UNIVERSITAT POLITÈCNICA DE VALÈNCIA

A DYNAMIC SNAPSHOT OF BUD DORMANCY IN PEACH

PhD. THESIS

Submitted by

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Abstract

Plant species from temperate climates have developed adaptive mechanisms in order to cope with seasonal changes in temperature and other environmental constraints that limit the normal growth. One example of these fascinating adaptations occurs during winter, when deciduous trees form buds in order to protect growing cells from unfavourable environmental conditions. In that period, trees cease growth and remain in a quiescent state, called dormancy. Buds are able to release dormancy only when a given amount of chilling is perceived. In that point, buds enter in a period known as ecodormancy in which they have the ability to sprout and flower only when conditions become favourable. Under a situation of climate change, the amount of available winter chilling is expected to decrease, altering dormancy release and flowering. The study of genes regulated during dormancy is crucial to understand the process with the final objective to develop new tools to adapt to new climatic conditions. Therefore, the general aim of this thesis is to study the dormancy process from a molecular point of view identifying mechanisms and targeting genes that control it. In order to do that we have focused on the study of three genes that are differentially expressed during reproductive bud development within the conceptual framework of the three major processes that converge spatially and temporally in a reproductive bud: dormancy, stress tolerance and flower development.

The first gene is down-regulated in dormancy release flower buds and encodes a STRESS ASSOCIATED PROTEIN (*PpSAP1*) that contains Zn-finger domains A20 and AN1. SAP proteins have been related to stress tolerance response in both plants and animals and in fact, we have shown that drought stress induces its expression in buds, resembling other SAP genes in plants. Moreover, the constitutive expression of *PpSAP1* in plum increases its tolerance to water stress by increasing water retention. Likewise, transgenic plum plants show leaf alterations related to reduced cell size concomitant with the down-regulation of genes involved in cell growth. All these studies suggest a dual role of *PpSAP1* in stress tolerance response and cell growth during peach dormancy.

The second gene is *PpeS6PDH,* coding for an enzyme with sorbitol-6-phosphate dehydrogenase activity. *PpeS6PDH* is differentially regulated during bud development, highly expressed in dormant buds consistently with sorbitol accumulation. Concomitantly with *PpeS6PDH* down-regulation in dormancy-released flower buds, chromatin around the translation start site of the gene shows changes in the methylation state of specific residues of histone H3 (H3K4 and H3K27). These data suggest the transcriptional regulation of *PpeS6PDH* expression by chromatin modification mechanisms. Moreover, abiotic stresses affect *PpeS6PDH* expression. Low temperature treatments induce gene expression in buds and leaves, whereas desiccation up-regulates *PpeS6PDH* in buds and represses the gene in leaves. These data suggest the participation of *PpeS6PDH* in tolerance against cold and water deficit stresses in buds.

Finally, the third gene is *PpeDAM6,* one of the major regulators of bud dormancy in peach. *PpeDAM6* is sharply down-regulated during bud development concomitantly with dormancy release events. This repression is in part due to the direct binding of PpeBPC1, a BASIC PENTACYSTEINE PROTEIN, to the GAGA motifs present in an intronic regulatory region of *PpeDAM6* gene that becomes enriched in H3K27me3 chromatin modification after dormancy release. In addition, the ectopic expression of *PpeDAM6* in *Arabidopsis* shows abnormal flower phenotypes resembling 35S::*SVP* plants. On the other hand, overexpression in plum causes stunted growth in the transgenic lines due to an altered hormonal homeostasis. The changes in hormone content are mediated by the modulation of genes involved in jasmonic acid, cytokinins and gibberellic acid metabolism and signalling pathways. These results suggest that *PpeDAM6* works as a master growth repressor maintaining dormancy, stress tolerance response and flowering inhibition by mainly modulating hormone homeostasis.

Therefore, this thesis provides a dynamic snapshot of different molecular mechanism that take place inside the bud. The studied genes have a crucial role regulating dormancy processes, stress tolerance response and flowering pathways and all of them are potential candidate genes for breeding new plants more adapted to the climate change.

Resumen

Las plantas que viven en climas templados han tenido que desarrollar mecanismos adaptativos para hacer frente a las distintas restricciones medioambientales, como por ejemplo los cambios estacionales de temperatura, que limitan su crecimiento normal. Un ejemplo de una adaptación fascinante ocurre durante los meses de invierno, cuando los árboles caducos forman unas estructuras denominadas yemas para proteger a las células en crecimiento de las condiciones medioambientales desfavorables. En ese periodo, el árbol cesa su crecimiento y permanece en un estado conocido como latencia. Estas yemas son capaces de salir de latencia únicamente cuando reciben una cantidad determinada de frío. En ese momento las yemas entran en un periodo conocido como ecolatencia, en el cual la yema ya es capaz de brotar pero únicamente lo hará cuando las condiciones medioambientales vuelvan a ser favorables. En un escenario de cambio climático, se espera que disminuya la cantidad de frío invernal disponible, alterando por tanto la salida de latencia y consecuentemente la floración de la mayoría de árboles frutales. El estudio de genes regulados durante la latencia es crucial para comprender este proceso, y así poder desarrollar nuevas herramientas que permitan adaptarnos mejor a las nuevas condiciones climáticas. Por esta razón, el objetivo general de esta tesis es el estudio de la latencia desde un punto de vista molecular, identificando mecanismos y genes diana que la controlen. Para ello, nos hemos centrado en el estudio de tres genes que se expresan de manera diferencial durante el desarrollo de una yema reproductiva en melocotón, bajo el marco conceptual de los tres procesos que convergen espacialmente y temporalmente en una yema reproductiva: latencia, tolerancia a estrés y desarrollo floral.

