. In agreement with the hypothesized role of *DAM* genes in maintenance of the dormant state, *DAM1* expression was very similar in both cultivars in dormant samples but drastically decreased in December in 'Zincal

5', roughly coinciding with dormancy release. Coherently, 'Springlady' maintained high levels of *DAM1* transcript until January, when buds break dormancy. Another MADS box transcription factor gene from the same family (*DAM6*), which was obtained in our SSH experiment under the name PpB17, followed an expression pattern different to *DAM1* but also correlated with the distinct chilling requirements of the studied cultivars. *DAM6* cDNA accumulated in November sample and abruptly fell in December in 'Zincal 5', whereas 'Springlady' levels decreased in January.

Other regulatory unigenes coding for a Zn-finger transcription factor (PpB19) and a putative GRAS-like (GIBBERELLIN ACID INSENSITIVE, REPRESSOR of GA1 and SCARECROW) regulator (PpB20) were selected for real time PCR validation. PpB19 expression showed a gradual decrease in 'Zincal 5' genotype from October to January, reaching a final amount of transcript about five times lower than in the first collected sample. However 'Springlady' maintained similar PpB19 values during the same period to finally drop in February. On the other hand, GRAS-like PpB20 led to a particular profile with punctual variations in expression. Unigene PpB29, coding for a forisome-like protein, was also down-regulated during progression and release of bud dormancy, with consistently lower cDNA levels in 'Zincal 5' than in 'Springlady' from November to January. PpB43 unigene, having no relevant similarities to any known gene in databases, maintained a low and constant expression level in 'Zincal 5' from October to January, whereas expression in 'Springlady' was higher and more variable during this period.

On the other hand, unigenes PpB62, PpB71, PpB87 and PpB88, respectively coding for a putative flavonoid 3-hydroxylase, a putative DNA-binding protein and two polypeptides with no homology in databases, showed a similar pattern of mRNA accumulation in January and February, just after budbreak, and much more intense signals in 'Zincal 5' than in the 'Springlady' background. In order to highlight the late increase in expression of these four genes in both genotypes, different graph scales have been employed for 'Zincal 5' and 'Springlady' data in Figure 2.3.

Discussion

Some details on the physiological behaviour of flower buds from 'Zincal 5' and 'Springlady' are shown. The difference in chilling requirement between both cultivars was estimated to be about 100 CH, which is relatively low when considering that certain peach genotypes have requirements higher than 1000 CH. However under the mild climate conditions where this experiment has been performed high chilling genotypes cannot be properly cultivated. Despite this limitation, the selected genotypes clearly showed different dormancy-related behaviour during several years, and

more relevantly, ‘Zincal 5’ but not ‘Springlady’ was able to budbreak to some extent before the fulfillment of its chilling requirement.

The SSH procedure was designed to achieve a list of genes related to dormancy maintenance and release. The DR and RD SSH samples were expected to yield transcripts respectively repressed and induced during the CH accumulation and dormancy release in the ‘Springlady’ background. On the other hand, the ZS and SZ samples were obtained to identify genotype-dependent differences in expression following dormancy release, which could account for some qualitative genetic differences between ‘Zincal 5’ and ‘Springlady’ cultivars related to dormancy release.

A low percentage of the 2496 clones contained in the four SSH libraries could be validated by microarray hybridization of SSH final products. By considering 2 fold differences as a threshold for the signal ratio between forward and reverse probes (M=1), the interval of validated clones ranged from 15 % to 48 %, similar to the 29 % of differentially expressed clones found in a recent SSH work on bud dormancy in apricot (Yamane et al., 2008). These results suggest that simple selection and sequencing of SSH obtained clones could not be a completely reliable procedure to identify differentially expressed genes. Alternatively, such low SSH efficiency may be due to the transcriptional similarity between compared samples, showing a low number of differential transcripts. Under this consideration the comparatively better efficiency of RD library could be due to the existence of a relevant number of up-regulated genes along flower bud development and dormancy release.

Overlapping of unigene groups shows a bias towards the coincidence of RD and ZS enriched unigenes on the one hand and DR and SZ on the other, which in fact confers a high degree of interest to those genes. Whereas unigenes contained exclusively into RD or DR groups could be regulated by flower development processes or by different environmental stresses, and ZS or SZ enriched unigenes could simply correspond to genotype specific genes; RD/ZS and DR/SZ overlapping unigenes are more likely related to bud dormancy due to their double, developmental and genotype dependent regulation.

A rough functional classification of unigenes highly similar to genes described in other species showed evident differences between the four groups. In group RD, sequences related to metabolism and oxidation-reduction were the most abundant, which fits well with the idea that some basic metabolic activation is required to restart growth and cell division following dormancy and with

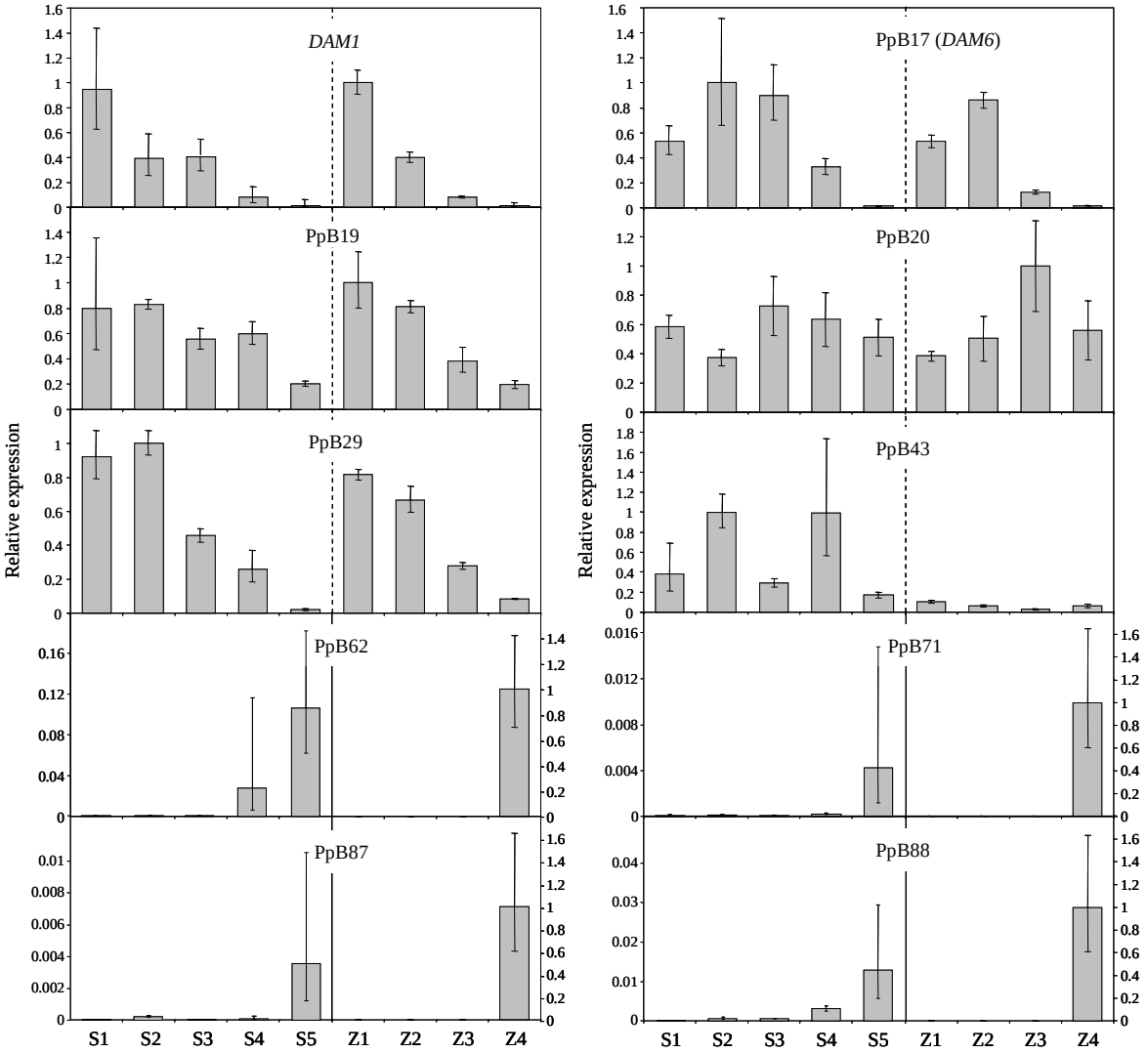

Figure 2.3. Real time RT-PCR analysis of selected unigenes. RNA samples were obtained from 'Springlady' and 'Zincal 5' flower buds harvested during the first days of October (S1 and Z1), November (S2 and Z2), December (S3 and Z3), January (S4 and Z4) and February (S5). Expression levels are relative to actin. An expression value of one is assigned to the sample with a higher accumulation of transcript. Different graph scales have been employed for 'Springlady' and 'Zincal 5' data in unigenes PpB62, PpB71, PpB87 and PpB88. Data are means from two biological replicates, with error bars representing standard deviations.

those previous works linking oxidative stress and dormancy release (Nir et al., 1986; Scalabrelli et al., 1991; Or et al., 2002). On the other side, group DR was rich in stress and defence genes, as dormant tissues must be prepared against drought and cold stresses appearing during winter time. Moreover, the high presence in this group of unigenes related to signalling and transcription suggests that certain developmental processes might be tightly regulated at the transcriptional level in the dormant stage. SZ and ZS groups reproduced respectively DR and RD tendencies in most of the categories.

Some of the genes found in this work were previously identified in a SSH approach to isolate genes regulated by low temperature and photoperiod in peach bark (Bassett et al., 2006), whereas a high percentage of them were isolated from two cDNA libraries enriched in cold treated fruit mesocarp (Ogundiwin et al., 2008). Respectively 9 and 24 unigenes are coincident with these works, coding for a late embryogenesis abundant protein, a dehydrin, two NAC-like transcription factors, a sorbitol transporter and distinct peroxidases among others. Such a high degree of coincidence with works performed on bark and fruit mesocarp suggests that cold and light regulated pathways share common elements between flower buds and these tissues.

The unigenes PpB16, PpB17 and PpB26 are part of six similar genes located in tandem in linkage group 1 (*DAM5*, *DAM6* and *DAM4* respectively), which are related to the early flowering genes *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS LIKE24* (*AGL24*) of *Arabidopsis thaliana*. A deletion affecting these *DAM* genes has been found linked to the dormancy induction defects observed in the *evergrowing* (*evg*) mutant of peach, showing constant growth in terminal meristems and a defect in terminal vegetative bud formation (Bielenberg et al., 2008). Additional evidences suggesting a role of *DAM*-like genes in regulating dormancy transitions have been obtained in transcriptomic studies in raspberry (Mazzitelli et al., 2007), japanese apricot (Yamane et al.,2008) and leafy spurge (Horvath et al., 2008). The real time RT-PCR analysis of *DAM1* and *DAM6* essentially confirms previously published data by Li et al. (2009) with some relevant genotype dependent particularities. *DAM1* and *DAM6* major down-regulation started about one month earlier in 'Zincal 5' that in 'Springlady', which is approximately coincident with their specific chilling requirements for dormancy release. Transcript accumulation of the *DAM6* gene followed that of the *DAM1* gene (Bielenberg et al., 2008; Li et al., 2009) suggesting a kind of sequential regulation between them that deserves further study. This work offers additional evidences on the relevant role of *DAM* genes in delaying dormancy release, but a deeper analysis of *DAM* genes expression and further biotechnological approaches are required to uncover molecular and physiological details on their function. In addition to *DAM*-like genes, SSH analysis of bud dormancy transcriptome of the related species *Prunus mume* (japanese apricot) provided a cytochrome P450 EST (AB437326) similar to PpB7 and two xyloglucan endotransglycosylase ESTs (AB437332, AB437340) related to PpB59 (Yamane et al., 2008). Such low number of coincidencies between species from the common genus *Prunus* may be due to the few ESTs described in the japanese apricot article (26) and to the nature of the SSH procedure that produces adjacent *RsaI* fragments proceeding from the same gene but not matching by BLAST analysis.

Circadian clock and dormancy have been associated by two recent articles in chestnut, showing that *CsTOC1* (*TIMING OF CAB, CHLOROPHYLL A/B BINDING PROTEIN EXPRESSION 1*), *CsLHY* (*LATE ELONGATED HYPOCOTYL*), *CsPRR5*, *CsPRR7* and *CsPRR9* (*PSEUDO-RESPONSE REGULATOR*) genes, coding for homologs to components of the *Arabidopsis* circadian oscillator, lose their circadian daily regulation during winter dormancy and under low temperature incubation (Ramos et al., 2005; Ibáñez et al., 2008). One of the peach unigenes accumulating in dormant buds (PpB25) showed high similarity to *CsPRR7*, which in addition to the observation by Horvath et al. (2008) that several circadian regulatory genes are up-regulated following the paradormancy-endodormancy transition, increases the accumulated evidence that connects circadian clock with dormancy.

In close linkage to circadian mechanisms are the light signal transduction networks triggered by phytochromes. PpB20 and PpB27 unigenes code for putative GRAS transcription factors that could proceed from the same locus and are very similar to the *PAT1* (*PHYTOCHROME A SIGNAL TRANSDUCTION*) gene from *Arabidopsis thaliana*. A truncated version of *PAT1* strongly reduces far-red light signaling mediated by phytochrome A (Bolle et al., 2000). Despite the fact that PpB20 average expression was slightly higher in December, when photoperiod is shorter, no reliable conclusions about the light regulation of this gene could be reached due to the absence of significant difference. Other transcription factors identified in this work have different structural or functional domains, as NAC (PpB18 and PpB21), Zn-finger (PpB19 and PpB22), AP2 (PpB24), AT-hook (PpB71), bHLH (PpB72) and PHD-finger (PpB73). The Zn-finger PpB19 showed an attenuated *DAM1*-like decreasing expression profile after quantitative analysis, which confers to this unigene a high interest for studies of transcriptional regulation related to the dormancy process.

The PpB29 and PpB32 unigenes, up-regulated in dormant buds, are very similar to components of the forisome, a protein aggregate of sieve elements of Fabacean plants that occludes them in a reversible and regulated manner (Noll et al., 2007; Pélissier et al., 2008). Real time analysis of PpB29 showed a gradual drop in expression from December to February, with a significant lower transcript accumulation in the 'Zincal 5' genotype. One attractive idea is that forisome-like structures could be mediating the temporary obstruction of vascular elements to reduce solute exchange and water mobility in dormant buds, but the absence of reports about forisome-like complexes in non-Fabacean plants raises obvious objections. Additional efforts to unravel the activity of these forisome-like elements in other species would help to hypothesize a new role for them in bud dormancy.

Among the unigenes up-regulated in dormancy released buds, abundant peroxidase-like sequences were found (PpB63, PpB64, PpB65 and PpB67). Previously, other peroxidases have been related to the fulfillment of the chilling requirement in *Vitis riparia* buds (Mathiason et al., 2009) and to dormancy release in buds of leafy spurge (Jia et al., 2006). Earlier studies suggest a role of hydrogen peroxide (H_2O_2) in budbreak regulation, based on catalase activity inhibition and H_2O_2 accumulation occurring during the natural or hydrogen cyanamide induced breaking of bud dormancy in grapevine (Nir et al., 1986; Pérez et al., 2008). This points to a putative detoxifying role of peroxidases by degrading H_2O_2 produced during budbreak. Other RD unigenes are most likely involved in flower development pathways, as the ones coding for the anthocyanin biosynthetic enzymes chalcone synthase (PpB51 and PpB57), dihydroflavonol 4-reductase (PpB53, PpB55 and PpB56) and flavonoid 3-hydroxylase (PpB62).

Comparison of microarray data obtained in leafy spurge with previous studies in different species contributed to identify general processes related to bud dormancy, as cold and stress responses, circadian regulation, flowering time, chromatin remodelling and hormone responses (Horvath et al. 2008). Similarity of some of our unigenes to several of those general factors suggests that such interspecific pathways and processes are most likely conserved in peach. For instance ICE1-like transcription factors involved in cold response were identified in at least three dormancy studies in different species and also in this work (PpB72). Similarly, late embryogenesis abundant proteins (PpB9), dehydrins (PpB11), peroxidases (PpB63, PpB64, PpB65 and PpB67), UDP-galactose 4-epimerases (PpB1), chalcone synthases (PpB51 and PpB57), xyloglucan endotransglucosylases (PpB59) and flavonoid 3-hydroxylases (PpB62) among others have been recurrently described in previous articles (Schrader et al., 2004; Jia et al., 2006; Mazzitelli et al., 2007; Rohde et al., 2007; Horvath et al., 2008; Xin et al., 2008; Yamane et al., 2008; Mathiason et al., 2009). Such ESTs and other related to plant dormancy for the first time in this work provide insight into the signals and processes regulating bud dormancy in general, and offer a series of gene sequences susceptible to be used for marker-assisted selection in peach breeding.

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Supplementary data

Supplementary Table S2.1. Accession number of ESTs.

Supplementary Table S2.2 List of primers used in this work.

This material is contained in the CD annexed to this thesis.

3. Histone modifications and expression of *DAM6* gene in peach are modulated during bud dormancy release in a cultivar-dependent manner

Leida C., Conesa A., Llácer G., Badenes M.L. and Ríos G. 2012 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.

Abstract

Bud dormancy release in many woody perennial plants responds to the seasonal accumulation of chilling stimulus. MADS-box transcription factors encoded by *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes in peach (*Prunus persica*) are implicated in this pathway, but other regulatory factors remain to be identified. In addition, the regulation of *DAM* gene expression is not well known at the molecular level.

A microarray hybridization approach was performed to identify genes whose expression correlates with the bud dormancy-related behaviour in ten different peach cultivars. Histone modifications in *DAM6* gene were investigated by chromatin immunoprecipitation in two different cultivars.

The expression of *DAM4-6* and several genes related to ABA and drought stress response correlated with the dormancy behaviour of peach cultivars. The trimethylation of histone H3 at K27 in the *DAM6* promoter, coding region and the second large intron was preceded by a decrease of acetylated H3 and trimethylated H3K4 in the region of translation start, coinciding with repression of *DAM6* during dormancy release.

Analysis of chromatin modifications reinforced the role of epigenetic mechanisms in *DAM6* regulation and bud dormancy release, and highlighted common features with the vernalization process in *Arabidopsis thaliana* and cereals.

Introduction

In different plant lineages, adaptation of flowering time to seasonal fluctuations in temperature has been achieved through similar mechanisms with lineage-specific features. Brassicaceae and cereals avoid premature flowering in the autumn by vernalization which inhibits the transition from the vegetative to the reproductive phase until exposure to a prolonged cold period. Similarly, during autumn and winter many perennial species keep the reproductive tissue in a dormant stage (endodormancy, abbreviated to dormancy in this work) inside specialized organs named buds, which are then activated by a period of chilling in a genotype-dependent fashion. These processes are regulated by a set of related MADS-box transcription factors (Hemming & Trevaskis, 2011).