El primer gen que se estudió codificó para una STRESS ASSOCIATED PROTEIN (*PpSAP1*) con dos dominios tipo Zn-finger, A20 y AN1 que disminuye su expresión durante la latencia. Las proteínas tipo SAP se han relacionado con resistencias a distintos tipos de estrés tanto en plantas como en animales. De hecho, se ha visto que *PpSAP1* aumentó su expresión en yemas de melocotón bajo condiciones de

estrés por sequía, de forma similar a como lo hacen otras SAP en distintas plantas. Además, la expresión ectópica de *PpSAP1* en ciruelos transgénicos ha permitido aumentar la tolerancia a estrés hídrico en estas líneas al incrementar la cantidad de agua retenida. Asimismo, estas plantas transgénicas también mostraron alteraciones en el tamaño de las hojas, provocadas principalmente por una menor área celular de las células que formaban parte de ellas y relacionadas con una represión de distintos genes implicados en crecimiento celular. Todo ello sugiere que *PpSAP1* probablemente tenga una doble función relacionada tanto con resistencia a estrés como con crecimiento celular durante la latencia de melocotonero.

El segundo gen de estudio fue *PpeS6PDH,* el cual codifica para una enzima con actividad sorbitol-6-fosfato deshidrogenasa. *PpeS6PDH* está diferencialmente regulado durante el desarrollo de la yema, aumentando su expresión en yemas latentes de manera consistente a la acumulación de sorbitol. Simultáneamente a la disminución de *PpeS6pDH* en las yemas no latentes, alrededor del sitio de inicio de la traducción del gen se mostraron cambios a nivel de cromatina en el estado de metilación de los residuos específicos de la histona H3 (H3K4 y H3K27). Estos datos apuntan a la existencia de una regulación transcripcional de *PpeS6PDH* a nivel de modificaciones de la cromatina. Además, también se ha visto que distintos tipos de estrés abiótico afectan a la expresión de *PpeS6PDH*. Tratamientos con bajas temperaturas inducieron su expresión tanto en yemas como en hojas, mientras que la desecación aumentó la expresión en yemas pero no en hojas. Estos estudios sugieren que la función de *PpeS6PDH* durante la latencia de melocotonero es dar tolerancia a estrés por frío y sequía.

Finalmente, el tercer gen de estudio fue *PpeDAM6*, uno de los mayores reguladores de la latencia en yemas de melocotonero. *PpeDAM6* está fuertemente reprimido durante el desarrollo de la yema con una relación directa con los eventos de salida de latencia. Esta represión se debe en parte a la unión directa de PpeBPC1, una BASIC PENTACYSTEINE PROTEIN, a dos motivos GAGA presentes en la región intrónica reguladora de *PpeDAM6.* Justamente esta región se encuentra modificada a nivel de cromatina con un enriquecimiento en

H3K27me3 después de la salida de latencia. Además, la expresión ectópica de *PpeDAM6* en *Arabidopsis* mostró fenotipos de floración anormal parecidos a los producidos en plantas 35S::*SVP*. Por otro lado, la sobreexpresión en ciruelos provocó retrasos en el crecimiento de las líneas transgénicas, debido a una alteración en los niveles hormonales. Así mismo, se determinó que estos cambios en la homeostasis hormonal estaban producidos por la regulación diferencial de genes involucrados en las rutas del ácido jasmónico, las citoquininas y del ácido giberélico en las plantas transgénicas. Estos resultados sugieren que *PpeDAM6* actúa como un represor máster del crecimiento, manteniendo la latencia, la respuesta de tolerancia a estrés y la inhibición floral a través de la regulación del equilibrio hormonal.

Con todo ello, esta tesis proporciona una instantánea dinámica de los diferentes mecanismos moleculares que tienen lugar dentro de la yema. Los genes estudiados tienen una función crucial regulando tanto el proceso de latencia como la respuesta de tolerancia a estrés y las rutas de floración, y todos ellos son potenciales candidatos para mejorar nuevas plantas más adaptadas al cambio climático.

Resum

Les plantes que viuen en climes templats han hagut de desenvolupar mecanismes adapatatius per fer front a les diferents restricciones mediambientales, com per exemple el canvis estacionals de temperatura, que limiten el creixement normal. Un bon exemple d'una adaptació fascinant ocurreix durant els mesos d'hivern, quan els arbres caducs formen estructures denominades gemmes per protegir a les cèl·lules en creixement de les condicions mediambientals desfavorables. En aquest període, l'arbre para el seu creixement i roman en un estat conegut com latència. Aquestes gemmes únicamente poden eixir de la latència quan reben una quantitat determinada de fred. En aquest moment les gemmes entren en un període conegut com ecolatència, en el qual la gemma té la capacitat de brotar però únicament ho farà quan les condicions mediambientals tornen a ser favorables. En un escenari de canvi climàtic, és esperable que disminuesca la quantitat de fred hivernal disponible, alterant per tant l'eixida de latència i conseqüentment la floració de la majoria dels arbres fruiters. L'estudi de gens regulats durant la latència és fonamental per comprendre aquest procés, i així poder desenvolupar noves eines que permetran adpatar-nos millor a les noves condicions climàtiques. Per aquesta raó, l'objectiu general d'aquesta tesi és l'estudi de la latència des d'un punt de vista molecular, identificant mecanismes i gens diana que la controlen. Per això, ens hem centrat en l'estudi de tres gens que s'expressen d'una manera diferencial durant el desenvolupament d'una gemma reproductiva en el préssec, sota el marc conceptual dels tres processos que convergeixen espacialment i temporalment en una gemma reproductiva: latència, tolerància a estrés i desenvolupament floral.

El primer gen d'estudi codifica per a una STRESS ASSOCIATED PROTEIN (*PpSAP1)* amb dos dominis tipus Zn-finger, A20 i AN1, i disminueix la seua expressió durant la latència. Les proteïnes tipus SAP s'han relacionat amb resistències a diferents tipus d'estrés tant en plantes com en animals. De fet, s'ha vist que *PpSAP1* va augmentar la seua expressió en gemmes de préssec sota condiciones d'estrés per sequia, de manera similar a com ho fan altres SAPs en

diferents plantes. A més, l'expressió ectòpica de *PpSAP1* en pruneres transgèniques ha permés augmentar la tolerància a estrés en aquestes línies en incrementar la quantitat d'aigua retinguda. Així mateix, aquestes plantes trnasgèniques també mostraren alteracions en la mida de les fulles, causades principalmente per una menor àrea cel·lular de les cèl·lules que formen part d'elles i relacionades amb una repressió de diferents gens implicats en el creixement cel·lular. Tot aço, suggereix que *PpSAP1* probablement tinga una doble funció relacionada tant amb resistència a estrés com amb creixement cel·lular durant la latència del préssec.

El segon gen d'estudi va ser una *PpeS6PDH,* la qual codificava per a un enzim amb activitat sorbitol-6-fosfato dehidrogenasa. *PpeS6PDH* està diferencialment regulada durant el desenvolupament de la gemma, augmentant la seua expressió en gemmes latents de manera consistent a l'acumulació de sorbitol. Simultàniament a la disminució de *PpeS6PDH* en les gemmes no latents, al voltant del lloc d'iniciació de la traducció del gen es van mostrar canvis a nivell de cromatina en l'estat de metilació dels residus específics de la històna H3 (H3K4 i H3K27). Aquestes dades assenyalen l'existència d'una regulació transcripcional de *PpeS6PDH* a nivell de modificacions de la cromatina. A més, també s'ha vist que diferents tipus d'estrés abiòtic afecten a l'expressió de *PpeS6PDH.* Tractaments amb baixes temperatures van induir la seua expressió tant en gemmes com en fulles, mentres que la desecació va augmentar l'expressió en gemmes però no en fulles. Aquests estudis suggereixen que la funció de *PpeS6PDH* durant la latència del préssec és donar tolerància a estrés per fred i sequia.

Finalment, el tercer gen d'estudi va ser *PpeDAM6,* un dels majors reguladors de la latència en gemmes de préssec. *PpeDAM6* està fortament représ durant el desenvolupament de la gemma amb una relació directa amb els events d'eixida de la latència. Aquesta repressió és deguda en part a la unió directa de PpeBPC1, una BASIC PENTACUSTEINE PROTEIN, a dos motius GAGA presents en la regió intrònica reguladora de *PpeDAM6.* Justament aquesta regió es troba modificada a nivell de cromatina amb un enriquiment en H3K27me3 després de l'eixida de latència. A més, l'expressió ectòpica de *PpeDAM6* en *Arabidopsis* va mostrar

fenotips de floració anormal semblants als produïts en plantes 35S::*SVP*. Per un altra banda, la sobreexpressió en pruneres va provocar retards en el creixement de les línies transgèniques a causa d'una alteració en els nivells hormonals. Aixi mateix, es va determinar que aquests canvis en l'homeostasi hormonal estaven produïts per la regulació diferencial de gens involucrats en les rutes d'àcid jasmònic, citoquinines i àcid gibberèl·lic en les plantes transgèniques. Aquests resultats suggereixen que *PpeDAM6* actua com un repressor master del creixement, mantenint la latència, la resposta de tolerància a estrés i la inhibició floral a través de la regulació de l'equilibri hormonal.

Com a conclusió, aquesta tesi proporciona una instantània dinàmica dels diferents mecanismes moleculars que tenen lloc dins de la gemma. Els gens estudiats tenen una funció fonamental, regulant tant el mateix procés de la latència com la resposta de tolerància a estrés i les rutes de floració i tots ells són potencials candidats per a millorar noves plantes més adaptades al canvi climàtic.

Abbreviation list

ABA	Absicisic acid
AbA	Aureobasidin A
BLASTN/P	Basic Local Alignment Tool Nucleotide/Protein
BPC	Basic Pentacysteine Protein
cDNA	complementary DNA
CDS	Coding sequence
CK	Cytokinin
COR	Cold-responsive
CV	Control 'Claudia Verde'
DAM	Dormancy Associated MADS-box
DEU	Differentially Expressed Unigenes
DNA	Deoxyribonucleic acid
evg	evergrowing
GA	Gibberellic acid
GO	Gene Ontology
H3K27me3	Trimethylated H3 at lysine 27
H3K4me3	Trimethylated H3 at lysine 4
iPA	Isopentyl-adenine
iPR	Isopentyl-adenosine
JA	Jasmonic acid
JA-Ile	JA-Isoleucine
KEGG	Kyoto Encyclopedia of Genes and Genomes
LSD	Least Significant Difference
miRNA	microRNA
OD	Optical density
OPDA	*cis*-(+)-12-oxo-phytodienoic acid
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time RT-PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
RWC	Relative water content
S6PDH	Sorbitol-6-phosphate dehydrogenase

SAM	Shoot apical meristem
SAP	Stress Associated Protein
SD	Minimal medium
UTR	Untranslated region
WT	Wild-type
Y1H	Yeast one-hybrid
Y2H	Yeast two-hybrid

GENERAL INTRODUCTION

Modulation of Dormancy and Growth Responses in Reproductive Buds of Temperate Trees

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Plants, unlike animals that can fly, swim or walk, are sessile organisms that need to adapt their growth and development to cope with a wide range of stresses and thus, ensure their survival. An interesting example of such fascinating adaptation is the annual growth cycle of trees in temperate climates. These regions present wide temperature ranges and seasonal changes along the year so trees adjust their pattern of growth to this environmental climate modulation. During spring and early summer, when the climatic conditions are favourable, most temperate trees actively grow, but before the advent of winter they stop growing, and form structures called buds in order to protect meristems of the harsh environmental conditions. This quiescent state is called dormancy and to reestablish the ability to grow, buds need to perceive an enough amount of cold, allowing development to resume once environmental conditions are suitable again.