In *Arabidopsis thaliana*, the MADS-box transcription factor FLOWERING LOCUS C (FLC) prevents the flowering transition by repressing the floral integrator genes *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (Searle et al., 2006). The *FLC* transcript decreases quantitatively during cold exposure by an epigenetic mechanism involving the synthesis of non-coding RNAs and the binding of the Polycomb group (PcG) proteins (De Lucía et

al., 2008; Swiezewski et al., 2009; Heo & Sung, 2011). PcG complexes ensure stable repression of *FLC* after subsequent cell divisions by means of chromatin modifications including trimethylation of histone H3 at lysine 27 (H3K27me3) (Doyle & Amasino, 2009). In cereals, vernalization is controlled by *VERNALIZATION1* (*VRN1*), a *FRUITFULL*-like MADS-box gene that contrarily to *FLC* responds to cold by increasing its expression. Up-regulation of *VRN1* during vernalization is associated with reduced H3K27me3 and increased histone H3 lysine 4 trimethylation (H3K4me3) levels at *VRN1*, a typical mark of transcriptionally active chromatin also found in *FLC*. These histone modifications suggest a participation of PcG-like complexes in repression of *VRN1* prior to winter (Oliver et al., 2009; Hemming & Trevaskis, 2011).

In peach (*Prunus persica*), a set of six tandemly repeated *SHORT VEGETATIVE PHASE*-like MADS-box genes, named *DORMANCY ASSOCIATED MADS-box1-6* (*DAM1-6*), have been found partially deleted in the *evergrowing* (*evg*) mutant showing non-dormant behaviour (Bielenberg et al., 2008). The expression of *DAM* genes is highly dependent on the establishment, maintenance and release of bud dormancy. Photoperiod length and chilling accumulation have been postulated as the major inputs conditioning seasonal fluctuations in the expression of these genes (Li et al., 2009). *DAM1* and *DAM6* are up-regulated during bud growth cessation, following a change from long to short day photoperiod (Jiménez et al., 2010a), and down-regulated during dormancy release in two different peach cultivars (Leida et al., 2010). In a recent work, the expression of *DAM5* and *DAM6* have been found associated to the dormancy status of peach plants treated with prolonged low temperature and with the dormancy-breaking reagent cyanamide (Yamane et al., 2011).

Other *DAM*-like genes showing dormancy-dependent expression in buds have been identified in poplar (Ruttink et al., 2007), raspberry (Mazzitelli et al., 2007), Japanese apricot (Yamane et al., 2008), leafy spurge (Horvath et al., 2008) and blackcurrant (Hedley et al., 2010), suggesting a similar control of bud dormancy in perennial plants. Moreover, the *DAM1* gene of leafy spurge shows altered levels of H3K27me3 and H3K4me3 at two different bud dormancy stages (Horvath et al., 2010) resembling the mechanisms of chromatin regulation observed in *FLC* and *VRN1*.

We have performed a genome-wide search for peach genes related to bud dormancy by hybridizing a cDNA microarray containing bud-enriched probes (Leida et al., 2010) with RNA samples from cultivars showing diverse dormancy behaviour, followed by an expression correlation analysis. The *DAM6* gene identified in this study was subjected to a detailed analysis by localizing histone H3 modifications associated to dormancy release in its promoter and coding region.

Materials and Methods

Plant material and growth conditions

The *Prunus persica* [L.] Batsch cv 'Red Candem', 'Flor Red', 'May Glo', '86-6', 'Precocinho', 'Sunraycer', 'Carolina', 'Crimson Baby', 'Rose Diamond' and 'Big Top' were grown in an orchard located at the Instituto Valenciano de Investigaciones Agrarias (IVIA), in Moncada (Spain), under standard agricultural practices. The samples required for microarray hybridizations were obtained from flower buds collected on 29 December 2008, after a chilling accumulation of 400 h below 7 ºC or chilling hours (CH). Buds were routinely pooled from shoots obtained from three different trees. Flower buds for chromatin immunoprecipitation assays were collected on the following dates of autumn-winter in 2009-2010: 3 November (0 CH), 29 December (276 CH), 12 January (385 CH), 16 February (634 CH), and 2 March (684 CH) for 'Big Top', and 3 November (0 CH), 1 December (50 CH), 15 December (187 CH) and 29 December (276 CH) for 'Red Candem'.

Incubation of shoots for the determination of bud break and dormancy parameters was performed in a phytotron set at 25 ºC, with a 12/12 h photoperiod and 70 % humidity.

Measurement of flowering time, bud break and chilling requirement

The flowering time of a certain cultivar in the field corresponded to the date in which at least half of flowers were fully open. This measurement was made relative to the earliest flowering date of 'May Glo', and expressed in days.

For measuring the percentage of bud break, 10 excised shoots with four to six flower buds remaining in their upper half were placed with their basal end in water and incubated in a phytotron. The basal ends of the shoots were cut and water was replaced every 2 to 3 d. Bud break was measured as the percentage of open flower buds, showing at least the green tip of the sepals, after 10 d incubation. Those cultivars with percentage of bud break < 50 % were considered to remain in dormant stage.

For estimating the chilling requirement, bud break was measured periodically during the cold season. Chilling requirement was the time in hours below 7 ºC (CH) recorded for a given cultivar when its percentage of bud break exceeded 50 %.

Isolation of RNA and mRNA purification

For microarray hybridization, total RNA was isolated from 1.5 g of flower buds by a guanidine thiocyanate-based protocol (Salzman et al., 1999). Poly(A)+ RNA was subsequently purified using the Oligotex mRNA Purification System (Qiagen) from 180 to 250 μg of total RNA, according to the manufacturer's instructions. The poly(A)+ RNA was concentrated by precipitation with two volumes of ethanol, in the presence of 33 mM NaCl and GlycoBlue Coprecipitant (Ambion), then washed with ethanol 80 % (v/v), and dissolved in RNase-free water. The poly(A)+ RNA concentration in the solution was measured with the Quant-iT RiboGreen RNA Assay Kit (Invitrogen).

For quantitative real-time reverse transcription PCR (RT-PCR) experiments, total RNA was isolated from 100 mg of flower buds using the RNeasy Plant Mini Kit (Qiagen), but adding 1 % (w/v) polyvinylpyrrolidone (PVP-40) to the kit extraction buffer before use.

Microarray hybridization

The poly(A)+ RNA (100 ng) was reverse transcribed, amplified and labelled with the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). Cy5 and Cy3 fluorescent dyes were coupled to the amino allyl modified RNA of each sample and a mix of the samples (for reference), respectively. Purified Cy5-labelled sample and Cy3-labelled reference (200 pmol each) were combined, diluted with water to a final volume of 500 μl, and concentrated until 40 μl in a microcon YM-30 filter (Millipore). Half of the mixture (20 μl) was vacuum-concentrated until about 4 μl, then heat-denatured for 2 min at 80 ºC, mixed with 20 μl of pre-heated hybridization buffer [5X SSC (75 mM trisodium citrate pH 7.0, 0.75 M NaCl), 50 % (v/v) formamide, 0.1 % (w/v) SDS, 0.1 mg mL^{-1} salmon sperm DNA], and finally applied to the microarray slide, which was previously incubated for at least 1 h at 42 ºC with prehybridization buffer [5X SSC, 0.1 % (w/v) SDS, 1 % (w/v) bovine serum albumin]. The microarray contained 2496 expressed sequence tags (ESTs) obtained by a subtraction procedure from dormant and non-dormant flower buds of peach, as described by Leida et al. (2010). Hybridization was performed overnight at 42 ºC. After hybridization, slides were washed twice at 42 ºC for 5 min in 2X SSC-0.1 % (w/v) SDS, followed by two washes at room temperature for 5 min in 0.1X SSC-0.1 % (w/v) SDS, then five washes at room temperature for 3 min in 0.1X SSC, and finally rinsed briefly with 0.01X SSC before drying by centrifugation at 300 rpm for 5 min.

Microarray data analysis

Arrays were scanned at 5-μm resolution. Cy3 and Cy5 fluorescence intensity was recorded by using a ScanArray Gx scanner (Perkin Elmer). The resulting images were overlaid and spots identified by the ScanArray Express program (Perkin Elmer). Spot quality was confirmed by visual test. Microarray and experiment data have been placed in ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/), with accession number E-MEXP-3201.

For statistical analysis of microarray data, the Gene Expression Profile Analysis Suite (GEPAS) package was employed (Montaner et al., 2006; http://www.gepas.org/). Normalizations within and between arrays were Loess and Scale, respectively. Signal intensities proceeding from duplicated spots were averaged. Identification of ESTs correlating with the different dormancy variables was performed by Pearson's test. The P-value to control the false discovery rate was adjusted with the Benjamini and Hochberg method. ESTs showing a P-value lower than 0.05 were selected for DNA sequencing with primers NP1 or NP2R (Table S3.3) and expression analysis. From 160 ESTs correlating with the chilling requirement variable, 46 showed a higher signal in dormant buds (positive correlation) and 114 in dormancy released buds (negative correlation). With respect to the percentage of bud break, 201 ESTs were found, of which 30 were more abundant in dormant buds and 171 in dormancy released buds. Similarly, from 154 ESTs correlating with the flowering time variable, 58 had a higher expression in dormant buds and 96 in dormancy released buds. After removing the clones producing a bad sequence and the ones having an internal *Rsa*I site, which was indicative of a chimeric rearrangement during the subtraction procedure, 242 ESTs were identified. The accession numbers of these ESTs are listed in Table S3.2. To identify the genes or transcript models containing the positive ESTs, a BLASTN analysis (Altschul et al., 1990) was performed on peach genome sequence database released by the International Peach Genome Initiative (IPGI). A BLASTP analysis of the deduced protein of the different genes or ESTs was made on the non-redundant protein sequence database, to find the closest annotated hits.

To identify the varieties with similar gene expression fingerprints we applied principal component analysis to the initial matrix of 2525 available data considering the 10 varieties as individuals and the genes as variables. The analysis was performed using Statgraphics 5.1 package for windows (Statistical Graphics).

Real-time RT-PCR

One microgram of total RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) in a total volume of 20 µl. Two microliter of a 40X diluted first-strand cDNA was used for each amplification reaction in a final volume of 20 µl. Quantitative real-time PCR was performed on a StepOnePlus Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primers shown in Table S3.3. Cycling protocol consisted of 10 min at 95 ºC, followed by 40 cycles of 15 s at 95 ºC for denaturation, and 1 min at 60 ºC for annealing and extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve after the amplification and through size estimation of the amplified product by agarose electrophoresis. We used as reference a peach actin gene amplified with specific primers (Table S3.3). Relative expression was measured by the relative standard curve procedure. Results were the average of two independent biological replicates repeated twice.

Chromatin immunoprecipitation (ChIP) assays

Cross-linking of 4 g of flower buds, and chromatin isolation and sonication were performed according to Saleh et al. (2008), with the following few modifications. For chromatin isolation, we added 5 ml of nuclei isolation buffer to 1 g of cross-linked frozen material. After homogenization and centrifugation at 11,000g for 20 min, we additionally washed the pellet with 5 ml of nuclei isolation buffer. The chromatin was resuspended in 0.5 ml of nuclei lysis buffer and the DNA sheared into fragments of about 500 bp (100 to 1000 bp interval) by sonicating 5 times for 10 s with 37 % amplitude, on a Vibra-Cell VCX-500 sonicator (Sonics and Materials). Protease inhibitor cocktail (PIC) for plant cell and tissue extracts (Sigma) was added to nuclei isolation buffer and nuclei lysis buffer, to a final concentration of 0.5 % (v/v) and 1 % (v/v) respectively. PVP-40 was added to nuclei isolation buffer (1 % w/v) shortly before use.

ChIP was performed according to Sandoval et al. (2004) and Ferres-Maso et al. (2009). The sonicated chromatin was centrifuged at 13,800g for 10 min, and the supernatant diluted 10-fold with dilution buffer [16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 0.01 % (w/v) SDS, 1.1 % (v/v) Triton X-100, 1.2 mM EDTA, 0.5 % (v/v) PIC]. Aliquots of 600 µl of diluted chromatin were incubated overnight at 4 ºC on a rotating platform with Dynabeads-Protein G (Invitrogen) previously washed with PBS buffer (1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl, 5 mg mL^{-1} bovine serum albumin) and saturated with 2 µg of the different antibodies. The employed antibodies were anti-trimethyl-histone H3 (Lys4) [07-473], anti-trimethyl-histone H3 (Lys27) [07-449], and

anti-acetyl-histone H3 [06-599] from Millipore, and anti-histone H3 [ab1791] from Abcam. Immunocomplexes were recovered using a DynaMag-2 magnetic particle concentrator (Invitrogen). Samples were washed twice with cold low-salt buffer [50 mM Hepes pH 8.0, 140 mM NaCl, 1 % (v/v) Triton X-100, 0.1 % (w/v) sodium deoxycholate, 1 mM EDTA], twice with cold high-salt buffer [50 mM Hepes pH 8.0, 500 mM NaCl, 1 % (v/v) Triton X-100, 0.1 % (w/v) sodium deoxycholate, 1 mM EDTA], twice with cold LiCl buffer [10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate, 1 mM EDTA], and finally once with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Washings were performed at 4 ºC for 4 min, under rotation. After discarding TE buffer, the immunoprecipitated chromatin was eluted from the Dynabead-protein G by adding 50 µl of elution buffer [98 mM $NaHCO_3$, 1 % (w/v) SDS], vortexing and incubating for 10 min at 65 ºC. A second elution step with additional 50 µl of elution buffer was performed. Formaldehyde cross-linking was reversed by incubating overnight at 65 ºC in the presence of proteinase K (0.4 mg mL^{-1}). DNA was purified with High Pure PCR Product Purification Kit (Roche) and resuspended in 100 µl of 10 mM Tris-HCl pH 8.0. Chromatin cross-linking and ChIP was made on two independent samples per condition.

Two microliter of DNA was used for each amplification reaction in a final volume of 20 µl. Quantitative real-time PCR was performed on a StepOnePlus Real-Time PCR System using PerfeCTa SYBR Green SuperMix ROX (Quanta Biosciences) and primers shown in Table S3.3. Cycling protocol consisted of 10 min at 95 ºC, followed by 45 cycles of 15 s at 95 ºC, and 1 min at 60 ºC. Specificity of the PCR reaction was assessed as above. The relative standard curve procedure was utilized for quantification. Results were made relative to the anti-histone H3 reaction. Results were the average of three PCR reactions for each sample.

Figure 3.1. Flower developmental changes during dormancy progression and bud break in peach. Two dormant flower buds flanking a vegetative bud are shown on the left panel. In the middle, several swollen flower buds are opening and starting to show the green sepals on their tips. A fully open flower is shown on the right.

Results

Dormancy-related behaviour of ten different peach cultivars

The particular response to climatic and environmental factors affecting bud dormancy in ten peach cultivars was studied by three related methods. The flowering time method integrated complex intrinsic and extrinsic inputs, such as cultivar specific chilling and heat requirements for endodormancy and ecodormancy releases, and the influence of light and meteorological conditions. On the other hand, the measurement of chilling requirement did not account in principle the ecodormancy-related factors and was thus expected to better respond to particular mechanisms overcoming winter. Finally, the percentage of bud break method, measured in samples exposed to 400 CH, allowed an additional estimation of cultivar-specific depth of dormancy in a fixed time condition. The peach phenological stages more relevant to this study are shown in Figure 3.1, depicting dormant buds, opening buds showing the green sepals utilized for chilling requirement and bud break estimation, and fully open flowers employed for flowering time determination.

Table 3.1. Dormancy variables measured for peach cultivars.

Cultivar	Chilling requirement (CH)	Bud break (%)[a]	Flowering time (d)[b]
'Red Candem'	<278	76.0	4
'Flor Red'	<278	82.1	11
'May Glo'	278-385	86.1	0
'86-6'	278-385	54.2	9
'Precocinho'	385-412	68.3	7
'Sunraycer'	385-412	66.7	9
'Carolina'	412-511	21.1	11
'Crimson Baby'	412-511	35.6	14
'Rose Diamond'	631-639	0.0	28
'Big Top'	674-712	0.0	39

[a]Measured after 10 d incubation of 400 CH shoots. [b]Days after 'May Glo'.

The three methods gave essentially different measurements, generating three distinct grading of cultivars according to their dormancy behaviour (Table 3.1). However, comparative analysis of flowering time, chilling requirement and percentage of bud break variables supported an overall classification of cultivars into three major groups. The earliest cultivars 'Red Candem', 'Flor Red',

'May Glo', '86-6', 'Precocinho' and 'Sunraycer' were not consistently separated by the three methods and showed similar responses to dormancy releasing factors. The varieties 'Rose Diamond' and 'Big Top' were clearly later than the rest of cultivars. Finally, a third group containing 'Carolina' and 'Crimson Baby' had an intermediate behaviour between the early and late groups. This broad classification was useful for a general overview of the plant material studied, however the different nature of the three methods precluded their fusion in a unique joint source of data. Instead of that, the three sets of measurements were utilized independently.

Identification of genes whose expression correlates with cultivar-dependent differences in dormancy

To search for genes responding to bud dormancy stage in peach, we compared gene expression in flower buds excised from the different cultivars at a fixed date, using a custom microarray. Buds were collected after 400 CH, which was supposed to be an intermediate value among the chilling requirements of the studied cultivars. The corresponding percentage of bud break after 10 d ranged from 0 % ('Rose Diamond' and 'Big Top') to 86.1 % ('May Glo'), a nearly uniform distribution that ensured the utilization of flower buds with diverse degrees of dormancy (Table 3.1). RNA obtained from these buds was labelled and hybridized to a microarray slide containing a set of dormancy-related cDNAs obtained by suppression subtractive hybridization (SSH) in Leida et al. (2010).

In order to determine whether the overall gene expression was conditioned by cultivar-specific factors, a principal component analysis was performed, showing that more than 60 % of the variability in the original data was explained by two principal components. Component two divided the varieties according to their dormancy stage, conferring positive values to the group of six early cultivars and negative values to the others (Figure 3.2). In addition, the intermediate 'Carolina' and 'Crimson Baby', and the relatively late 'Rose Diamond' and 'Big Top' varieties were placed closely, reproducing properly the three-groups classification described above. This result suggested that the dormancy stage of collected buds had a wide influence on gene expression, which in fact validates the use of this transcriptomic approach for identification of dormancy related genes.

A correlation analysis of hybridization signals and the three dormancy variables shown in Table 3.1 was performed using a false discovery rate of 0.05 (Table S3.1). Both positive and negative correlations were obtained for ESTs, which were either more or less abundant in dormant tissues.

Following the guidelines outlined in Materials and Methods, 242 ESTs were finally identified (Table S3.2).