An exhaustive study of this process is essential for commercial purposes, since the growth habits will affect the production of trees. For this reason, this thesis has focused on the study of the molecular mechanisms that control dormancy process in a temperate tree model like peach.

1. Dormancy phases and annual growth cycle.

Dormancy has been originally defined as the absence of visible growth in a meristematic structure. Traditionally, three types of dormancy have been

distinguished according to physiological cues leading to growth inhibition: paradormancy, imposed by another part of the plant; endodormancy, when signals are intrinsic to the meristem and ecodormancy, due to environmental factors (Lang, 1987). More recently, dormancy has been reformulated as "the inability to initiate growth from meristems under favorable conditions" (Rohde and Bhalerao, 2007) or "a state of self arrest of the shoot apical meristem (SAM) which is maintained under growth-promoting conditions" (Paul et al., 2014). Because of that nowadays when we apply this term to buds, the current general definition covers only endodormancy and paradormancy, but not bud growth inhibition by environmental factors (ecodormancy).

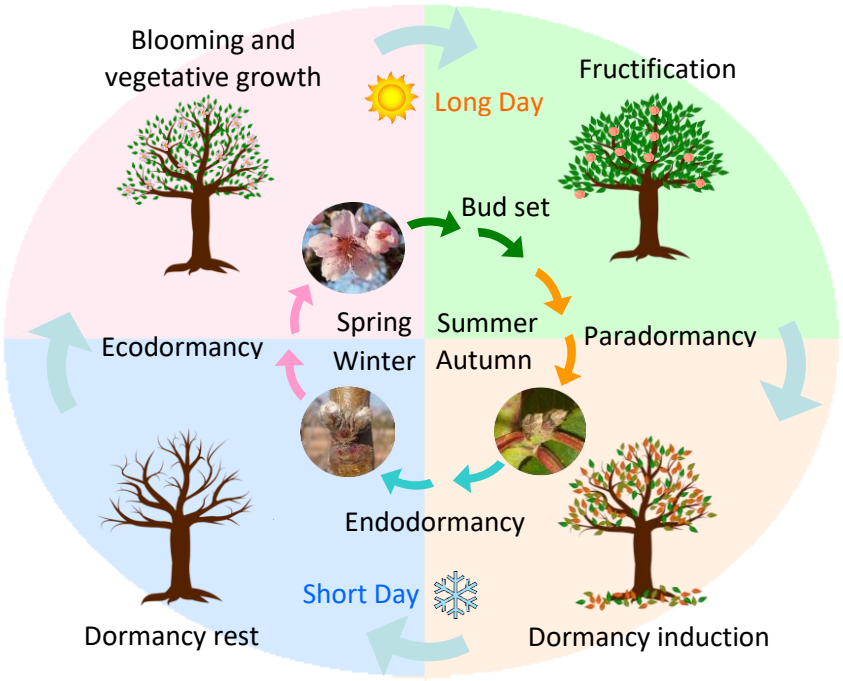


Figure 1. Peach annual growth cycle.

In temperate trees before growth cessation, paradormancy marks the first step to a deeper dormant state. In this stage, buds are competent to grow but they are inhibited by hormones and competition among other organs (Horvath, 2010). At the end of the autumn, the reduction of the photoperiod and the exposure to low temperatures induce growth cessation and the endodormancy establishment (Heide and Prestrud, 2005). During this stage, bud growth is inhibited by internal signals, which will be only resumed after an enough amount of cold is perceived by the bud. The chilling requirement is a quantitative character dependent on the genotype and an incomplete accomplishment of it affects dormancy release and floral and vegetative development (Topp et al., 2008). Once this chilling requirement is fulfilled, buds enter in a period known as ecodormancy in which they have the ability to sprout, but they won't do it until environmental conditions become favourable. This step concludes with the advent of spring, when flowering and vegetative growth are reestablished. (Fig. 1)

In this thesis, we use the term dormancy only referring to endodormancy.

2. Dormancy from a molecular point of view

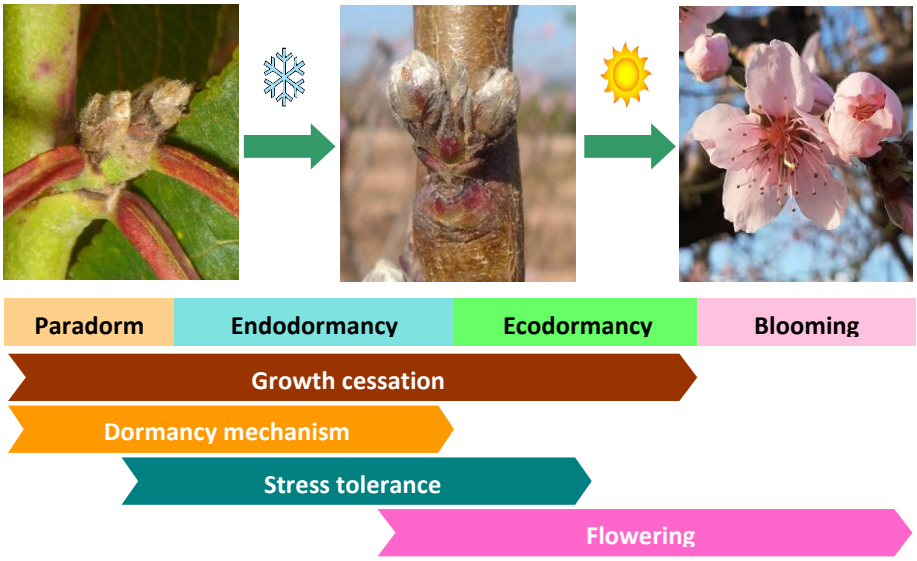
Although a dormant tree or a dormant bud could seem a completely inactive organ this is not exactly true and inside them there are active processes taking place, as verified by physiological and gene expression studies. Several reviews have also addressed the known mechanisms of bud dormancy control in perennial plants from a molecular perspective (Rohde and Bhalerao, 2007; Allona et al., 2008; Anderson et al., 2010; Yamane, 2014; Maurya and Bhalerao, 2017; Singh et al., 2017). Several of these studies have focused on the molecular control of growth arrest in apical vegetative meristems. Growth cessation and dormancy induction in those meristems are regulated by endogenous and environmental signals, being photoperiod shortening and temperature lowering major determinants of dormancy setup in forest species and *Rosaceae* fruit trees, respectively (Heide and Prestrud, 2005; Cooke et al., 2012). By contrast, the

development of flower lateral meristems is usually determined by apical dominance and other factors, which compel the buds to overwinter in an already differentiated immature stage, preceding dormancy release and blooming on next season. Even though diverse anatomical and physiological particularities are found, differentiated vegetative and reproductive meristems cease growth in a well defined stage and form a bud surrounded by protective scales in a similar fashion in different tree species. However, in this thesis we focus on the study of dormancy in flower lateral buds specifically.

The transition to reproductive growth starts around early spring in *Populus* (Boes and Strauss, 1994) and summer in many *Rosaceae* (Kurokura et al., 2013), like *Purnus Persica,* where flower bud induction occurs in axillary meristems. Then, flower organ differentiation starts and is substantially accomplished before mid-autunm when dormancy initiates. Growth arrest and seasonal dormancy are induced specifically by either photoperiod in *Populus* or temperature in *Prunus persica* (Hänninen and Kramer 2007, Cooke et al., 2012). In parallel, cold, freezing and desiccation tolerance is increased by an acclimation mechanism (Welling and Palva, 2006). Subsequent production of reproductive gametes and resumption of flower organ growth requires dormancy release triggered by the quantitative perception of chilling accumulated during the dormancy period (Coville, 1920; Couvillon and Erez, 1985). After dormancy release, buds remain cold-acclimated until a period of warm temperatures results in de-acclimation and bud break (Welling and Palva, 2006). The whole succession of events from flower bud induction to blooming can be interpreted as a trade-off between defense factors leading to cold acclimation and dormancy and growing factors leading to dormancy release and flowering.

Globally, three major molecular processes including dormancy, stress tolerance and flower development converge spatially and temporally in a reproductive bud, playing an active and relevant role in bud dynamics and determining plant survival and growth resumption under favorable conditions (Fig. 2). These processes determine bud phenology and development through

their reciprocal interaction (Kurokura et al., 2013; Vitasse et al., 2014; Singh et al.,



2017).

Figure 2. Active molecular mechanisms during bud development

2.1 Dormancy process

We know that bud dormancy is dynamically modulated by environmental, intrinsic and hormonal factors (Cooke et al., 2012) but the exact molecular mechanisms that control dormancy maintenance and dormancy release are practically unknown. Several RNA-seq experiments along bud development have been performed in order to have a transcriptome-wide view about what is exactly happening inside the bud (Zhu et al., 2015; Zhang et al., 2018; Bai et al., 2013; Kumar et al., 2016; Khalil-Ur-Rehman et al., 2017; Hao et al., 2017). In spite of their different experimental approach, there are two GO categories that are mainly represented in all of them: biosynthesis and catabolism of hormones and regulation of gene expression.

2.1.1. Hormone pathways

Plant hormones are signal molecules that regulate growth and developmental processes in plants and play an important role in the growth-dormancy trade-off (Cooke et al., 2012, Liu and Sherif, 2019). Although practically all the groups of hormones have been related to dormancy (Liu and Sherif, 2019), in this section we focus on gibberellins (GAs) acting as promoters of cell growth and abscisic acid (ABA) that associates with growth arrest and dormancy maintenance.

GAs promote vegetative and reproductive growth, thus a decrease of GA content is a prerequisite for growth cessation and dormancy induction and an increase is necessary for dormancy release (Liu and Sherif, 2019). In *Prunus mume,* GA content changes across bud dormancy phases, in concordance with the expression of biosynthetic *GA20ox* genes (Wen et al., 2016). Moreover, application of exogenous active GA increases bud break (Zhuang et al., 2013), and induces shoot elongation under short-days in *Salix pentandra* (Junttila and Jensen, 1988). A set of transgenic *Populus* plants with altered GA metabolism and signaling show faster growth cessation in response to short photoperiod, early bud set and delayed bud break as compared with the wild type (Zawaski et al., 2011; Zawaski and Busov, 2014). On the contrary, hybrid aspen plants with increased GA concentration by overexpression of *AtGA20ox1* continue to grow under short-day conditions (Eriksson et al., 2015).