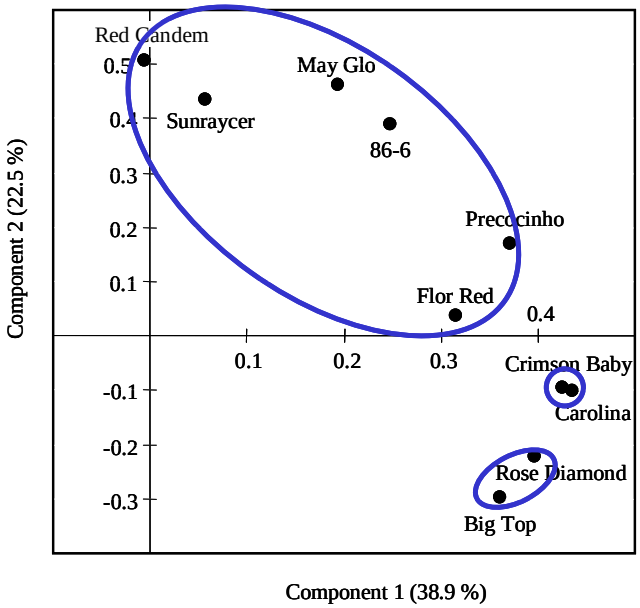

Figure 3.2. Principal component analysis of ESTs signal ratio for the different cultivars assayed. The percentage of the explained variance is shown in parentheses.

A Venn diagram representing the number of ESTs whose expression level correlated with each of the three dormancy-related variables is shown in Figure 3.3(a). Both positive and negative correlations were accounted. The results showed the high degree of overlap between them, with 59 ESTs (from a total of 242) common to chilling requirement, percentage of bud break and flowering time variables, and 39 coincident ESTs in chilling requirement and percentage of bud break analyses. In conclusion, the three variables offered similar results with certain particularities that should be taken into consideration. The hybridization signals of ten ESTs showing better positive and negative correlation with the chilling requirement were respectively plotted in Figure 3.3(b,c) to illustrate their overall cultivar dependence.

Six of these 242 ESTs did not match any of predicted gene models in the peach genome database released by the International Peach Genome Initiative (http://www.rosaceae.org/node/365). Also, two sequences corresponding to the same cDNA were not present in the peach v1.0 genome assembly. The rest of the ESTs matched to 68 transcript models; 45 of them were associated with a higher expression level in dormant buds, and the remaining 23 with higher expression in dormancy released buds.

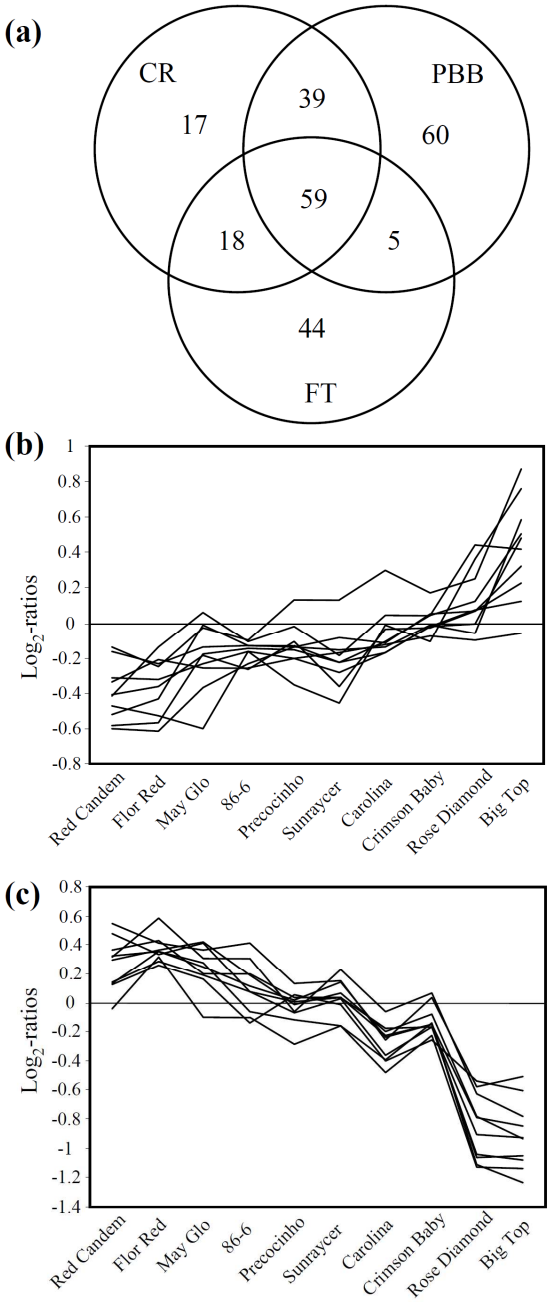

Figure 3.3. Analysis of ESTs correlating with bud dormancy variables. A Venn diagram showing the number of ESTs found under a false discovery rate of 0.05 for chilling requirement (CR), percentage of bud break (PBB) and time to flowering (FT) parameters (a). The normalized log2 signal ratios of the ten ESTs having better positive (b) and negative (c) correlation values for chilling requirement are plotted.

Table 3.2. Genes showing higher expression in dormant buds.

Transcript name	Representative EST	No. of ESTs	BLASTP hit	BLASTP hit annotation	*E* value	Variable[a]
ppa008651m	GR410432	4	1601521B	LEA D-29 gene	6×10^{-36}	FT
ppa010714m	JK006283	4	ABJ96360	DAM6 [*P. persica*]	2×10^{-132}	FT
ppa014312m	JK006309	4	ACG24938	Hypothetical protein [*Zea mays*]	9×10^{-25}	CR, FT
ppa010822m	GR410442	3	ABJ96359	DAM5 [*P. persica*]	2×10^{-128}	CR, PBB, FT
Not found	GR410720	2				PBB, FT
ppa005514m	JK006287	2	AAC49658	Dehydrin [*P. persica*]	3×10^{-169}	CR, FT
ppa009007m	GR410685	2	ABQ45405	Sorbitol-6-phosphate dehydrogenase [*P. persica*]	0	FT
ppa010086m	JK006300	2	EEF35690	Ferritin, putative [*Ricinus communis*]	2×10^{-95}	CR
ppa011123m	GR410688	2	ABJ96358	DAM4 [*P. persica*]	2×10^{-122}	CR, PBB
ppa011831m	JK006295	2	CAB85625	Putative ripening-related protein [*Vitis vinifera*]	8×10^{-67}	CR
ppa012373m	GR410435	2	ABI31653	Zinc finger protein [*Camellia sinensis*]	2×10^{-62}	CR, FT
ppa001989m	JK006292	1	AAL91171	Low-temperature-induced 65 kD protein [*A. thaliana*]	2×10^{-28}	PBB, FT
ppa002102m	JK006378	1	AAQ23899	RSH2 [*Nicotiana tabacum*]	0	PBB, FT
ppa003017m	JK006373	1	NP_179869	AtGRF1 (GROWTH-REGULATING FACTOR 1) [*A. thaliana*]	2×10^{-97}	PBB
ppa003327m	JK006380	1	CAA48630	4-alpha-glucanotransferase precursor [*Solanum tuberosum*]	0	PBB
ppa005713m	JK006285	1	AAK96816	Putative B-box zinc finger protein [*A. thaliana*]	7×10^{-75}	FT
ppa005802m	JK006375	1	NP_194274	ZFWD1 (zinc finger WD40 repeat protein 1) [*A. thaliana*]	7×10^{-124}	CR
ppa006008m	JK006366	1	NP_564673	Peptidoglycan-binding LysM domain-containing protein [*A. thaliana*]	8×10^{-13}	CR, PBB
ppa006974m	JK006374	1	NP_564956	AFP (ABI FIVE BINDING PROTEIN) [*A. thaliana*]	2×10^{-62}	CR, PBB
ppa007137m	JK006313	1	AAG01381	Alcohol dehydrogenase 1 [*Vitis vinifera*]	0	CR, PBB, FT
ppa007415m	JK006372	1	XP_002266388	Similar to B2 protein [*Vitis vinifera*]	6×10^{-118}	PBB
ppa007606m	JK006297	1	ADE41131	AP2 domain class transcription factor [*Malus x domestica*]	2×10^{-103}	CR, FT
ppa007666m	JK006369	1	EEF30918	Palmitoyl-protein thioesterase 1 precursor, putative [*Ricinus communis*]	7×10^{-124}	CR, PBB
ppa008311m	JK006299	1	ABI34650	bZIP transcription factor bZIP68 [*Glycine max*]	4×10^{-44}	CR
ppa008849m	JK006284	1	ACF06448	Annexin [*Elaeis guineensis*]	1×10^{-129}	FT
ppa008859m	JK006379	1	ACM45713	Class I chitinase [*Pyrus pyrifolia*]	2×10^{-148}	FT
ppa008979m	JK006331	1	EEF52342	R2R3-MYB transcription factor, putative [*Ricinus communis*]	8×10^{-92}	FT
ppa009032m	JK006286	1	EEF52567	2-hydroxyacid dehydrogenase, putative [*Ricinus communis*]	6×10^{-109}	CR, FT
ppa009498m	JK006370	1	EEF42166	Homeobox protein, putative [*Ricinus communis*]	4×10^{-136}	CR, PBB
ppa010299m	JK006382	1	BAG09366	Peroxisomal short-chain dehydrogenase/reductase family protein [*Glycine max*]	1×10^{-118}	CR, PBB, FT
ppa010931m	JK006367	1	ABN08437	Ribosomal protein L10 [*Medicago truncatula*]	6×10^{-98}	FT
ppa011776m	JK006357	1	EEF50502	Remorin, putative [*Ricinus communis*]	2×10^{-60}	CR
ppa012188m	JK006293	1	NP_563710	AWPM-19-like membrane family protein [*A. thaliana*]	1×10^{-72}	FT
ppa012329m	JK006304	1	EEF35031	Transcription initiation factor iia (tfiia), gamma chain, putative [*Ricinus communis*]	4×10^{-55}	FT
ppa012578m	JK006359	1	EEF30224	Conserved hypothetical protein [*Ricinus communis*]	3×10^{-46}	CR
ppa012801m	JK006290	1	NP_195570	ATFP6 (FARNESYLATED PROTEIN 6) [*A. thaliana*]	8×10^{-69}	CR
ppa012915m	JK006288	1	CBY94070	Early responsive to dehydration [*Fagus sylvatica*]	5×10^{-41}	CR, PBB
ppa013063m	JK006291	1	NP_197518	Ribosomal protein L36 family protein [*A. thaliana*]	7×10^{-28}	CR, PBB
ppa013625m	JK006296	1	NP_568818	Eukaryotic translation initiation factor SUI1, putative [*A. thaliana*]	2×10^{-49}	CR
ppa013723m	JK006360	1	EEF34837	Small nuclear ribonucleoprotein sm d2, putative [*Ricinus communis*]	3×10^{-51}	CR, PBB, FT
ppa014118m	JK006303	1	AAK73280	Drought-induced protein [*Retama raetam*]	1×10^{-3}	FT
ppa014358m	JK006376	1	EEF30268	Conserved hypothetical protein [*Ricinus communis*]	3×10^{-24}	FT
ppa015914m	JK006294	1	ABK94181	Unknown [*Populus trichocarpa*]	1×10^{-07}	FT
ppa017425m	JK006335	1	NP_193292	MAA3 (MAGATAMA 3) [*A. thaliana*]	0	FT
ppa020191m	JK006368	1	BAG80556	UDP-glucose:glucosyltransferase [*Lycium barbarum*]	4×10^{-149}	CR, PBB, FT
ppa024188m	JK006365	1	NP_564673	Peptidoglycan-binding LysM domain-containing protein [*A. thaliana*]	3×10^{-36}	CR, PBB
Not found	JK006298	1				CR, PBB
Not found	JK006306	1				CR

[a]Variables correlating with the expression of at least one EST of the gene.

Table 3.2 lists transcript models and ESTs with increased expression in dormant buds of the late cultivars. A gene coding for a putative late embryogenesis abundant protein (LEA) was previously reported to depend on the dormancy stage (Leida et al., 2010), and identified also in a transcriptomic approach defining peach mesocarp genes affected by chilling (Ogundiwin et al., 2008). Other genes associated with dormancy in peach are: ppa005514m, coding for a dehydrin from bark tissue that has been described to have a restricted expression pattern in dormancy-defective genotypes of peach (Artlip et al., 1997); and *DAM4-6* genes, part of a six-member gene family of MADS-box transcription factors that have been postulated to cause dormancy-related alterations in the *evg* mutant of peach (Bielenberg et al., 2008). The presence of seven additional genes coding for putative transcription factors (ppa003017m, ppa005713m, ppa007606m, ppa008311m, ppa008979m, ppa009498m and ppa012329m) suggests that multiple regulation pathways are involved in dormancy maintenance and bud development processes.

Table 3.3. Genes showing higher expression in non-dormant buds.

Transcript name	Representative EST	No. of ESTs	BLASTP hit	BLASTP hit annotation	*E* value	Variable[a]
ppa020321m	JK006332	69	EEF52630	Peroxidase 9 precursor, putative [*Ricinus communis*]	3×10^{-146}	CR, PBB, FT
ppa008309m	GR410503	27	ABW82528	Class III peroxidase [*Gossypium hirsutum*]	9×10^{-119}	CR, PBB, FT
ppa020886m	GR410508	26	EEF51430	MEN-8 protein precursor, putative [*Ricinus communis*]	5×10^{-21}	CR, PBB
ppa018509m	GR410669	9	EEF49202	Lipid binding protein, putative [*Ricinus communis*]	3×10^{-32}	CR, PBB
ppa008548m	GR410674	5	EEF38791	Cinnamoyl-CoA reductase, putative [*Ricinus communis*]	4×10^{-153}	PBB
ppa017856m	GR410555	5	ACG41003	Carboxyl-terminal peptidase [*Zea mays*]	8×10^{-86}	CR, PBB, FT
ppa005535m	JK006364	4	AAL26909	Dehydration-responsive protein RD22 [*P. persica*]	1×10^{-81}	CR, PBB, FT
ppa005767m	JK006334	4	AAL26909	Dehydration-responsive protein RD22 [*P. persica*]	6×10^{-82}	CR, PBB, FT
ppa006739m	GR410750	3	EEF45922	Dehydration-responsive protein RD22 precursor, putative [*Ricinus communis*]	1×10^{-78}	CR, PBB, FT
ppa014645m	GR410516	3	ACG41003	Carboxyl-terminal peptidase [*Zea mays*]	1×10^{-84}	CR, PBB, FT
ppa025857m	GR410576	3	NP_177530	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein [*A. thaliana*]	1×10^{-20}	PBB, FT
Not found	JK006315	3				CR, PBB
ppa009789m	GR410684	2	NP_196821	SAG29 (SENESCENCE-ASSOCIATED PROTEIN 29) [*A. thaliana*]	2×10^{-85}	PBB
ppa021109m	JK006325	2	EEF51426	Nonspecific lipid-transfer protein precursor, putative [*Ricinus communis*]	9×10^{-32}	PBB
ppa003039m	JK006342	1	EEF48818	Proteasome-activating nucleotidase, putative [*Ricinus communis*]	0	CR, PBB
ppa003411m	JK006371	1	EEF32187	L-ascorbate oxidase, putative [*Ricinus communis*]	0	CR, PBB, FT
ppa003797m	GR410504	1	EEE82643	Acyl:coa ligase [*Populus trichocarpa*]	0	FT
ppa004872m	JK006302	1	XP_002268893	Hypothetical protein [*Vitis vinifera*]	0	CR, PBB, FT
ppa006506m	GR410648	1	AAO42227	Putative strictosidine synthase [*A. thaliana*]	0	CR
ppa006852m	JK006362	1	EEE85993	Chs-like protein [*Populus trichocarpa*]	0	CR
ppa010924m	JK006328	1	NP_181525	Microsomal signal peptidase 25 kDa subunit, putative (SPC25) [*A. thaliana*]	1×10^{-71}	CR, FT
ppa020936m	JK006318	1	AAL26909	Dehydration-responsive protein RD22 [*P. persica*]	5×10^{-94}	FT
ppa025137m	JK006336	1	XP_002277756	Hypothetical protein [*Vitis vinifera*]	2×10^{-22}	CR, PBB, FT
ppb012876m	GR410653	1	EEF42354	Conserved hypothetical protein [*Ricinus communis*]	3×10^{-38}	CR, PBB
Not found	JK006311	1				PBB

[a]Variables correlating with the expression of at least one EST of the gene.

Transcripts accumulated in buds after dormancy release are shown in Table 3.3. The transcript models of ppa020321m and ppa008309m, encoding peroxidase-like proteins, were the most represented genes in the experiment, with 69 and 27 ESTs respectively. Up-regulation of these and other related peroxidase genes has been reported in non-dormant buds of peach (Leida et al., 2010), coincidently with bud-break induced by chilling accumulation in *Vitis riparia* (Mathiason et al., 2009), and during dormancy release in leafy spurge (Jia et al., 2006). Peroxidases have been proposed to counteract the production of H_2O_2, a signal molecule exerting a dormancy breaking effect in grapevine buds and *A. thaliana* seeds (Pérez et al., 2008; Liu et al., 2010). Other transcripts related to dormancy release in Table 3.3 coded for putative lipid-transfer proteins (ppa020886m, ppa018509m, ppa025857 and ppa021109m), peptidases (ppa017856m, ppa014645m and ppa010924m), and dehydration-responsive proteins (ppa005535m, ppa005767m, ppa006739m and ppa020936m).

Real-time PCR validation of microarray data

Several genes selected from Tables 3.2 and 3.3 were analyzed by quantitative real-time RT-PCR using bud samples after 400 CH (Figure 3.4). Genes from the tandemly repeated family of MADS box transcription factors *DAM4*, *5* and *6* showed a pattern of higher expression in 'Rose Diamond' and 'Big Top' cultivars, and lower expression in early cultivars, with maximum differences of about 100 fold. Genes ppa008651m, ppa012373m, ppa006974m, ppa007606m, ppa009498m and ppa012188m, listed in the Table 3.2 of dormancy-related transcripts, showed an expression profile similar to the *DAM* genes, but with much lower differences between cultivars. Five of these genes showed a slightly higher expression level in 'Flor Red' and 'Precocinho' compared to other cultivars with proximate dormancy behaviour.

The most striking feature of transcripts associated to dormancy release in Table 3.3 is their almost null expression in the late varieties 'Rose Diamond' and 'Big Top' (Figure 3.4). Interestingly, three of these genes (ppa020886m, ppa018509m and ppa008548m) showed a specific pattern of overexpression in 'Red Candem', '86-6' and 'Sunraycer', resembling cultivar-specific alterations described above for five dormancy-related genes, which supports an involvement of common transcription regulatory mechanisms in case of a subset of genes.

DAM6 expression decreases concomitantly with dormancy release

DAM proteins are the major known regulatory factors of bud dormancy processes, based on the analysis in the *evg* mutant of peach and different genomic studies in other species (Bielenberg et al., 2008; Horvath et al., 2008). From the six *DAM* genes, we chose *DAM6* for subsequent expression analysis due to its recurrent identification in peach-dormancy genomic approaches (Jiménez et al., 2010a; Leida et al., 2010), and a close correlation of its expression with dormancy release and bud break found by Jiménez et al. (2010b), Yamane et al. (2011), and in this work.

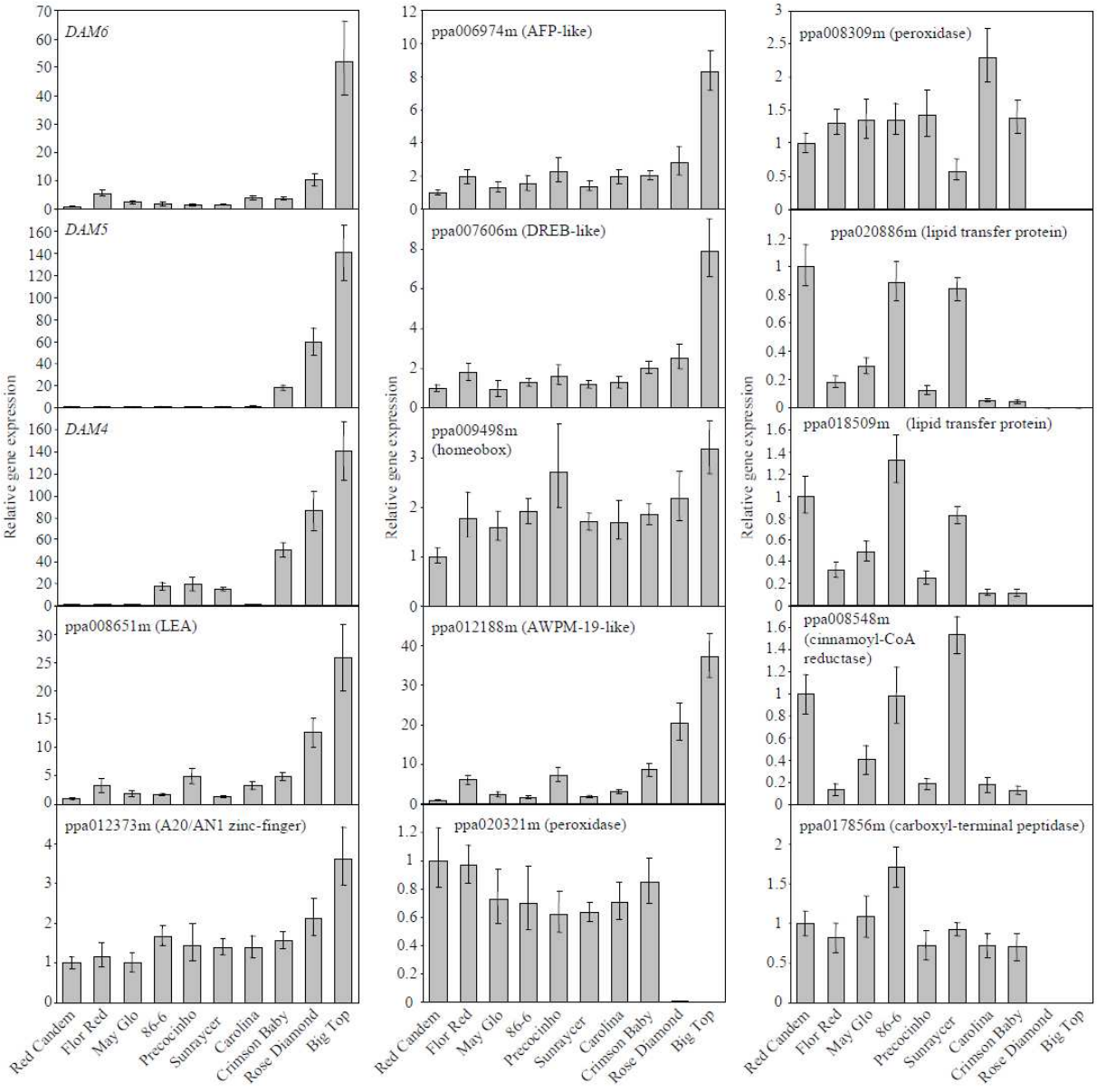

Figure 3.4. Real-time RT-PCR of selected genes. RNA samples were obtained from flower buds harvested after 400 chilling hours (CH). The name of the gene or transcript model is shown in the upper left corner of the graph. Expression levels are relative to actin. An expression value of one is assigned to the 'Red Candem' sample. Data are means from two biological replicates, with error bars representing standard deviations.

We measured *DAM6* expression in two representative cultivars of early ('Red Candem') and relatively late ('Big Top') cultivars at different dates during bud dormancy release. In both cultivars, *DAM6* transcript level was strongly reduced coincidently with a rise in growth competence of flower buds measured in the bud break assay (Figure 3.5a,b). Due to their distinct behaviour with respect to dormancy, such bud growth competence occurred after very different periods of chilling accumulation, 276 CH for 'Red Candem' and 684 CH for 'Big Top'. Thus, in agreement with previous works, *DAM6* expression was correlating well with the dormancy release stage of two different cultivars. An expression peak was observed in 'Red Candem' after 50 CH, which could be due to the combination of light and chilling effects, following a peaked pattern similar to semi-quantitative measurements of *DAM6* expression in Li et al. (2009).

H3K4me3 and acetylated H3 around the translation start of *DAM6* decrease during gene repression and dormancy release

We conducted a ChIP assay in order to define histone modifications of *DAM6* chromatin during dormancy release. Three genomic fragments corresponding to *DAM6* promoter ('PR'), the translation start site ('ST'), and a region of second large intron ('IN') were selected for ChIP analysis (Figure 3.5c) using 'Big Top' and 'Red Candem' bud samples shown in Figure 3.5(a,b). Real-time quantitative PCR data were standardized to histone H3 levels to calculate the relative ratio of modified H3 shown in Figure 3.5(d,e).

H3K4me3 was in general more abundant in the 'ST' region than the upstream promoter and downstream intron in both cultivars, suggesting that the zone around the translational start could be susceptible to regulation (Figure 3.5d,e). Indeed, H3K4me3 was reduced in 'Big Top' buds in parallel to dormancy release in two independent experiments, reproducing accurately *DAM6* down-regulation shown in Figure 3.5(a). A significantly lower rate of H3K4me3 in 'Red Candem' after 276 CH (RC4; Figure 3.5e) was also coincident with a lower expression level of the gene and the concomitant end of bud dormancy measured as bud break competence (Figure 3.5b). The rise in *DAM6* expression observed after 50 CH (RC2) was however not accompanied by a corresponding increase in H3K4me3. This discrepancy could be due to the presence of a yet unknown chromatin modification contributing to gene expression, the binding of a transcriptional activator, or alternatively could have a posttranscriptional origin, as a differential rate of mRNA degradation.

H3ac was found around the 'ST' region during dormancy progression of 'Big Top' and 'Red Candem' samples (Figure 3.5d,e). In 'Big Top' a significant reduction of H3ac level occurred in

BT3, a dormant sample previous to dormancy release, but no events timing could be established in 'Red Candem' due to certain discrepancies in the decreasing pattern observed between independent experiments.

Similar results were observed when measuring the relative level of H3 acetylation (H3ac). H3ac was not consistently regulated during bud development in 'PR' and 'IN' fragments, but a significant decrease in H3ac level occurred in BT3, a dormant sample before dormancy release, but no events timing could be established in 'Red Candem' because of certain discrepancies in the decreasing pattern observed between independent experiments.

H3K27me3 increases along *DAM6* gene after dormancy release

H3K27me3 followed an opposite pattern to H3K4me3 and H3ac modifications. A relevant accumulation of H3K27me3 was only detected in the last 'Big Top' sample (BT5), when buds had already passed the dormancy period (Figure 3.5d). Interestingly, H3K27me3 occurred to a similar extent in the three genomic regions of *DAM6*, that supports the overall gene modification rather than the modulation of a short regulatory element. A certain increase of H3K27me3 was noted in the non-dormant RC4 sample of 'Red Candem', but this alteration was not significant and about 10-fold lower than in BT5 (Figure 3.5e). Consequently, H3K27me3 modification, unlike H3K4me3 and H3ac, correlated positively with bud break competence following the dormancy period in a cultivar-dependent manner, and showed a wide distribution along *DAM6* gene.

Collectively, these results emphasize the occurrence of specific chromatin histone modifications in the *DAM6* gene during bud dormancy progression and release, and offer a plausible mechanism for the transcriptional regulation of this relevant gene in dormancy processes.

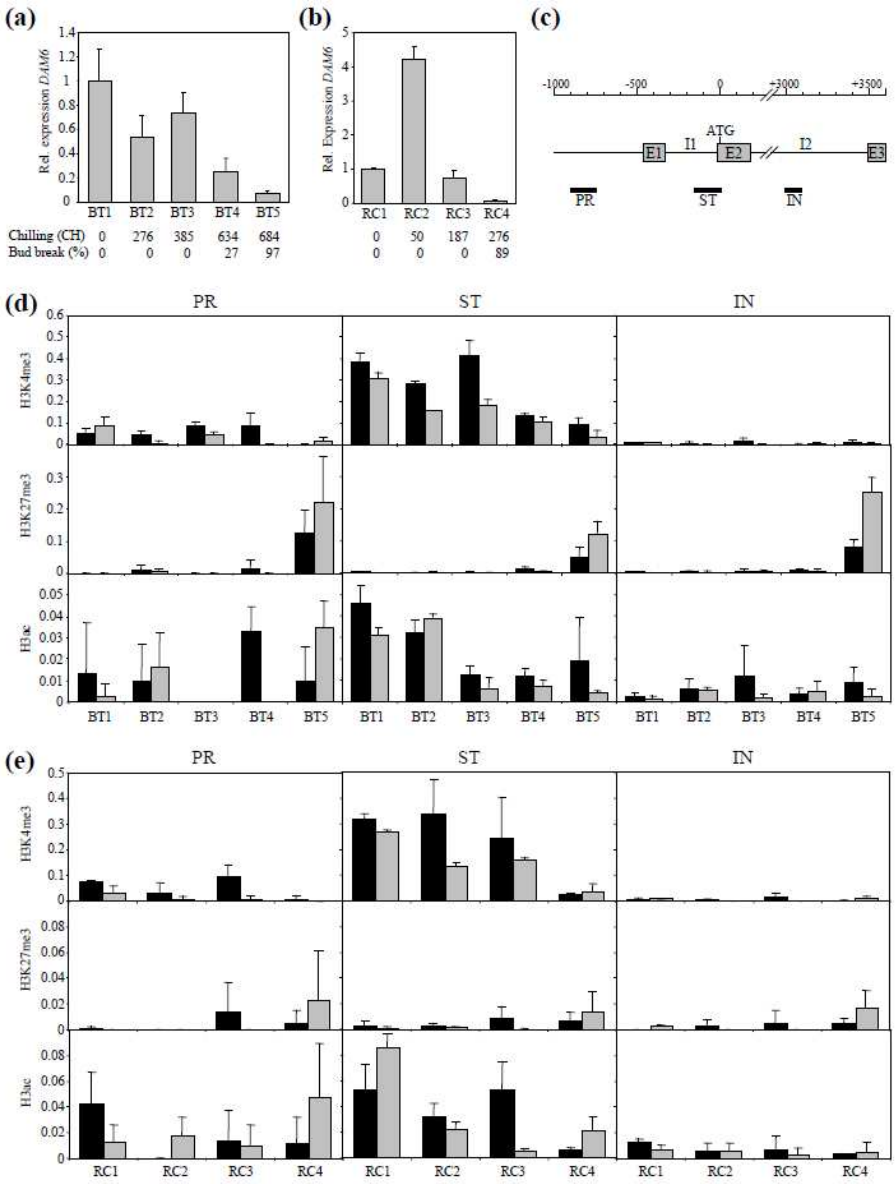

Figure 3.5. Chromatin immunoprecipitation (ChIP) analysis of *DAM6* gene during dormancy progression. Relative expression of *DAM6* by real-time RT-PCR in the late 'Big Top' (a) and the early 'Red Candem' (b) cultivars. Flower bud samples of 'Big Top' (BT1-5) and 'Red Candem' (RC1-4) were collected at different chilling exposure times or chilling hours (CH), as shown in the lower part of the figure. The dormancy stage of these buds was assessed by measuring the percentage of bud break, which is also shown. Expression values are relative to actin and to the first sample (BT1 or RC1). Data are means from two biological replicates repeated twice, with error bars representing standard deviations. A diagram showing the localization of three fragments on the promoter (PR), translation start (ST) and second large intron (IN) of *DAM6* gene, employed in the ChIP experiment, is depicted (c). The first three exons (E1-3, grey boxes) and the first two introns of the gene are shown. The gene diagram and the corresponding ATG-centered base-pair scale are discontinued on the second intron. Quantification of histone modifications in PR, ST and IN fragments was performed by real-time PCR subsequently to ChIP in the 'Big Top' (d) and 'Red Candem' (e) samples. The levels of H3K4me3, H3K27me3, and H3ac are normalized for histone H3 occupancy. Two independent ChIP experiments starting with biological replicates were performed (black and grey bars). Data are means from three replicates, with error bars representing standard deviations.

Discussion

Complementary transcriptomic approaches find *DAM* genes related to bud dormancy establishment and release

The aim of this work was to identify peach genes whose expression in flower buds at a single developmental stage (400 CH) correlated with the chilling requirement and other dormancy variables of ten different cultivars. Previous studies employed the SSH procedure for the isolation of bud dormancy-related genes in peach. Jiménez et al. (2010a) used SSH to compare wild type and *evg* mutant gene expression after bud growth cessation mediated by transfer to short day conditions. From 23 genes found by Jiménez and coworkers, only one (*DAM6*) has been also obtained in this work, most likely due to differences in the experimental design. Whereas Jiménez and colleagues examined dormancy entrance by modulating photoperiodic conditions, our work is mostly devoted to dormancy break processes based on cultivar diversity. Thus, the identification of *DAM6* in both studies confers an increasing interest to this gene.

On the other hand, Leida et al. (2010) employed SSH to identify genes associated to bud dormancy release processes, which served to design the microarray used in this work. In spite of the experimental differences between both approaches, the number of coincidences with our work is higher in this case (25). The set of common genes includes three members of the *DAM* family (*DAM4-6*), and genes coding for a LEA, a Zn-finger protein, peroxidases, lipid transfer proteins and peptidases among others. The percentage of cDNAs correlating with cultivar-specific differences may seem unexpectedly low if we consider that microarray probes were obtained by subtractive hybridization of dormant versus non-dormant samples, however previous results show that the ratio of genuine differentially expressed clones after SSH may be very low, and an additional validation step is required in most cases (Yamane et al., 2008; Leida et al., 2010).

The role of DAM transcription factors in regulating bud dormancy entrance and release in peach is not only supported by transcriptomic analyses, but also by expression data (Jiménez et al., 2010b; Yamane et al., 2011), and genetic studies. A deletion of several *DAM* genes was tightly linked to the non-dormant and defective terminal bud formation phenotype of the *evg* mutant (Bielenberg et al., 2008). In addition, quantitative trait loci (QTL) mapping of chilling requirement, heat requirement and bloom date in peach led to a major QTL in linkage group 1, overlapping with the *EVG* locus containing *DAM* genes (Fan et al., 2010). Thus, in agreement with our results DAM factors are consequently considered the main candidates to control bud dormancy and meristem

growth cessation, through regulation of gene expression, in peach and other perennial plants. *DAM4-6* and other genes found in this work could be employed as expression markers for comparing the chilling requirements and dormancy aptitudes of different cultivars. In our opinion, a single time RT-PCR assay using few genes could facilitate the phenotypic evaluation of large collections of individuals, as the segregating population of a cross for plant breeding purposes.

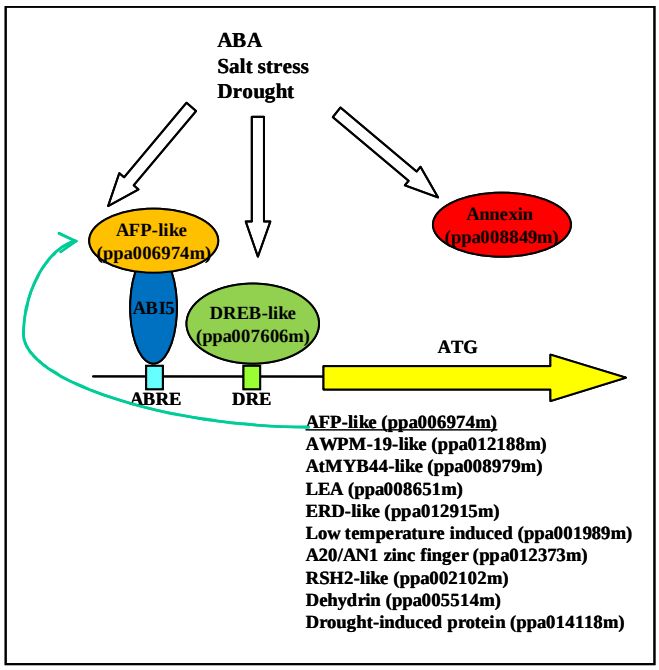

Figure 3.6. ABA and drought related proteins. White arrows symbolize the transduction of ABA and abiotic stress signals. White boxes represent ABRE and DRE elements on the promoter of ABA and drought responsive genes. Proteins coded by genes responding to these signals are located below the filled arrow. The transcript models of genes described in this work are shown in parentheses**.**

ABA and drought responding genes are dormancy regulated

It is remarkable that many genes expressed in dormant buds correspond to ABA and drought-related genes in other species. On this matter, Figure 3.6 lists some proteins identified in this work with homologues described as inducible by abiotic stresses or ABA. One of these proteins encoded by ppa006974m is similar to ABA-INSENSITIVE5 (ABI5) binding protein (AFP), involved in ABA signal transduction in *A. thaliana*. AFP binds to and promotes proteolytic degradation of ABI5, a basic leucine zipper (bZIP) transcription factor that regulates ABA-dependent genes by binding to the ABA-responsive element, ABRE (López-Molina et al., 2003). In addition to ABRE, abiotic stresses affect gene expression through the dehydration-responsive element (DRE) and their respective DRE binding proteins (DREB; Liu et al., 1998). The transcript model ppa007606m found in this work encodes a DREB-like factor that could contribute to the dormancy-specific expression of ABA and drought responsive genes. On the other hand, calcium-binding annexins

related to the product of ppa008849m have been found involved in ABA and osmotic stress signal transduction in *A. thaliana* (Lee et al., 2004).

These observations are in agreement with recent findings by Jiménez et al. (2010a), showing that genes encoding a LEA and KEEP ON GOING (KEG)-like proteins are misregulated in *evg* mutant during short day induction of bud dormancy. KEG is an E3 ligase which regulates ABI5 abundance by means of its ubiquitination and subsequent proteasome-mediated degradation in *A. thaliana* (Stone et al., 2006).