On the other hand, ABA has a function opposite to that of GA. ABA is involved in the maintenance of dormancy, increasing its content at dormancy establishment and decreasing at dormancy release. Modification of ABA signaling by overexpression and down-regulation of a poplar ortholog of *ABA INSENSITIVE 3* (*ABI3*) alters bud formation in response to short-days (Rohde et al., 2002; Ruttink et al., 2007). Interestingly, ABI3 protein interacts with FLOWERING LOCUS D 1 (FDL1), pointing to an orchestrated control of bud development by photoperiod and ABA pathways (Tylewicz et al., 2015; Singh et al., 2017). However, recently it

has been proved that in an aspen mutant with less ability to respond to ABA by down-regulation of *ABA INSENSITIVE 1 (ABI1)*, photoperiodic pathways are insufficient to control dormancy induction and confirm that the ABA content is needed for dormancy maintenance, affecting cell-to-cell communications (Tylewicz et al., 2018). Similarly to *ABI3* overexpressing lines, birch (*Betula pendula*) plants made insensitive to ethylene by expressing the dominant mutation *etr1-1* of the ethylene receptor *ETR1,* show alterations in bud formation (Ruonala et al., 2006). In the same study, *etr1-1* plants fail to accumulate ABA in response to short-days, which suggests an interplay of both hormones in bud development mechanisms. On the other hand, in grapevine, ABA has been postulated to affect bud dormancy development through the modulation of the expression of cell cycle genes (Vergara et al., 2017).

Although there are many studies that expose the close relationship between ABA/GA content and dormancy, it is important to highlight that functional studies have been mainly performed in non *Rosaceae* species and an effort focused on the analysis of these hormones in this family is still required.

2.1.2. Regulation of gene expression

2.1.2.1 *DAM* genes

There are a group of genes that recently emerged as potential regulators of the dormancy cycle in tree species. These genes, called *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*), were firstly found in peach (Bielenberg et al., 2008) and leafy spurge (Horvath et al., 2008) and after that, in many other species: *Malus x domestica* (Falavigna et al., 2014; Wu et al., 2017), *Pyrus pyrifolia* (Saito et al., 2015), *Prunus armeniaca* (Balogh et al., 2019), *Prunus avium* (Rothkegel et al., 2017), *Prunus mume* (Sasaki et al., 2011), and *Prunus pseudocerasus* (Zhu et al., 2015).

These genes are considered the major factors affecting dormancy due to the study of a natural mutant of peach called *evergrowing* (*evg*) that shows a non-

dormant phenotype, maintaining apical growth and persistent leaves in response to dormancy inducing conditions. This phenotype was related to a partial deletion that affects the expression of *DAM* genes (Bielenberg et al., 2008). *DAM* genes are specifically expressed in dormant vegetative and reproductive buds, and down-regulated concomitantly with dormancy release events. In spite of that, several *DAM* family members show gene expression particularities (Li et al., 2009; Jiménez et al., 2010a; Yamane et al., 2011). For example, in peach, *DAM5* and *DAM6* are proposed the main quantitative repressors of bud dormancy release (Jimenez et al., 2010).

This special pattern of expression is transcriptionally modulated by environmental cues, mainly by low temperature (Li et al., 2009; Leida et al., 2010; Jiménez et al., 2010a; Yamane et al., 2011). In *Arabidopsis*, MYC transcription factors encoded by *INDUCER OF CBF EXPRESSION 1-2* (*ICE1-2*) are activated by specific cold-dependent post-translational modifications, causing up-regulation of *C-REPEAT BINDING FACTOR1-3* (*CBF1-3*) genes. Subsequently, CBFs regulate most of cold-responsive (COR) targets by binding to the C-repeat/drought-responsive element (CRT/DRE) (Knight and Knight, 2012). In fact, CRT/DRE regulatory elements have been found in the *DAM* genes promoters in different species (Leafy spurge, Horvath et al., 2010; Japanese pear, Saito et al., 2015; Niu et al., 2016; apple, Mimida et al., 2015; Wisniewski et al., 2015; Japanese apricot, Zhao et al., 2018). In addition, the ectopic expression of a peach *CBF* in apple alters the expression of *DAM*-like and *EBB*-like (*EARLY BUD BREAK*-like) genes in buds, providing an explanation for its prolonged dormancy period through the regulation of key transcription factors involved in dormancy regulation (Wisniewski et al., 2015). Finally, direct binding and activation of *DAM* promoters by *CBF* has been confirmed by yeast one-hybrid and transient expression experiments in pear and Japanese apricot (Saito et al., 2015; Niu et al., 2016; Zhao et al., 2018b). These examples show the close relationship between dormancy and low temperature tolerance mechanisms, and suggest that CBF, apart from its role in cold acclimation in plants, as we will show later, participates in the up-regulation of *DAM* genes during dormancy induction. Recently, another

transcription factor*, TEOSINTE BRANCHED/CYCLOIDEA/ PROLIFERATING CELL FACTOR 20* (*PpeTCP20*), has been found to down-regulate *DAM5* and *DAM6* expression and interact with their promoter in *Prunus persica* (Wang et al., 2020b). TCP are transcription factors that have been associated with morphogenesis novelty, like control of leaf and flower size and shape as well as the suppression of shoot branching (Nicolas and Cubas, 2016), which suggests an important role of these factors in dormancy maintenance.

But this is not the only known mechanism that controls the expression of *DAM* genes, epigenetic modifications also alter their transcripts levels. Concomitantly with cold accumulation and gene down-regulation, the chromatin in regulatory regions of *DAM* genes in leafy spurge and peach shows a decrease in trimethylation of histone H3 at lysine 4 (H3K4me3) and an increase of trimethylated H3 at lysine 27 (H3K27me3). Both changes are associated with gene repression and silencing (Horvath et al., 2010; Leida et al., 2012b). A reduction in H3K4me3 was also observed in *PpyMADS13-1* promoter during endodormancy release in Japanese pear, however no differences were found in H3K27me3 (Saito et al., 2015). Moreover, *PpyDAM* transcripts are targeted and degraded by miR6390 microRNA, thus contributing to *DAM* down-regulation in the bud dormancy release transition (Niu et al., 2016). Finally, in sweet cherry, the fulfillment of chilling requirements associates with an increase of *de novo* DNA methylation and the abundance of matching small interfering RNAs in the promoter region of *PavMADS1* (Rothkegel et al., 2017).