ABA has been proposed for long time to promote and maintain bud dormancy, although only few consistent molecular data support this prediction (Arora et al., 2003; Horvath et al., 2003; Rohde & Bhalerao, 2007). More relevantly, the poplar homologue of *A. thaliana ABSCISIC ACID INSENSITIVE 3* (*ABI3*) gene was found expressed in buds during bud set, coinciding with an increase in ABA content (Rohde et al., 2002). Moreover, overexpression and down-regulation of *PtABI3* in poplar caused developmental alterations in bud formation and misregulation of numerous genes during bud induction and dormancy (Ruttink et al., 2007). In a recent work, ectopic expression of the *A. thaliana* mutant *abscisic acid insensitive 1* (*abi1*) gene in poplar was shown to modify the dormancy response of lateral buds to exogenous ABA (Arend et al., 2009).

Some of the proteins shown in Figure 3.6 could contribute to cold hardening processes improving frost tolerance of buds. Interestingly, overexpression of *AtMYB44* (Jung et al., 2008), rice A20/AN1 zinc-finger protein (Mukhopadhyay et al., 2004), and *A. thaliana* DREB2C gene similar to ppa007606m (Lee et al., 2010) conferred tolerance to cold or freezing temperatures in transgenic plants. In addition, dehydrins and AWPM-19-like plasma membrane proteins have been associated to cold tolerance in peach and wheat respectively (Artlip et al., 1997; Koike et al., 1997). In conclusion, our results support a role of ABA and drought responses in bud dormancy and cold acclimation processes, and contribute to identify several genes encoding putative regulatory factors of these pathways in peach.

DAM6 is regulated at the chromatin level

The chromatin modifications H3K4me3, H3ac and H3K27me3 had a different timing for ‘Big Top’ and ‘Red Candem’, coinciding with their specific patterns of gene repression and increase in bud break competence. This argues for a potential role of chromatin modification in modulating *DAM6* expression and subsequently the state of bud dormancy. Nevertheless, H3K27me3 was not significantly increased in the ‘Red Candem’ buds just after dormancy release (RC4), which in fact

suggests a sequential chain of molecular events affecting the local state of *DAM6* chromatin as outlined in Figure 3.7. In dormant buds (BT1 and RC1 samples), transcriptionally active *DAM6* gene would contain H3K4me3 and H3ac in a short chromatin region around its ATG (Figure 3.7a). Following demethylation of H3K4 and deacetylation of H3, *DAM6* repression would contribute to release dormancy in samples BT4/BT5 and RC4 (Figure 3.7b). Finally, H3K27me3 in a region of at least 4 Kb including promoter, coding sequence and introns, would mediate stable epigenetic repression of the gene through subsequent cell cycles (Figure 3.7c). This last stage corresponds to the BT5 sample, but does not have a 'Red Candem' counterpart in this work, which in fact confirms the temporal separation of H3K4me3 and H3ac events from H3K27me3. Two scenarios are possible in 'Red Candem' after the induction stage detected in RC4: i) *DAM6* chromatin is not trimethylated on H3K27, which could in fact contribute to cultivar specific differences in dormancy response, and ii) H3K27 trimethylation occurs in a subsequent step, and thus phenotypic differences between cultivars could simply depend on the distinct chilling time required for triggering the whole process.

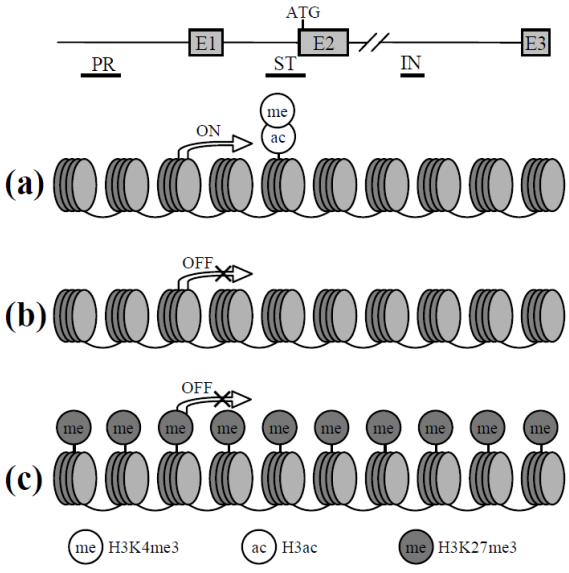

Figure 3.7. Possible sequence of *DAM6* chromatin events during dormancy release. In dormant buds, transcriptionally active *DAM6* has H3K4me3 and H3ac modifications around the translation start region (a). Buds undergoing dormancy release lose H3K4me3 and H3ac modifications concomitantly with gene repression (b), as observed in 'Red Candem' cultivar. Finally, overall H3K27me3 modification could facilitate long-term gene inactivation (c).

Changes in the methylation state of H3K27 and H3K4 have been recently observed in the promoter of *DAM1* gene of leafy spurge when comparing buds in different dormancy stages (Horvath et al., 2010). These chromatin modifications have been related to the down-regulation of *DAM1*, as observed for *DAM6* in this work. Both genes encode MIKC^{c}-type MADS-box proteins belonging to the SVP/StMADS11 clade (Becker & Theißen, 2003; Jiménez et al., 2009; Horvath et al., 2010), but are not strictly orthologous. Leafy spurge *DAM1* is more similar to the peach transcript

ppa022274m, which has not been related to dormancy processes. They may have been originated in an ancestral duplication event followed by a functional diversification.

Modification of *DAM6* chromatin in a similar way to *FLC* and *VRN1*, as a consequence of the prolonged exposure to low temperatures (Bastow et al., 2004; Sung & Amasino, 2004; Oliver et al., 2009), confirms the existence of mechanistic similarities between vernalization and bud dormancy processes. The coincidence of H3K27me3 and gene repression in these three models suggests the common concurrence of methyl-transferases activities associated to related PcG multi-protein complexes in *FLC*, *VRN1* and *DAM6*. Thus, PcG complexes are potentially responsible for the stable repression of *DAM6* at the end of the seasonal dormancy until the next period of bud formation, which would require yet unknown mechanisms of *DAM6* activation for the initiation of a new dormancy cycle. Additional genetic and biochemical approaches are required to identify these and other regulatory elements implicated in DAM-dependent pathway.

Supporting Information

Table S3.1 Analysis of correlation for the variables chilling requirement, flowering time, percentage of bud break.

Table S3.2. Accession numbers of ESTs.

Table S3.3. List of primers used in this work.

This material is contained in the CD annexed to this thesis.

4. Gene expression analysis of chilling requirements for flower bud break in peach

This work was accepted in Plant Breeding:

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

Abstract

Dormancy has been defined as the inability to initiate growth from meristem under favourable environmental conditions. The length of dormancy is a genotype-specific trait that limits the climatic adaptability of temperate crops, as peach. A better knowledge of the genes involved in dormancy may provide genetic tools for an early assessment of the trait in breeding programs. Recent studies on the molecular aspects of dormancy provided an initial description of candidate genes involved in bud dormancy maintenance and release in peach. In this paper, we compare the chilling requirement for dormancy release of five peach cultivars with the expression of five genes and ESTs related to bud dormancy: *DAM5*, DB396 (ppa007606m), DB247 (ppa012188m), SB280 (ppa006974m) and PpB63 (ppa008309m). Results indicated that gene expression analysis could contribute to estimate the chilling requirement for dormancy release of new cultivars.

Introduction

Dormancy has been defined as the inability to initiate growth from meristem under favourable conditions (Rohde & Bhalerao, 2007). Perennial plants have developed a dormancy mechanism that ensures the survival of meristems in specific structures (buds) during the low temperatures of winter. Bud dormancy is only released after exposure to a prolonged period of cold, which helps avoid freezing injury of actively growing tissues. The length of cold exposure required by a particular genotype is quantitative and heritable (Hauagge & Cummins, 1991; Fan et al., 2010). Incomplete fulfilment of this quantitative chilling requirement may lead to bud break delay (Murray et al., 1989, Heide 1993), and irregular floral and vegetative development (Coville 1920; Erez et al., 1979; Erez & Couvillon, 1987; Oukabli & Mahhou, 2007; Topp et al., 2008), with a subsequent reduction in yield. Consequently, chilling requirement is an adaptive genetic trait that restricts fruit production to specific climatic zones, and thus has become an important objective in different plant breeding initiatives worldwide (Monet & Bassi, 2008). In peach, chilling requirements and dormancy duration are specific for each cultivar (Ruiz et al., 2007; Viti et al., 2010). The introduction of germplasm with less chilling requirements for dormancy release has expanded the growing area of this species to mild-winter climates and has increased the fruit harvest period, allowing more competitive prices. Interest in low chill requiring varieties in a wide range of perennial species has increased due to the onset of climatic warming (Topp et al., 2008; Luedeling et al., 2011).

Several empirical models for chilling requirement fulfilment have been developed. These models differ in the effective temperatures for chilling fulfilment and the possible negation of chilling accumulation by warm temperatures. Three models are most widely used. The Weinberger considers temperatures below 7ºC as equally effective in chilling fulfilment (Weinberger 1950). The Utah model has a narrow range of effective temperatures (3ºC to 9ºC) and assigns negative values to temperatures higher than 16ºC and lower than 0ºC (Richardson et al., 1974). Finally, the Dynamic model developed by Fishman et al. (1987a, 1987b), introduces the effect of different temperature cycles and weighted effective temperatures, based on studies developed by Erez and collaborators (Erez et al., 1979; Couvillon & Erez, 1985; Erez & Couvillon, 1987). While these models tend to agree in relatively cold winter climates, they vary widely in warm winter climates. Assignment of a chilling requirement to a given genotype is made by repeated sampling of cuttings throughout the winter. The cuttings are then incubated at growth conducive temperatures and evaluated for bud break. The accumulated chilling exposure determined by one of the above models at the sampling date where a threshold of bud break occurs is then considered to be the chilling requirement of that specific genotype. Unfortunately, these procedures are costly and time-consuming when analyzing a large number of genotypes, and the reliability depends on environmental conditions, showing wide variations during consecutive years (Balandier et al., 1993). From a practical point of view, plant breeding programs that need to determine chilling requirements of a large number of genotypes simplify the phenotyping by only measuring bloom date as an indicator of dormancy release. Unfortunately this introduces additional variables in the assessment such as the genetic variation of heat requirement for bud break (Topp et al., 2008).

A better knowledge of dormancy processes at the physiological and genetic level may provide key information for an early assessment of chilling requirement. Several reviews highlight the role of external (day-length shorteningand temperature) and endogenous factors (growth regulators, cell cycle regulation, water status and chromatin modification) as regulators of bud dormancy onset and release (Arora et al., 2003; Horvath et al., 2003; Baurle & Dean, 2006; Rohde & Bhalerao, 2007; Allona et al., 2008).

Mapping of bud dormancy variables in *Prunus* species has been approached by QTL analysis in almond (Sánchez-Pérez et al, 2007), apricot (Olukolu et al., 2009) and peach (Fan et al., 2010). Interestingly, the major QTLs located in linkage groups 1, 5 and 7 were common to apricot and peach. On the other hand, the trait ‘blooming date’ has been mapped in linkage group 4 in almond (Sánchez-Pérez et al., 2007).

Undoubtedly, the characterization of the peach non-dormant mutant *evergrowing* (*evg*), carrying a deletion affecting several members of a family of six tandem repeated MADS-box genes, named *DAM1-6* (*DORMANCY ASSOCIATED MADS-BOX*), represented a valuable tool for genetic and molecular assessment of growth cessation and dormancy processes (Bielenberg et al., 2004, Bielenberg et al., 2008). Furthermore, the EVG genomic region coincided with the major QTL mapped for chilling requirement (Fan et al, 2010). Consequently, *DAM* genes are considered major candidates to control bud dormancy and meristem growth cessation. However the dormancy trait is a complex character linked to many biochemical processes and further studies are needed. Recent studies on the molecular aspects of dormancy gave an initial description of additional candidate genes involved in bud dormancy maintenance or release in raspberry, grapevine, peach and apricot (Bassett et al., 2006; Mazzitelli et al., 2007; Yamane et al., 2008; Mathiason et al., 2009; Leida et al., 2010).

In this paper we evaluate the chilling requirements for dormancy release of five peach cultivars by applying the Weinberger, Utah and Dynamic models, and study the differential expression of five genes and ESTs related to dormancy processes: *DAM5*, DB396 (peach transcript ppa007606m), DB247 (ppa012188m), SB280 (ppa006974m) and PpB63 (ppa008309m) (Bielenberg et al., 2004; Leida et al., 2010; 2011). Finally we discuss the usefulness of relative gene expression analysis as a tool for assisting in the evaluation of the chilling requirement of peach varieties in breeding programs.

Materials and Methods

Plant material

The plant material consisted of five peach cultivars differing in chilling requirements: ‘Big Top’, ‘Catherina’, ‘Fergold’, ‘Maruja’ and ‘Springlady’. The plants were grown in Murcia region (37º 59’ N- 1º 07’ W) at IMIDA experimental fields, located in four different locations. The plot consisted in a collection of varieties planted at 4 x 4 m that received standard cultural practices. Data were obtained from three trees per cultivar.

Quantification of chilling requirements

Flower bud break was determined weekly from October 2009 to February 2010 using a shoot cutting test. Thirty one-year old shoots of 25 to 40 cm in length were harvested weekly from each

cultivar and grouped into three sets of ten shoots, placed in bottles containing distilled water with 3% sucrose and incubated in a growth chamber with a 12 h photoperiod and 22ºC constant temperature. The basal 1 cm of shoots were cut back and the water renewed twice per week to prevent disease development. After 10 days, we scored the number of buds that reached the green stage (stage C) according to the Baggiolini code (Baggiolini 1980). The percentage of bud break was calculated for each sampling date and a variety was classified as non-dormant when 25% of flowering buds were at stage C.

Air temperatures experienced by the five varieties in the field were recorded from the beginning of November until the end of April at the SIAM (Murcia Agriculture Information System, www.siam.es) weather station, located at the experimental plot. The air temperature data was used to calculate the accumulated chill hours (Weinberger 1950), chill units (Utah model, Richardson et al., 1974) and chill portions (Dynamic model, Fishman et al., 1987a; 1987b) at each shoot sampling date. Each variety was assigned a chill hour, chill unit, and chill portion value based upon the sampling date at which the variety reached the 25% bud break threshold.

Measurement of the expression of selected genes by quantitative real-time RT-PCR

Flower buds were sampled when they accumulated 400 chilling hours according to Weinberger model, which were reached from 18th December 2009 to 11th February 2010. Plant material was stored at -80ºC until RNA isolation. Total RNA was isolated from approximately 100 mg of bud tissue with the RNeasy mini kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions but with the addition of 1% PVP (w/v) to the extraction buffer. Approximately three µg of total RNA was reverse transcribed with the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 µl. One microlitre of a 10× diluted first strand cDNA was used for each amplification reaction in a final volume of 20 µl. Quantitative real-time PCR was performed on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), using PerfeCta™ SYBR® Green SuperMix ROX (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's instructions. Primer pairs used are listed in Table 4.1.

The cycling protocol consisted of 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC for denaturation and 60 s at 60ºC for annealing and extension. Specificity of the reaction was assessed by the presence of a single peak in the melting curve after the amplification and through size estimation of the amplified product on a 1% agarose gel. The comparative C_T ($\Delta\Delta C_T$) method was used to estimate cDNA abundance and the standard curve regression was applied when amplification efficiencies were not equivalent to that of the reference actin gene. Results were the average of two independent biological replicates repeated three times.

Table 4.1. Primers employed in the quantitative RT-PCR.

	Primer sequence forward (5'-3')	**Primer sequence reverse (5'-3')**
Actin	CAGATCATGTTTGAGACCTTCAATGT	CATCACCAGAGTCCAGCACAAT
DAM5	CCACATCAAACTGAGTAAGGAACTC	CTGCCTTAGCTGGTTGTTAGCTTCAACT
DB396	TGAAGAGGGATGATGTTACTGGCGA	CACAAAATGACCAGACATGACAAGG
DB247	CCCAGCCAATATGGCGAATATCAGAA	CATAGTGAGCAGTAAGTTTGTGCT
SB280	TTCCGTTGGTGGTGGAGTGGATGCA	TTACTAGCAGGGCTTCTTGCTTCAC
PpB63	TCTCCCTTCGTCCCAGTAAATGGTC	TTTCTGGGGAGGGTTTGCTTCCATC

Results

Chilling accumulation in field conditions

The cultivars ‘Catherina’, ‘Fergold’ and ‘Maruja’ displayed higher chilling requirements, with values ranging between 800 and 1200 chilling units, 570 and 920 chilling hours, and 50 and 65 portions (Table 4.2). These cultivars were classified as ‘high-chill’. ‘Springlady’ and ‘Big Top’ showed medium requirements with values ranging between 500 and 700 chilling units, 300 and 400 chilling hours, and 30 and 45 portions, and were considered as ‘medium-chill’ (Table 4.2).

A high correlation was observed for calculated effective chilling accumulation between Utah and Dynamic models (R^2=0.96), which supports the high similarity between both methods in Mediterranean mild winters (Figure 4.1). The Weinberger model showed lower correlation coefficients with both the Utah and Dynamic models, with R^2=0.77 and 0.76 respectively (Figure 4.1).

Table 4.2. Chilling requirement to overcome dormancy.

Cultivar	Dormancy breaking date	Utah model (chilling units)	Weinberger model (chilling hours)	Dynamic model (portions)
'Springlady'	27th January 2010	626	331	40.8
'Big Top'	2nd February 2010	716	363	45.2
'Maruja'	10th February 2010	809	572	51.8
'Catherina'	16th February 2010	1220	793	62.4
'Fergold'	2nd February 2010	861	921	52.8

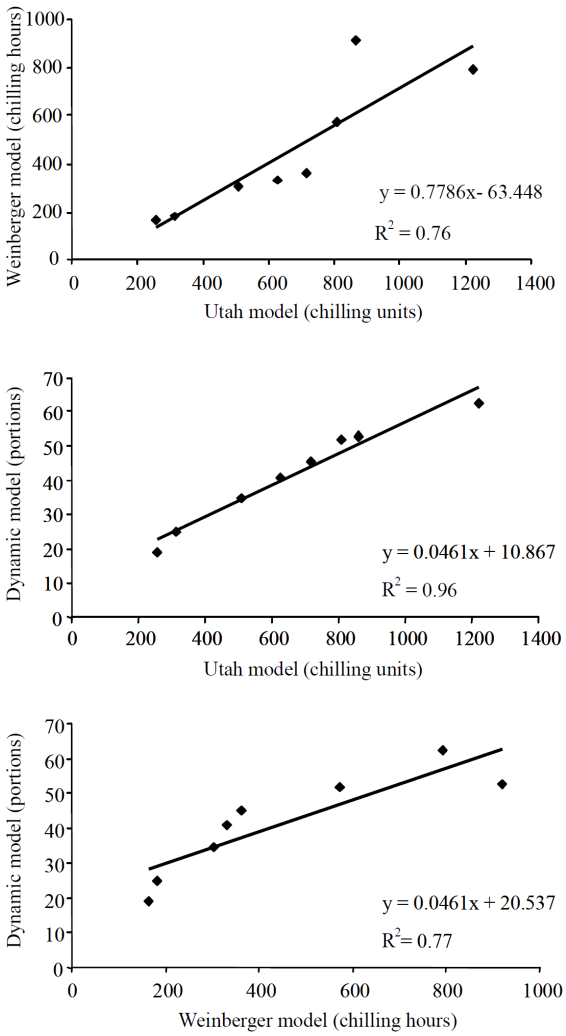

Figure 4.1 Correlations between the Weinberger, Utah, and Dynamic models for calculation of the chilling requirement for dormancy release.