Paradoxically, *DAM* genes expression depends on two cold-dependent mechanisms that act antagonistically. Firstly, low temperatures activate *DAM* genes transcription by CBFs, inducing endodormancy entrance. Later, long time exposition to these low temperatures causes the opposite effect, silencing *DAM* genes by epigenetic modification and inducing endodormancy release (Horvath, 2010; Falavigna et al., 2019).

Regarding their function, *DAM* genes are homologs of *SVP* and *AGL24* genes encoding MADS-box transcription factors related to flowering in *Arabidopsis*. Specifically, SVP is a transcriptional repressor that inhibits flowering by direct

repression of the floral integrators *FLOWERING LOCUS T* (*FT*) and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) (Li et al., 2008; Jang et al., 2009). In fact, *DAM* genes have been proposed to directly repress *FT* in leafy spurge and in pear (*Pyrus pyrifolia*) (Hao et al., 2015; Niu et al., 2016) despite this interaction is not supported by an independent study (Saito et al., 2015). MADS-box transcription factors form multimeric complexes with different targets and roles depending on their interacting proteins (de Folter et al., 2005). These interactions have been already described in *Prunus mume* where PmuDAMs proteins are able to form homodimeric and heterodimeric complexes between them (Zhao et al., 2018). In addition, PmuDAM6 interacts with PmuSOC1 (Kitamura et al., 2016), resembling the interaction of SVP and SOC1 from *Arabidopsis*. These studies confirm the phylogenetic relationship between *DAM* genes and *SVP* and suggest conserved regulation pathways between perennial trees and herbaceous plants.

But *FT* is not the only target that has been associated with *DAM*s genes. In Japanese pear, it has been shown that *DAM* genes activate a 9-cis-epoxycarotenoid dioxygenase gene (*PpNCED3*) (Tuan et al., 2017). *NCED* encodes for an enzyme that participates in ABA biosynthesis pathway providing specific mechanisms for ABA accumulation in dormant buds and showing the close relationship between ABA and dormancy maintenance.

In summary*, DAM* genes play a central role in reproductive bud development, contributing to dormancy maintenance as shown in *evg* mutant and the recent studies of *NCED* activation, as well as flowering repression trough FT down-regulation.

2.1.2.2 Epigenetic mechanisms

As we highlighted in *DAM* genes, epigenetic modifications including chromatin histone methylation and acetylation, and small RNA regulation have been postulated to mediate chilling dependent release of dormancy (Horvath et al., 2003; Ríos et al., 2014). The *EARLY BUD-BREAK 1 (EBB1)* gene, that encodes an

AP2 type transcription factor associated with bud break events in different species (Yordanov et al., 2014; Busov et al., 2016), has been found differentially enriched in H3K4me3 modification when dormancy is released and flowering starts in Japanese pear (Tuan et al., 2016). Indeed, the level of many histone modifiers changes during winter affecting dormancy regulation (Conde et al., 2019). A transgenic hybrid aspen expressing an RNAi that suppresses the *FERTILIZATION INDEPENDENT ENDOSPERM (FIE),* a component of the polycomb repressive complex 2 (PRC2) which catalyzes H3K27me3 modification, prevents dormancy establishment (Petterle et al., 2011). In addition, down-regulation of the *CHROMODOMAIN/HELICASE/DNA-BINDING DOMAIN (CHD3) PICKLE*, a known antagonist of H3K27me3 modification in *Arabidopsis* (Aichinger et al., 2009), restores plasmodesmata closure and photoperiod-dependent bud dormancy in ABA response defective plants, suggesting that ABA promotes bud dormancy by repressing *PICKLE* (Tylewickz et al., 2018).

Also, methylation of DNA affects both gene-specific expression and chromatin structure, and thus it may potentially account for large transcriptomic rearrangements observed in developmental transitions. Global and specific levels of genomic DNA cytosine methylation change during bud development in chestnut (Santamaría et al., 2009), apple (Kumar et al., 2016a), sweet cherry (Rothkegel et al., 2017) and almond (Prudencio et al., 2018). In addition, recent functional studies reveal the important role of DNA methylation enzymes in seasonal dormancy regulation. Overexpression of a chestnut *DEMETER-like* (*CsDML*), a DNA demethylase accelerates photoperiodic-dependent bud formation (Conde et al., 2017b), whereas down-regulation of poplar *DEMETER-like* (*PtaDML10*) delays bud break in poplar (Conde et al., 2017a).

Finally, modification of transcript stability by microRNA action has been also hypothesized to participate in bud dormancy regulation. The aspen microRNA ptr-MIR169 represses the expression of *HEME ACTIVATOR PROTEIN 2* (*ptrHAP2*) in dormant buds (Potkar et al., 2013). HAP2 is a component of nuclear factor Y (NF-Y) complexes involved in regulation of flowering in *Arabidopsis* by modulating the epigenetic state of target genes (Hou et al., 2014) like for example *FT*. Another

case of dormancy regulation by microRNAs is found in tea plant where csn-miR319c represses *CsnTCP2* affecting dormancy release (Liu et al., 2019).