Differential expression of genes related to dormancy release

Five genes with expression profiles correlating with chilling fulfilment in previous studies were selected for expression analysis in this study (Li et al., 2009; Leida et al., 2010; 2012). *DAM5* belongs to the cluster of *DAM* genes that have been found deleted in the *evg* mutant and associated with the major QTL for bud dormancy in peach (Bielenberg et al., 2008; Fan et al., 2010). The protein encoded by EST DB396 has an AP2 DNA-binding domain found in transcriptional regulators such as APETALA2 and EREBP, an ethylene responsive element binding protein (Tacken et al., 2010). The product of EST DB247 is similar to a membrane protein that accumulates during induction of freezing tolerance in wheat (Koike et al., 1997). EST SB280 codes for a protein with homology to ABI5 binding protein from *Arabidopsis thaliana*, involved in abscisic acid signalling (Lopez-Molina et al., 2003). Finally, EST PpB63 codifies for a protein similar to a peroxidase described in cotton (Chen et al., 2009).

When expression of the above genes was analyzed at 400 chilling hours in the each of the five cultivars, two clear patterns emerged. *DAM5*, DB396, DB247 and SB280 were expressed at higher levels in the varieties with the highest chilling requirements while PpB63 was up-regulated in lowest chill requiring varieties (Figure 4.2).

The expression level of *DAM5* was lower in ‘Springlady’ and ‘Big Top’ than in ‘Maruja’, ‘Fergold’ and ‘Catherina’ (Figure 4.2). DB247, SB280 and particularly DB396 were expressed at higher levels in ‘Catherina’, which showed respectively about 5, 2.5 and 15-fold higher transcript abundance than the other cultivars.

PpB63 gene expression was ten-fold higher in ‘Springlady’ than in ‘Fergold’, the cultivar with the lowest expression. This is in close agreement with published data showing a pattern of PpB63 accumulation at the end of dormancy (Leida et al., 2010), considering that at the sampling date (400 chilling hours) ‘Springlady’ had already fulfilled the chilling requirement for bud break. (Table 4.2).

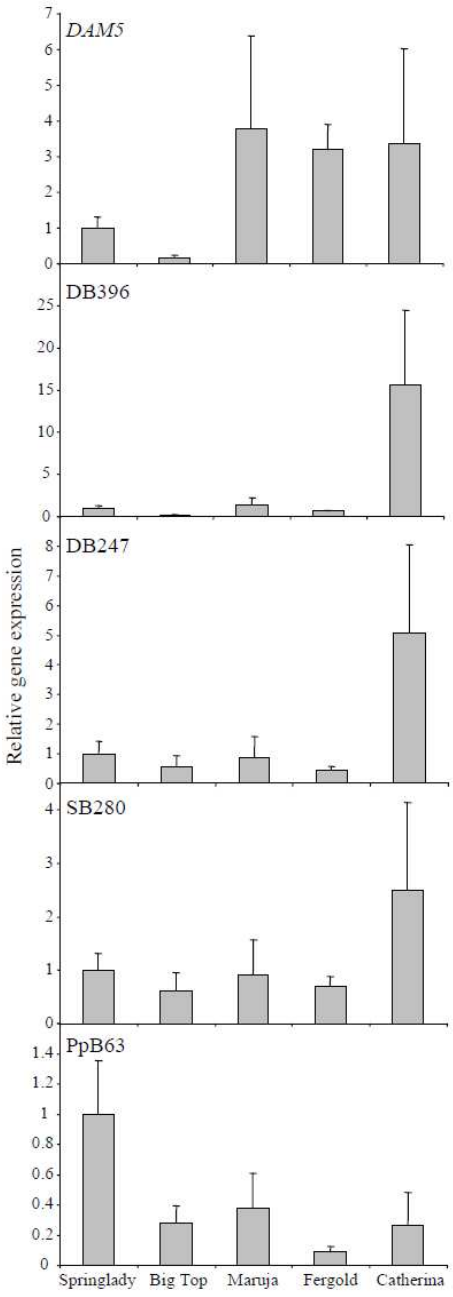

Figure 4.2 Quantitative real-time RT-PCR analysis of five genes related to dormancy. RNA samples were obtained from flower buds of 'Springlady', 'Big Top', 'Maruja', 'Fergold' and 'Catherina'. The cultivars are arranged by increasing chilling requirement in the graph. Expression levels are referred to Actin and a relative expression of one is assigned to 'Springlady'. Data are means of two biological replicates with error bars representing standard deviations.

Discussion

In peach, many cultivars are released every year. The description of new cultivars usually includes common pomological characteristics, but detailed information about chilling requirement is often absent , mainly due to the difficulties inherent to the measurement of dormancy variables. A method allowing the quick and accurate determination of chilling requirements and consequently providing

information about the adaptability of a cultivar to a given environment would be very valuable. The current methods are based on indirect temperature measures with low reproducibility among areas with different climatic conditions. A comparative study between the three models in two different locations reported wide environmental and temporal variations (Balandier et al., 1993).

We have calculated the chilling requirement of selected cultivars using temperature models and compared them with results obtained in other areas by other authors. The inaccuracy of current methods is confirmed by the interval of values obtained for the same variety by different authors. For instance, Valentini et al. (2001) found chilling requirements for 'Springlady' that doubled the value determined in this study, and Okie (1998) reported for 'Springlady' and 'Catherina' 750 and 900 CU respectively, much different than our measurements of 626 and 1220 CU.

Under our experimental conditions we obtained a high correlation between Utah and Dynamic models, which was also observed by other authors (Erez et al., 1990; Linsley-Noakes & Allan, 1994; Erez & Fishman, 1998; Ruiz et al., 2007), and a low correlation between Weinberger and both Utah and Dynamic models.

The use of molecular markers for the analysis of large collections of individuals for breeding purposes has increased during the last years, due to their improved robustness and simplicity when compared with traditional phenotyping methods. These molecular techniques have been made possible by the progressive identification of genes related to different physiological processes, favoured by the broad application of genomic tools to crop species. A previous use of gene expression markers in the dormancy field was approached by Yooyongwech et al. (2009), who measured the mRNA levels of two aquaporins (Pp-γTIP1 and Pp-γPIP1) in high and low-chill varieties, and showed that their increased expression can be used to indicate the state of dormancy release in peach buds. In this work we propose the use of five peach genes as gene expression markers for the evaluation of the dormancy stage of any known or new cultivar in a particular time. We chose 400 chilling hours for this single point evaluation, due to its rough intermediate position among the chilling requirement interval of peach varieties, however it could be changed according to the dormancy attributes of the assayed genotypes. This unique sample collection would reduce the complexity of evaluation of dormancy release by the excised shoots method since simple phenotyping methods are required in breeding programs .

Our group previously identified the five genes shown in this study as changing expression during the progression of bud dormancy. *DAM5* is one of the six *DAM* genes identified as candidates for the *evg* mutation in peach (Bielenberg et al., 2008). This gene was also found in several

transcriptomic approaches studying peach bud dormancy (Leida et al., 2010; Jiménez et al., 2010a). Its expression is higher after bud set in late summer/autumn (Li et al., 2009) and during the dormancy period. This pattern is coherent with the role of *DAM5* in the maintenance of the endodormant state, as suggested by Li et al. (2009). The ESTs DB396, DB247 and SB280 and the unigene PpB63 were identified in a library enriched in bud dormancy related transcripts obtained by suppression subtractive hybridization, and their abundance correlated with the chilling requirements of 10 peach cultivars after a microarray hybridization experiment (Leida et al., 2010; 2012). PpB63 codes for a peroxidase-like protein similar to other peroxidases found in *Vitis riparia* and leafy spurge related to bud break (Jia et al., 2006; Mathiason et al., 2009). Peroxidases have been proposed to have a role in H_2O_2 detoxification during the metabolic activation initiated by bud break. The up-regulation of PpB63 at this point supports this protective function and its use as an expression marker of dormancy release in buds.

According to the results obtained in this work, the expression values of these five genes correlate with the chilling requirement measurements performed following the Utah and Dynamic models. Thus, these genes could be used as expression markers for single point evaluation of the dormancy stage of different peach genotypes, amenable for use in plant breeding programs aiming to obtain varieties with modified climatic requirements. We recommend this molecular approach for the characterization of the natural diversity of chilling requirements among peach varieties. The analysis of gene expression could offer new additional tools for classifying new cultivars according to their adaptability to a given area. A better knowledge of the biochemical pathways involved in dormancy processes is needed in order to increase the number of dormancy-related genes available for screening. Expression of additional marker genes would increase the accuracy of a chilling determination method based on gene expression.

Acknowledgements

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5. Chilling-dependent release of seed and bud dormancy in peach associates to common changes in gene expression

This work has been submitted to review:

Leida C., Conejero A., Arbona V., Gómez-Cadenas A., Llácer G., Badenes M.L., Ríos G. “Chilling Chilling-Dependent Release of Seed and Bud Dormancy in Peach Associates to Common Changes in Gene Expression”.

Abstract

Reproductive meristems and embryos display dormancy mechanisms in specialized structures named respectively buds and seeds that arrest the growth of perennial plants until environmental conditions are optimal for survival. Dormancy shows common physiological features in buds and seeds. A genotype-specific period of chilling is usually required to release dormancy by molecular mechanisms that are still poorly understood. In order to find common transcriptional pathways associated to dormancy release, we analyzed the chilling-dependent expression in embryos of certain genes that were previously found related to dormancy in flower buds of peach. We propose the presence of short and long-term dormancy events affecting respectively the germination rate and seedling development by independent mechanisms. Short periods of chilling seem to improve germination in an abscisic acid-dependent manner, whereas the positive effect of longer cold treatments on physiological dwarfing coincides with the accumulation of phenylpropanoids in the seed.

Introduction

Perennial plants protect important and delicate tissues as reproductive meristems and embryos in specialized structures respectively designated buds and seeds. Growth of bud and embryo meristems leading respectively to blooming and germination is strictly regulated by dormancy mechanisms, which impose a physiological constraint to this growth until environmental conditions are optimal for long-term survival. Dormancy is released by dry storage of seeds (after-ripening), moist chilling of seeds (stratification) or a prolonged period of chilling in buds. In stone-fruit species, a high correlation between the chilling requirements for seed and bud dormancy release has been observed (Kester 1969; Pasternak et al., 1980), which suggests the presence of common regulatory mechanisms. Insufficient cold stratification of seeds in peach (*Prunus persica*), almond (*Prunus dulcis*), and other rosaceous plants may cause, in addition to low germination rates, a shoot development abnormality called physiological dwarfing (Hartmann et al., 2011). Physiological dwarfs are characterized by a temperature-dependent rosette-type habit of growth, with short internodes, and deformed leaves (Pollock 1962).

Seed dormancy has been observed throughout higher plants with physiological and morphological particularities in different species (Finch-Savage et al., 2006). In most popular model organisms, physiological dormancy integrates contributions from the embryo and the seed coat, being the coat

component at least partially due to the mechanical resistance to breakage of endosperm and testa layers. The ratio of the hormones abscisic acid (ABA) and gibberellins (GA) is considered a relevant factor regulating seed dormancy processes. Several genetic approaches utilizing mostly mutant and transgenic lines of *Arabidopsis thaliana* and Solanaceae species have established that ABA is involved in induction and maintenance of dormancy, whereas GAs release dormancy and promote germination (Kucera et al., 2005). Other hormones as ethylene, brassinosteroids, auxin and cytokinins have been also proposed to affect dormancy and germination. The molecular factors and pathways conditioning seed dormancy status have been enumerated in several recent reviews (Bentsink et al., 2007; Finkelstein et al., 2008; Holdsworth et al., 2008; North et al., 2010). Early studies showed that the orthologous B3 class transcription factors encoded by *VIVIPAROUS 1 (VP1)* in maize and *ABA-INSENSITIVE 3 (ABI3)* in *A. thaliana* are involved in seed development and dormancy (McCarty et al., 1991; Giraudat et al., 1992). The basic leucine zipper (bZIP) transcription factor encoded by *ABA-INSENSITIVE 5 (ABI5)* interacts with ABI3 and mediates its effect on the expression of ABA responding genes thought the ABA-response element ABRE (Nakamura et al., 2001; Carles et al., 2002).

Bud dormancy in perennial plants resembles seed dormancy at the hormonal level (Powell 1987). The involvement of ABA in bud dormancy events is suggested by multiple physiological and transcriptomic studies (Arora et al., 2003; Horvath et al., 2003; Rohde et al., 2002; 2007), though few genetic approaches support this statement (Ruttink et al., 2007; Arend et al., 2009). The DORMANCY ASSOCIATED MADS-box (DAM) group of transcription factors related to SHORT VEGETATIVE PHASE (SVP) of *A. thaliana*, have been proposed to regulate bud dormancy processes in peach (Bielenberg et al., 2008), leafy spurge (Horvath et al., 2010) and Japanese apricot (Sasaki et al., 2011). *DAM* gene expression correlates with the dormancy state of buds, with higher transcript accumulation during the cold season followed by chilling-dependent down-regulation prior to dormancy release (Horvath et al., 2010; Jiménez et al., 2010b; Yamane et al., 2011). Different transcriptomic approaches have been conducted in order to identify *DAM*-like and other genes related to dormancy at the expression level (Ruttink et al., 2007; Jia et al., 2006; Mazzitelli et al., 2007; Rohde et al., 2007; Horvath et al., 2008; Yamane et al., 2008; Mathiason et al., 2009; Jiménez et al., 2010a; Leida et al., 2010).

In order to find common molecular features between seed and bud dormancy processes, we investigated the expression of *DAM* and other bud dormancy-dependent genes during the stratification of seeds in peach. The elucidation of general regulation pathways in both seed and bud

structures may contribute to improve our basic knowledge on dormancy mechanisms, and be employed in plant breeding projects that profit from an early prediction of chilling requirements for blooming of new genotypes.

Materials and Methods

Plant material and in vitro culture

The *Prunus persica* (L. [Batsch]) cv 'Big Top' was grown in an orchard located at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Moncada (Spain) under standard agricultural practices. Mature fruits were collected and immediately broken to eliminate the endocarp with special scissors. Seeds were disinfected and flamed with alcohol, and then the coats were removed under sterile conditions. Embryos were cultured in a sterile Woody Plant Medium (Lloyd et al., 1981), solidified with 0,8 % Bacteriological Agar and distributed in 20 ml aliquots into 25 x 150 mm culture tubes. Chilling treatment or stratification was performed by storing the tubes at 4ºC in continuous darkness for 0, 1, 3, 7 or 9 weeks. After the stratification period, 10 embryos were frozen with liquid nitrogen and stored at –80ºC for RNA extraction, and 48 embryos were placed in a culture chamber at 24ºC. The embryos were maintained in darkness during the first week and then in 16 hours light-photoperiod conditions for the rest of the development. The germination and shoot emergence rates, height of seedlings, and dwarfing phenotype were noted once per week.

Quantitative real-time RT-PCR

Total RNA was isolated from 100 mg of seeds deprived of their coats using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), but adding 1% (w:v) polyvinylpyrrolidone (PVP-40) to the extraction buffer before use. From 8 to 10 individuals were pooled for each sample. One microgram of total RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 µl. Two microliter of a 40X diluted first-strand cDNA were used for PCR reactions in a final volume of 20 µl. Quantitative real-time PCR was performed on a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA), using Perfecta SYBR Green SuperMix ROX (Quanta Biosciences, Gaithersburg, MD, USA). Primer pairs are listed in Table 1. Cycling protocol consisted of 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC for denaturation, and 1 min at 60ºC for annealing and extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve after the

amplification and through size estimation of the amplified product by agarose electrophoresis. We used as reference a peach Actin transcript amplified with specific primers. Relative expression was measured by the relative standard curve procedure. Results were the average of two independent biological replicates repeated twice.

Table 5.1. Primers employed in the quantitative real-time RT-PCR.

Transcript (protein)	Forward primer	Reverse primer
DAM1	GGGGACGATGAAAATGACGAGGGAG	CAATCACCCGGCCAAGGCTTGCATC
DAM4	GAAGAGCTGGATCTGGATGAGTTGC	TCTGATTGTTGGCTTCTACCAGCTCAGT
DAM5	CCACATCAAACTGAGTAAGGAACTC	CTGCCTTAGCTGGTTGTTAGCTTCAACT
DAM6	TACTGGACCTGCGTTTGTGGAGCC	TGTTGCAGCTGGTGGAGGTGGCAATT
ppa012373m (SAP-like)	ACACAGGCTTCCTCTACTCCATCTTT	GAACCCTCATTCCGAGACATTTATCAG
ppa012188m (AWPM19-like)	CCCAGCCAATATGGCGAATATCAGAA	CATAGTGAGCAGCAGTAAGTTTGTGCT
ppa007606m (DREB2-like)	TGAAGAGGGATGATGTTACTGGCGA	CACAAAATGACCAGACATGACAAGG
ppa008651m (LEA protein)	TCATCTTCCGCTGCCTTTGTAGCCT	GACACTGCCAAGAACACCAAGGACA
ppa006974m (AFP-like)	TTCCGTTGGTGGTGGAGTGGATGCA	TTACTAGCAGGGCTTCTTGCTTCAC
ppa008548m	GAGCATGAAAGTGCTCATGGTCGATA	GCGTAACTAGAGAGTAAGATGACCTTG
ppa001608m (ABI3-like)	ATGAGGTTAGGGTCTTCAGCTACGA	TAGTGCAATTCAGATCGGCTGCGTT
ppa012320m (FT-like)	TCGGCAATTGGGTAGGCAAACA	TTATCTTCTCCTCCCTCCAGAG
Actin	CAGATCATGTTTGAGACCTTCAATGT	CATCACCAGAGTCCAGCACAAT

Plant hormone and metabolite analyses

Plant hormones and phenolics were analyzed by LC/ESI-MS-MS essentially as described (Durgbanshi et al., 2005). Briefly, fresh frozen plant material was extracted in ultrapure water using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany) after addition of 50 µl of a mixture of internal standards (see (Durgbanshi et al., 2005) for further details). After extraction and centrifugation, pH of the supernatant was adjusted to 3.0 and partitioned twice against di-ethyl-ether (Panreac, Barcelona, Spain). The organic layers were combined and evaporated in a centrifuge vacuum evaporator (Jouan, Saint-Herblain, France). The dry residue was thereafter resuspended in a water:methanol (9:1) solution, filtered and injected in a HPLC system (Alliance 2695, Waters Corp., Milford, MA, USA). Analytes were then separated in reversed-phase Kromasil 100 C18

column (100 x 2.1 mm, 5 µm particle size, Scharlab, Barcelona, Spain) using methanol and 0.01% acetic acid in water as solvents at a flow rate of 300 µl min^{-1}. The mass spectrometer, a triple quadrupole (Quattro LC, Micromass Ltd., Manchester, UK) was operated in negative ionization electrospray mode using N_2 as nebulization and desolvation gas and set at 100 and 800 l h^{-1}, respectively. During measurements, capillary voltage was set at 3.5 kV whereas cone voltage was adjusted for every analyte. The precursor and product ions as well as cone and collision voltages were selected after direct injection of pure commercial standards into the mass spectrometer.

Promoter analysis

The promoter sequence of transcript models was obtained from peach genome database at phytozome (http://www.phytozome.net/cgi-bin/gbrowse/peach/). We selected 1-kb upstream sequence from the transcriptional start site when known, and from the translation start ATG in ppa006974m and ppa008548m genes. The ABRE element was located in these sequences applying a matrix-based procedure (Gómez-Porras et al., 2007). The core sequence of the CRT/DRE element (G/A)(C/T)CGAC was screened manually (Xue 2002). Finally, the RY-repeat element involved in seed-specific expression was searched using the Plant Cis-acting Regulatory DNA Elements Database (PLACE) (Higo et al., 1999).

Results

Effect of stratification on seed germination and seedling development in peach

An *in vitro* culture experiment was performed in order to characterize the response of peach embryos to different periods of cold stratification. The early variables germination rate, defined as the rate of embryos showing an apparent radicle, and shoot emergence were measured after 0, 1, 3, 7 and 9 weeks of chilling treatment. After few days, the germination rate was nearly identical and total in embryos with periods of cold stratification of one week and longer, whereas about 80 % of non-stratified embryos also showed root elongation (Figure 5.1A). The stratification also improved shoot growth but to a lesser extent. Embryos with three weeks and longer periods of chilling showed complete shoot emergence, whereas one-week and non-stratified samples had lower rates of emergence (Figure 5.1B).

In addition to these early observations on germination and shoot emergence, germinated embryos showed long-term effects of chilling on seedling development. At the end of the *in vitro*

experiment, seedlings with 0-1 weeks of stratification were small and not viable in most cases, whereas those with 3-9 weeks of chilling had higher size with variable rates of physiological abnormalities, as dwarfing (Figure 5.2A). The height of seedlings grown *in vitro* was radically improved by the cold treatment, with optimal values in plants stratified for 7 and 9 weeks (Figure 5.2B). The rate of dwarfed individuals was also lower after 7 and 9 weeks of stratification (Figure 5.2C). A qualitative classification of physiological dwarfs attending to the height of the plant, the presence of rosettes and the size and form of leaves was performed, assigning dwarfing values from 1 (those dwarfs more similar to normal plants) to 4 (those with deeper symptoms) (Figure 5.2D).

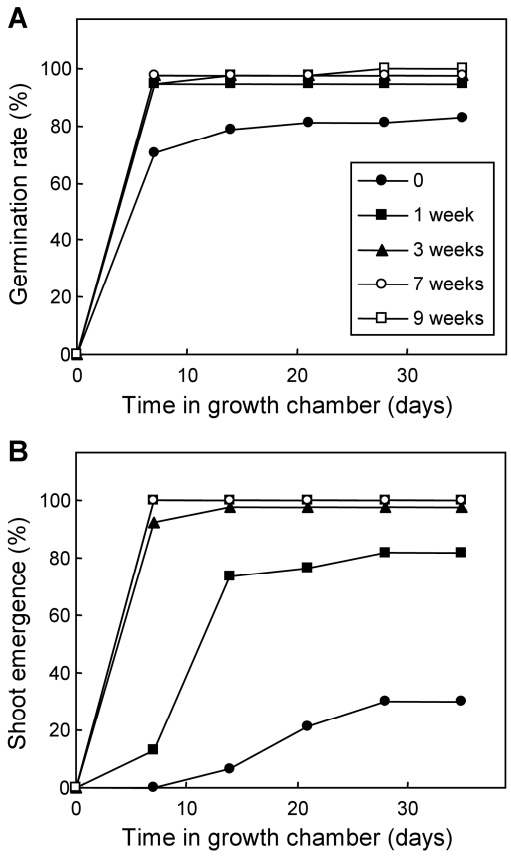

Figure 5.1. Effect of chilling on seed embryo germination and shoot emergence. The germination rate (A) and percentage of shoot emergence (B) were measured at different times after discrete periods of stratification: one week (black squares), three weeks (black triangles), seven weeks (white circles), nine weeks (white squares) and no stratification (black circles).

According to this classification, we found that chilling reduced the qualitative dwarfing level in a similar way to the dwarfing rate (Figure 5.2E). Plants stratified for 7 and 9 weeks grew actively during a time interval of three weeks, while those stratified for 3 weeks hardly increased their average height during the same period (Figure 5.2F). Interestingly, plants stratified for 7 and 9 weeks also reduced their rate of dwarfing in this time interval, due to an overall recovery of growth by the appearance of lateral shoots with normal development (Figure 5.2G).

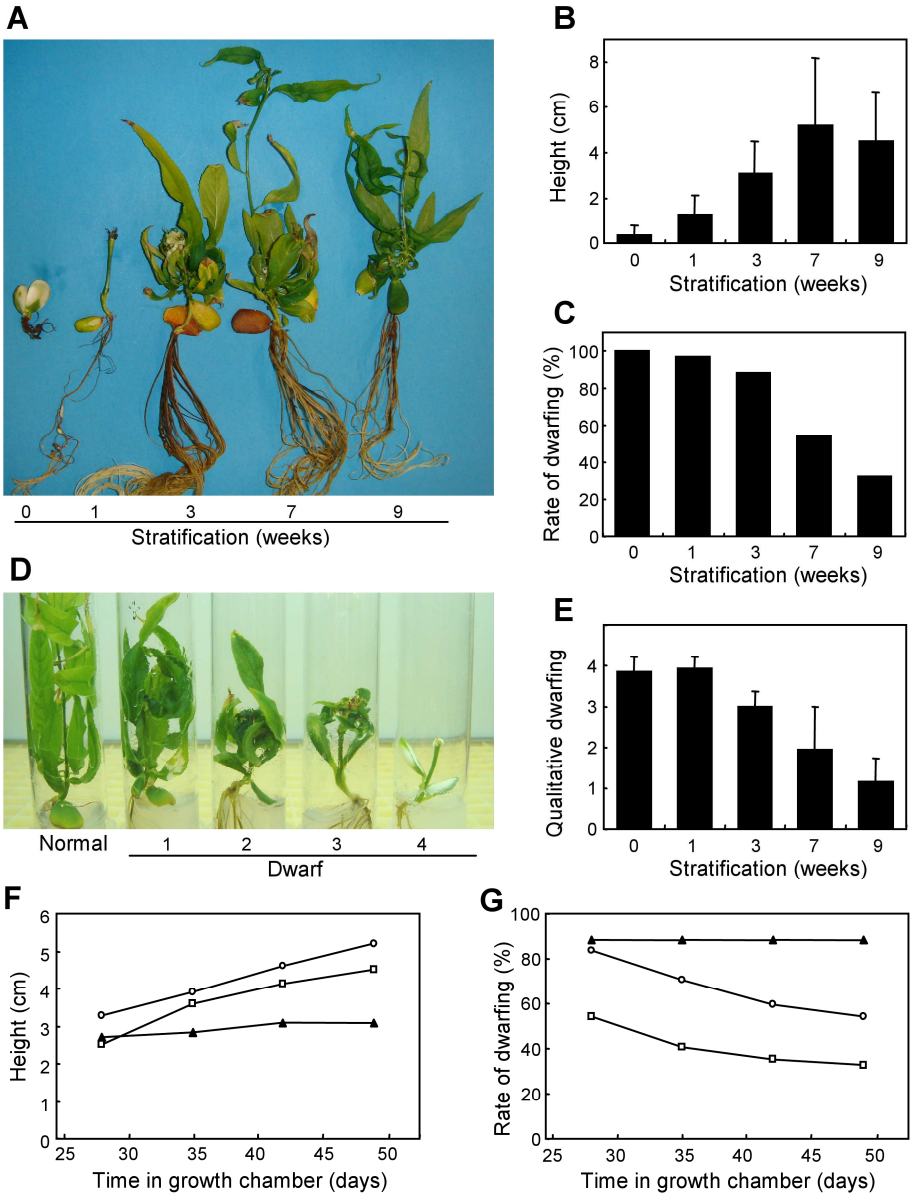

Figure 5.2. Effect of chilling on the physiological dwarfing of seedlings. (A) Representative plants subject to different stratification periods were photographed at the end of the in vitro experiment. (B) Average height of seedlings after seven weeks in growth chamber. (C) Percentage of dwarfed plants after seven weeks in growth chamber. (D) Representative seedlings showing different qualitative levels of dwarfism: level 1 individuals were slightly shorter than normal ones and had both normal and curved leaves; level 2 seedlings had shorter internodes and mostly abnormal leaves; level 3 dwarfism led to small deformed leaves grouped in a rosette-like structure; and finally level 4 dwarfs had a drastic reduction of growth and barely recognizable leaves. (E) Average qualitative dwarfing of seedlings grown for seven weeks. The height (F) and rate of dwarfing (G) of seedlings obtained from embryos stratified for three (black triangles), seven (white circles) and nine weeks (white squares), were measured at different times. In (B) and (E), error bars represent standard deviation, n>35.

Regulation of gene expression in stratified seeds

Previous transcriptomic approaches performed in our group, based on transcript enrichment by suppression subtractive hybridization (SSH) and cDNA microarray hybridization, led to a set of genes differentially expressed during bud dormancy release in peach (Leida et al., 2010; 2012). We selected several of these genes, previously validated by quantitative real-time RT-PCR on buds, for its expression analysis in peach embryos subject to the stratification treatments described above. The genes *DAM1*, *DAM4*, *DAM5* and *DAM6* are components of the *DAM* multigene family coding for MADS-box transcription factors involved in bud dormancy regulation in peach and other

species. *DAM1* and *DAM6* reduced gradually their expression during the chilling treatment to reach their lowest values in 7 and 9 weeks samples (Figure 5.3). *DAM5* gene showed a sharp and permanent down-regulation after one week under stratification, in contrast with the increase of *DAM4* expression in the same period. However, *DAM4* transcript accumulation experienced a slight reduction in consecutive time samples.

We assayed other genes down-regulated during bud dormancy release, related to elements of the ABA and drought stress response in *A. thaliana* (Leida et al., 2012). The genes coding for STRESS ASSOCIATED PROTEIN (SAP)-like (peach transcript model ppa012373m), AWPM19-like (ppa012188m), DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2 (DREB2)-like (ppa007606m), and LATE EMBRYOGENESIS ABUNDANT (LEA) protein (ppa008651m) reduced drastically their expression in stratified embryos (Figure 3), as previously observed in peach buds subject to environmental chilling. The *ABA-INSENSITIVE FIVE BINDING PROTEIN (AFP)*-like gene showed an early down-regulation in the first chilling week, followed by a slow recovery to reach initial expression values. Most of transcripts accumulating in buds after dormancy release had a negligible expression in embryos, but ppa008548m coding for a putative cinnamoyl-CoA reductase showed detectable expression after 7 and 9 weeks of stratification.

We analyzed by quantitative real-time RT-PCR two additional transcripts that were not previously identified in our transcriptomic experiments, but were considered putative candidates to affect dormancy responses in seeds and buds. The *ABA-INSENSITIVE 3 (ABI3*)-like transcript (ppa001608m) strongly declined during the first three weeks of chilling, whereas *FLOWERING LOCUS T (FT)*-like (ppa012320m) had higher expression values in last stratification stages (Figure 5.3).

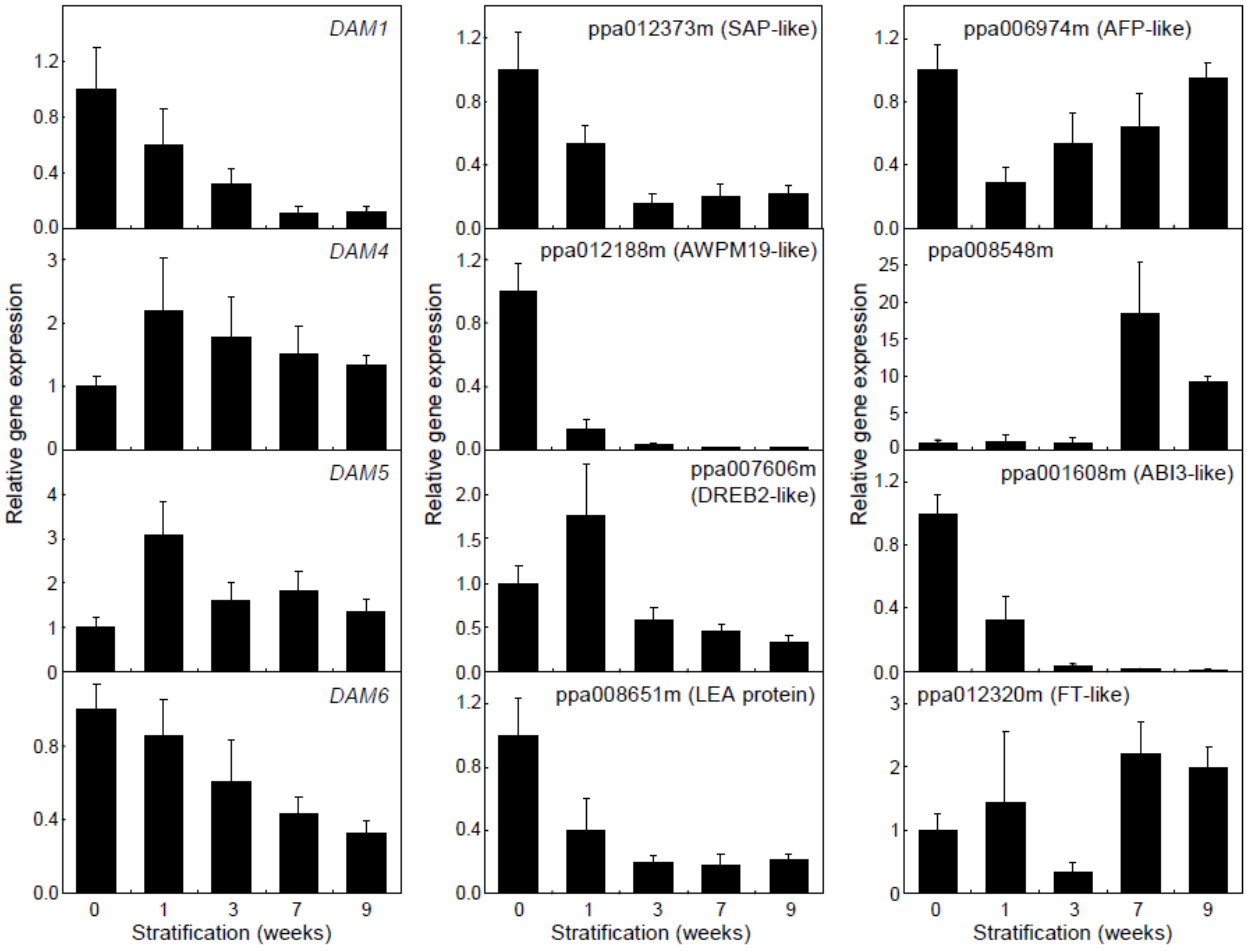

Figure 5.3. Expression of selected genes in stratified embryos. Relative expression of bud-dormancy related genes was determined by quantitative real-time RT-PCR with specific primers (Table 5.1). The name of the gene or transcript model is shown in the upper side of the graph. Expression levels are relative to Actin. An expression value of one is assigned to the non-stratified sample. Data are means from two biological replicates, with error bars representing standard deviation.

ABA content rapidly decreases during stratification

The ABA content in stratified embryos decreased from about 140 ng per gram (fresh weight) to less than 10 ng after one week of chilling. The hormone content did not change relevantly after longer chilling treatments (Figure 5.4).

In order to identify cis-regulatory elements responding to ABA and drought stress in the genes analyzed by quantitative real-time RT-PCR, we examined 1 kb sequence of their promoters beginning from the transcription start when known, otherwise the translation start ATG was used. We performed a search of ABA-responsive elements (ABRE), C-repeat/dehydration-responsive elements (CRT/DRE), and RY repeats as described in Materials and Methods. Genes repressed by chilling treatment had at least one of these three elements in their promoters with the exception of

DAM1 gene, and four of them had the three elements (Figure 5.5). We did not find any of these regulatory sequences in the promoter of the ppa008548m gene induced by cold stratification.

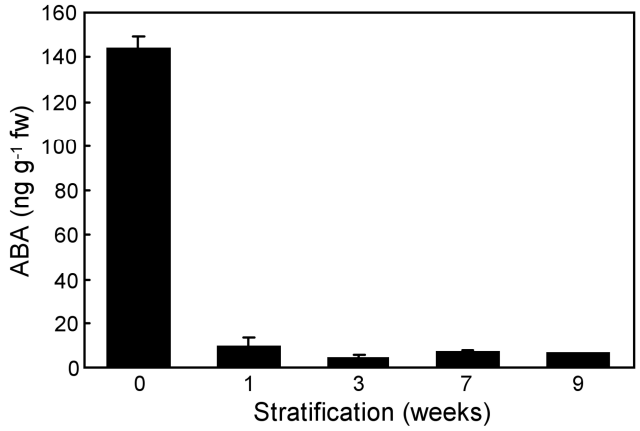

Figure 5.4. Abscisic acid content in stratified embryos. Abscisic acid content was determined as detailed in Materials and Methods. Data are means from three biological replicates, with error bars representing standard deviation.

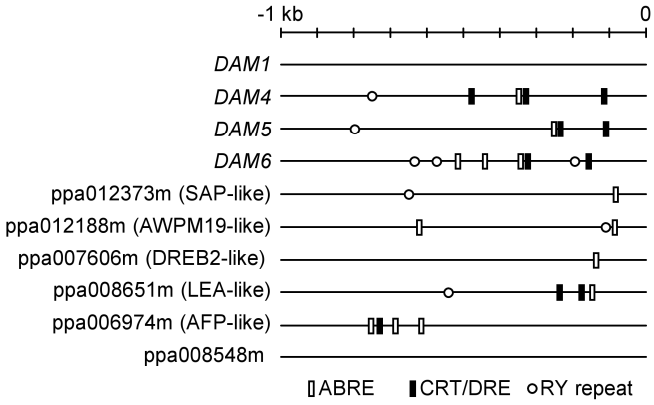

Figure 5.5. Predicted cis-elements in the promoter of chilling-regulated genes. The ABA-response element (ABRE, white rectangles), C-repeat/dehydration-responsive element (CRT/DRE, black rectangles), and seed-specific RY repeats (white circles) were localized in the 1-kb upstream sequence from the transcriptional start site when known, and from the translation start (in ppa006974m and ppa008548m), of chilling affected genes, as described in Materials and Methods.

Phenylpropanoid metabolites accumulate during prolonged cold treatment

A systematic analysis of metabolites was performed in stratified embryos. We found several intermediate metabolites of the phenylpropanoid biosynthesis pathway accumulating in embryos stratified for 7 and 9 weeks. Among them, the ferulic acid approximately doubled its initial content during long chilling treatments (Figure 5.6). Over-accumulation of other compounds from this pathway was even more pronounced. Caffeic, coumaric and cinnamic acids increased respectively 40-fold, 100-fold and 4-fold after a stratification period of 7 weeks, followed by a slight decrease two weeks later. Interestingly, the hormone salicylic acid increased its content during the chilling treatment in a similar way to phenylpropanoid metabolites (Figure 5.6). However, the addition of salicylic acid to the culture medium at different concentrations did not ameliorate the germination or physiological dwarfing defects observed in peach seeds (data not shown).

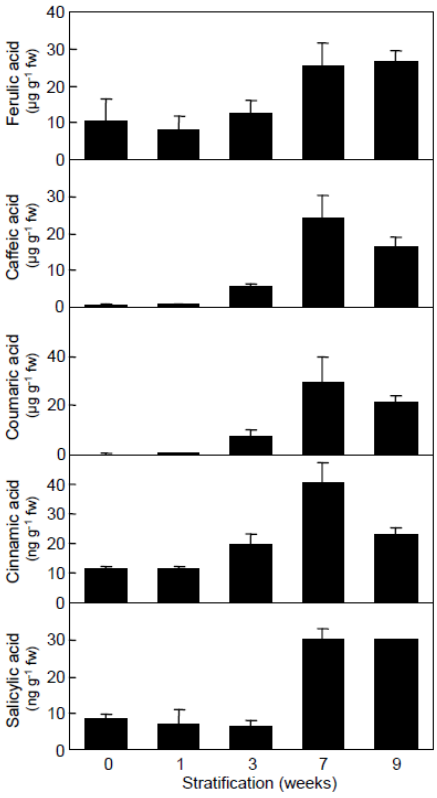

Figure 5.6. Accumulation of phenylpropanoids in stratified embryos. Ferulic, caffeic, coumaric and cinnamic acids, and the phytohormone salicylic acid were determined in embryos after different chilling treatments. Data are means from three biological replicates, with error bars representing standard deviation.

Discussion

Stratification has short and long-term effects on peach germination and development

Short chilling treatments of one and three weeks were sufficient for optimal germination and shoot emergence, respectively. These chilling requirements for radicle and shoot growth were lower than those reported by other physiological studies (Davies 1983; Frisby & Seeley, 1993), which may be due to genotype-based differences or most likely to previous removal of the seed coat in our samples. Coat excision helped to discard the mechanical and physiological contribution of the coat to seed dormancy, which in consequence was exclusively dependent on the embryo component. This experimental procedure was essential to distinguish two separate effects of cold stratification on seed germination and seedling development. Thus, in addition to early benefits of chilling on germination and shoot emergence, longer chilling treatments of seven and nine weeks contributed to prevent the physiological dwarfing of seedlings. This double effect of stratification on germination and subsequent development of seedlings has been noted previously in stone-fruit species (Hartmann et al., 2011; Martínez-Gómez & Dicenta, 2001), but no molecular mechanisms have been proposed to explain it. The improved germination ratio observed after one week of chilling was associated to a drastic reduction in ABA content, in close agreement with the known role of

this hormone in the induction and maintenance of dormancy in seeds. However, the alleviation of dwarfing abnormalities at long chilling treatments was not related to changes in ABA, which precludes a function of ABA in post-germinative dormancy events.

Other compounds as salicylic acid and the phenylpropanoids ferulic, caffeic, coumaric and cinnamic acids accumulated in embryos stratified for seven to nine weeks leading to seedlings with low rate of dwarfing. Overproduction of these compounds could benefit the normal development of seedlings or simply be a biochemical feature of dormancy-released embryos prepared to develop normally, however we have not obtained experimental evidences supporting any of these hypotheses. The phenylpropanoids pathway results in the synthesis of the monolignols p-coumaryl, coniferyl and sinapyl alcohols, which are the main precursors of lignin polymers, through the sequential activity of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) enzymes (Boerjan et al., 2003). Lignin deposition contributes to the secondary thickening of the cell wall, and takes part in xylem cell differentiation. The synthesis of this complex polymer becomes detectable in early stages of seedling development in *A. thaliana*, associated to the developing vasculature (Dharmawardhana et al., 1992). Interestingly, a triple *A. thaliana* null mutant in one CCR and two CAD genes, leading to a strong reduction in lignin deposition, displayed a severe dwarf phenotype and abnormal leaf morphology (Thévenin et al., 2011), resembling the symptoms of physiological dwarfing described in this work. These observations and the high-chilling dependent induction of ppa008548m in stratified embryos (Figure 5.3), coding for a predicted protein similar to CCR, suggest the participation of phenylpropanoids and lignin biosynthesis pathways in the alleviation of anomalies associated to physiological dwarfing.

Bud and seed dormancy regulate a common set of genes

The quantitative real-time RT-PCR analysis of genes previously related to bud dormancy revealed a parallel pattern of gene expression in buds and embryos. A relevant number of genes down-regulated during bud dormancy release after the fulfilment of cultivar-specific chilling requirements were also repressed by cold stratification in embryos. This suggests the presence of common regulatory pathways in dormancy release mechanisms of buds and seeds. The *in silico* search of known homologous genes in other species and cis-elements in their promoters pointed to a common effect of ABA and drought signalling on such genes. However ABA could only account for transcript accumulation changes observed during the first week of chilling, due to the stabilization

of ABA content after this time. Consequently, later down-regulation of the expression of these genes should be assigned to additional regulatory pathways.

DAM genes are particularly interesting among this set of genes. *DAM* have been related to bud dormancy maintenance by expression and functional studies in multiple species, and are considered the major known regulators of this process. The fact that *DAM1*, *DAM5* and *DAM6* are also significantly repressed during chilling treatment of the embryo suggests their participation in mechanisms of transcriptional regulation associated to release of seed dormancy by stratification. Additional evidences of *DAM* function in seed dormancy would be obtained by further analyses of the *evergrowing* mutant in peach carrying a deletion in *DAM* genes (Bielenberg et al., 2008) and transgenic plants expressing constitutively these genes (Sasaki et al., 2011).

Previous molecular studies relating seed germination and flowering in *A. thaliana* have been recently published. The *FLOWERING LOCUS C (FLC*) gene coding for a MADS box transcription factor involved in flowering time regulation through the vernalization pathway, also affected the temperature-dependent germination of dormant seeds (Chiang et al., 2009). The effect of FLC on seed germination was most likely mediated by *FLOWERING LOCUS T (FT)*, which also takes part in the flowering pathway. Interestingly, a peach gene similar to *FT* increased its expression after stratification during 7-9 weeks (Figure 5.3), which points to the presence of a related signalling pathway in peach. The RNA Polymerase II Associated Factor 1 Complex (PAF1C) of *A. thaliana* has been also proposed to have a dual role in flowering and seed dormancy (Liu et al., 2011). Moreover, a poplar orthologue of *ABSCISIC ACID INSENSITIVE 3 (ABI3)* gene, involved in the ABA-dependent expression of many seed-specific genes in *A. thaliana*, is expressed in buds during bud set and causes some alterations in bud development when overexpressed and silenced (Rohde et al., 2002).

The transcriptional similarities between bud and seed dormancy highlighted in this work may also be relevant for plant breeding purposes. The selection of early and late flowering genotypes from a segregating population usually requires the arduous evaluation of large collections of individuals, which could be improved by a previous selection of the desirable trait at the seed level. Previous studies found a positive correlation between the chilling requirements for seed germination and blooming in almond and apple (Kester et al., 1977; Mehlenbacher & Voordeckers, 1991). This work contributes to characterize the molecular bases underlying these and other physiological observations with high interest to plant breeders.

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6. GENERAL DISCUSSION

DAM genes are relevant regulators of dormancy processes

The peach transcriptional models ppa011123m, ppa010822m and ppa010714m, corresponding to MADS-box genes *DAM4, DAM5* and *DAM6,* were identified in an initial transcriptomic study based on the SSH procedure (Leida et al., 2010) and in the subsequent microarray hybridization experiment comparing 10 cultivars with different chilling requirements (Leida et al., 2012). Real-time RT-PCR analysis confirmed that *DAM* gene expression changes during flower bud development according to the dormancy stage, and correlates with dormancy-related parameters of different cultivars. Other authors, working independently, observed similar season and dormancy-dependent modifications of *DAM*-like gene expression in peach buds (Jiménez et al., 2010b; Yamane et al., 2011), and in other species as raspberry (Mazzitelli et al., 2007), Japanese apricot (Yamane et al., 2008), poplar (Druart et al., 2007), and leafy spurge (Horvath et al., 2008).

The central role of DAM genes in dormancy regulation of vegetative and reproductive buds has been confirmed by additional functional approaches. Four of the six tandemly arrayed *DAM* genes were found deleted in the *evg* mutant of peach showing non-dormant phenotype, suggesting that they are required for dormancy setting up (Bielenberg et al., 2008). Moreover, a genetic mapping approach using a family of peach, segregating for chilling requirements, heat requirement and flowering time identified a major QTL for these traits that fairly overlapped with *DAM* locus on linkage group I (Fan et al., 2010). Transgenic poplar plants expressing constitutively a *DAM*-like gene from Japanese apricot showed growth cessation and terminal bud set under favourable conditions (Sasaki et al., 2011), whereas overexpression of peach *DAM6* in plum, resulted in accentuated branching and dwarfing of plants suggesting a growth inhibitory effect of these genes (Fan, 2010).

Interestingly, the deletion of four of the *DAM* genes in the evg mutant caused the transcriptional inhibition of the other two structurally intact genes of the family (Bielenberg et al., 2008). This observation and the apparently sequential activation of *DAM1* and *DAM6* genes (Leida et al., 2010) suggest a kind of reciprocal regulation between these genes, which could only be tested by biochemical ways.

In order to know the mechanism of dormancy regulation we study the molecular mechanisms of *DAM6* down-regulation concomitant with dormancy release in flower buds. A ChIP analysis of *DAM6* promoter and structural gene revealed chromatin modification events similar to those observed in vernalization of *Arabidopsis* and cereals.

Certain biannual *Arabidopsis* lines require a prolonged period of cold accumulation during winter prior to flowering named vernalization. Vernalization represses the expression of the *FLC* gene, coding for a MADS-box transcription factor that inhibits flowering transition. The mechanism of *FLC* repression involves numerous epigenetic events, including micro RNA synthesis and covalent modification of histones. The N-terminal tail of histone H3 located on *FLC* promoter is highly acetylated and trimethylated at the lysine K4 when the gene is transcriptionally active. Changes triggered by vernalization on H3 include demethylation of K4, deacetylation of K9 and K14, and methylation of K27 and K9. Trimethylation of H3K27, catalyzed by PcG complexes, plays a critical role in maintenance of *FLC* stable repression. Some similarities between vernalization gene repression in Arabidopsis and PcG-mediated gene repression in animals are evident despite certain differences (Sung & Amasino, 2005).

In cereals, vernalization response involves epigenetic regulation of the MADS-box gene *VRN1*, targeted by histone modification complexes similar to those repressing *FLC*. H3K27 trimethylation at the *VRN1* locus causes mitotically stable repression of *VRN1* until plants experiment cold conditions. Vernalization activates protein complexes that reverse these modifications to activate *VRN1* expression, by increasing the rate of trimethylated H3K4 and decreasing the rate of trimethylated H3K27, as described in barley *HvVRN1* locus (Oliver et al., 2009).

We showed that the MADS-box gene *DAM6* is transcriptionally active in dormant peach flower buds collected in October. At this point a short chromatin region around its ATG is trimethylated at H3K4 and acetylated at the N-terminal tail of H3. Concomitantly with *DAM6* repression, H3K4 became demethylated and H3 deacetylated. Later H3K27 was trimethylated along a genomic region larger than 4kb, including promoter, coding sequence and intron. We postulated a possible sequence of *DAM6* chromatin modification events during dormancy regulation: 1) Trimethylation of H3K4 and acetylation of H3 are modifications of the chromatin structure associated to active gene expression. 2) Buds undergoing dormancy lose the trimethylation mark at H3K4 and H3 acetylation, leading to gene repression. 3) Finally, trimethylation of H3K27 mediates the stable repression of the gene.

These observations suggest that *DAM6* responds to chilling accumulation by epigenetic mechanisms similar to *FLC* and *VRN1*. The common enrichment of trimethylated H3K27 along the transcriptionally repressed chromatin of *DAM6*, *FLC* and *VRN1* supports the presence of related PcG-like multiprotein complexes with methyl-transferase activity. Thus, PcG complexes are

potentially involved in the stable repression of *DAM6* after dormancy release until the next dormancy cycle.

Due to their relevance in dormancy regulation, *DAM* genes could be utilized as expression markers to assess the dormancy stage of an individual plant and to evaluate the chilling requirements of new cultivars. In fact, we studied the expression pattern of *DAM5*, together with other transcripts (BD396, DB247, SB280 and PpB63), they correlated well with chilling requirements values of five varieties differing in chilling requirements ('Big Top', 'Catherina', 'Fergold', 'Maruja' and 'Springlady') measured following Utah and Dynamic models.

A further characterization of the function of these genes will be essential for understanding and future manipulation of dormancy in perennial trees. A deeper analysis of transgenic plants overexpressing these genes will contribute to understand their influence in plant development and dormancy regulation. Additional molecular approaches as the two-hybrid system and chromatin immunoprecipitation using specific antibodies may help to clarify the protein interactions required for proper function of these factors, and to identify target promoters. These and other biochemical and molecular approaches will improve our basic knowledge on the signal transduction pathways involved in dormancy regulation.

Dormancy in bud and seed has common regulatory pathways

Some of the genes identified in our transcriptomic experiments using flower buds, as *DAM1*, *DAM5* and *DAM6*, were also regulated during the cold stratification of peach seeds, suggesting the presence of common regulatory pathways in the dormancy process of buds and seeds. These similarities between bud and seed dormancy have potential implications in the evaluation of bud chilling requirements of peach genotypes at seed level. The measurement of the relative expression of *DAM* and other genes in seeds could provide a method for evaluating chilling requirements of plant material at very early stages of development greatly reducing the time needed for evaluating plant material in breeding programs. A positive correlation between seed germination and blooming date has been found in apple and almond, supporting this link between dormancy in buds and seeds at the phenotypical level (Kester et al., 1977; Mehlenbacher et al., 1991).

Many ABA dependent genes are down-regulated during dormancy release

ABA has been proposed to promote and maintain bud dormancy (Arora et al., 2003; Horvath et al., 2003; Rohde & Balherao, 2007) although few molecular data support this prediction. A significant number of genes identified in this work were homologous to ABA and drought related genes from

other species. Ppa006974m codes for a protein similar to ABA-INSENSITIVE5 (ABI5) binding protein (AFP) involved in signal transduction in Arabidopsis. AFP binds and promotes the degradation of ABI5. ABI5 is a basic leucine zipper (bZIP) transcription factor that regulates ABA-dependent genes by binding to the ABA-responsive element, ABRE (Lopez-Molina et al., 2003). Additionally, drought stress modulates gene expression through the dehydration-responsive element (DRE) and their DRE-binding proteins (DREB; Liu et al., 1998), similar to the transcript model ppa007606m encoding a DREB2C-like factor. The product of ppa008849m is similar to calcium binding annexins involved in ABA and osmotic stress signal transduction in *Arabidopsis* (Lee et al., 2004).

Our results contributed to the identification of peach genes regulated by ABA, drought and salt stress as ppa008979m (AtMYB44-like), ppa008651m (LEA), ppa012373m (A20/AN1 zinc-finger), ppa005514m (dehydrin) and ppa012188m (AWPM-19-like), involved in cold hardening and frost tolerance. Several orthologues of these genes conferred increased cold resistance in different transgenic plants (Jung et al., 2008; Mukhopadhyay et al., 2004; Lee et al., 2010; Artlip et al., 1997; Koike et al., 1997).

For instance, ppa012373m protein shows A20/AN1 zinc-finger domains that have been already described in other eukaryotes. In animals, ZNF216 protein regulates nuclear factor NFkB, involved in the immune and inflammation response reaction (Hishaya et al., 2006). The same protein, which plays a critical role in muscle atrophy, contains an A20 domain that binds ubiquitin and participates in the ubiquitin-proteasome pathway of protein degradation (Huang et al., 2004). In plants, proteins related to ppa012373m that are expressed under cold and salt stress conditions have been grouped into the stress associated protein (SAP) family. Experiments on *Arabidopsis* showed that modifications in the cytosolic redox state, as the one induced by stress conditions, cause a conformational change in SAP proteins. The fast up-regulation of SAP12 in *Arabidopsis* after 6 h of stress and its subsequent down-regulation after 48 h, suggests a possible role in rapid transmission of redox information under stress conditions (Ströher et al., 2009). If these proteins are part of an ABA or stress-dependent pathway for regulation of dormancy events may only be assessed after deep biochemical and genetic studies.

7. CONCLUSIONS

1. In two different transcriptomic approaches, we obtained 137 cDNAs related to dormancy release in flower buds of peach. Some of them, as ppa011123m, ppa010822m and ppa010714m (*DAM4*, *DAM5* and *DAM6*) were previously found associated to dormancy processes.
2. The isolation of certain transcripts involved in ABA, salt and drought stress regulation, such as ppa007606m (DREB2C-like), ppa006974m (AFP-like), and ppa008849m (annexin-like), supports the role of ABA and drought responses in bud dormancy events, and contributes to identify putative regulatory factors of both pathways in peach.
3. *DAM6* gene expression is regulated at the chromatin level. Demethylation of H3K4 and deacetylation of H3 in the region around the ATG, and trimethylation of H3K27 in a large genomic stretch of at least 4 kb, associate to *DAM6* repression, concomitantly with dormancy release.
4. The expression profile of five cDNAs (*DAM5*, DB396, DB247, SB280 and PpB63) correlated well with the chilling requirements of five cultivars (‘Big Top’, ‘Catherina’, ‘Fergold’, ‘Maruja’ and ‘Springlady’) measured following the Utah and Dynamic models. These genes were proposed as expression markers for single point evaluation of the dormant stage of peach genotypes.
5. Cold stratification had a short-term effect on germination of peach seeds, coincident with a decrease in ABA content.
6. We observed that a long-term (7-9 weeks) seed stratification have a positive effect in decreasing the developmental abnormalities on seedlings named "physiological dwarfing". This amelioration was associated with the accumulation of several intermediate metabolites of the phenylpropanoid biosynthesis pathway (salicylic, ferulic, caffeic, coumaric and cinnamic acids), involved in xylem synthesis.
7. A set of genes regulated during dormancy release shared by buds and seeds indicated the presence of common regulatory pathways of dormancy release in both organs.

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