2.2 Stress tolerance response

Overwintering buds must deal with low and freezing temperatures leading to different forms of physiological and cellular injury. In addition to physical damage caused by ice nucleation and propagation, a dehydration stress is induced by changes in water potential due to the formation of extracellular ice, and the water loss inherent to bud dormancy progress. Plants may actively enhance their tolerance to low temperatures and desiccation via gene expression modification by a cold acclimation process (Wisniewski et al., 2003). Several reviews describe in detail the molecular and genetic control of cold acclimation in trees (Welling and Palva, 2006; Preston and Sandve, 2013; Fennell, 2014; Wisniewski et al., 2014), which is broadly similar to cold acclimation mechanisms reported in herbaceous plants (Thomashow, 1999; Thomashow, 2010; Knight and Knight, 2012).

Seasonal cold acclimation and bud dormancy are related processes since both are induced by similar low temperature and photoperiod conditions (Welling et al., 2002), and both are incompatible with active plant growth, which suggests the presence of common regulatory mechanisms. In fact, the effect of temperature on seasonal growth cessation and cold acclimation invokes the same COR pathway (Wingler, 2015). Although COR genes have been essentially described in *Arabidopsis*, COR components and functions are conserved in perennials (Fennell, 2014; Wingler, 2015). In Japanese apricot, *PmCBFs* of COR pathway are up-regulated by low temperature (Zhao et al., 2018). In addition, the ectopic expression of *Arabidopsis CBF1* increases freezing tolerance in poplar and induces transcriptomic changes overlapping with *Arabidopsis* COR regulon (Benedict et al., 2006). On the other side, constitutive expression of birch *BpCBF1* increases freezing tolerance and induces known targets of *CBF* genes in *Arabidopsis* (Welling and Palva, 2008). Moreover, the ectopic expression of a peach *CBF* gene in apple

induces short-day dependent dormancy, improves freezing tolerance, and delays bud break in field studies (Wisniewski et al., 2011; Artlip et al., 2014). Not only low temperature triggers stress tolerance responses and dormancy establishment, but also impairment of the photoperiodic response by overexpression of *PHYA (*phytochrome A) and down-regulation of clock *LHY (LATE ELONGATED HYPOCOTYL1)* genes reduces the critical daylength for growth cessation and also prevents cold acclimation in hybrid aspen (Olsen et al., 1997; Ibáñez et al., 2010).

Epigenetic mechanisms have also been postulated to participate in the control of both, bud phenology and cold acclimation traits. In Norway spruce, the environmental temperature during embryogenesis and seed maturation affects the duration and intensity of bud dormancy and cold acclimation in the progeny, by an "epigenetic memory" process (Johnsen et al., 2005). This epigenetic mechanism has been proposed to modify the expression of certain miRNAs and genes related to bud break, such as *EBB1*, leading to different epitypes with the same genotype (Yakovlev et al., 2010, 2011; Carneros et al., 2017).

However, cold deacclimation and bud dormancy release are not concurrent events; winter buds remain cold-acclimated after dormancy release under appropriate low temperature conditions as long as meristem growth is not resumed, after which deacclimation is not any longer reversible (Kalberer et al., 2006). Thus, although dormancy and stress tolerance response could share common regulatory mechanisms, their downwards response is different.

CBF-dependent cold acclimation response includes synthesis of chaperones, dehydrins and other protective proteins, change in lipid composition of membranes, alteration of sugars metabolism, and production of storage and antioxidant compounds, among other responses aiming at alleviate cold, drought and oxidative stresses (Welling and Palva, 2006). Dehydrins are abundant cold-responsive proteins belonging to the late embryogenesis abundant (LEA) family that have been proposed to protect cell structures and enzymes against freezing and dehydration (Graether and Boddington, 2014). Seasonal up-regulation of a dehydrin gene in bark tissue is lower and restricted to a shorter period in the *evg* mutant of peach, in concordance with its lower cold tolerance (Arora et al., 1992;

Arora and Wisniewski, 1994; Artlip et al., 1997). Diverse chitinases have been also suggested to act as antifreeze, storage and defense proteins induced during the transition to dormancy in spruce (González et al., 2015).

Soluble sugars and other compounds potentially able to act as compatible solutes accumulate in dormant tissues in order to confer tolerance to cold and desiccation stresses. Low temperature up-regulates *DUAL SPECIFICITY PROTEIN PHOSPHATASE 4* (*DSP4*), most likely involved in starch dephosphorylation and degradation to increase the synthesis of oligosaccharides during winter dormancy in chestnut (Berrocal-Lobo et al., 2011). Raffinose family oligosaccharides (RFOs) including raffinose and stachyose are compatible solutes synthesized in seeds and plant tissues undergoing abiotic stresses (Sengupta et al., 2015). Genes coding for the enzyme galactinol synthase (GolS) catalyzing the first step in the synthesis of RFOs are up-regulated in dormant buds and other tissues of woody perennials (Ko et al., 2011; Ibáñez et al., 2013), and an apple *MdGolS2* gene confers tolerance to water deficit when is expressed in *Arabidopsis* (Falavigna et al., 2018).

2.3 Flowering pathways

Flowering pathways and genes are broadly conserved between herbaceous and perennial plants, in spite of their evident phenological particularities. In perennials, a period of seasonal dormancy usually interposes between flower induction and blooming (Boes and Strauss, 1994; Kurokura et al., 2013), which forces the mutual coordination of flowering, dormancy and stress tolerance processes. Under these circumstances, preexisting components of flowering pathways have apparently evolved to acquire new functionalities adapted to the growth of perennials in temperate climates. The proposed functions of *FT1* in flower induction and dormancy release and *FT2* in the regulation of photoperiodic growth cessation in poplar constitute a paradigmatic case of neo-functionalization after a gene duplication event in trees, in contrast to the main role of *FT* in the transition to flowering in *Arabidopsis* (Pin and Nilsson, 2012). A role for *FT* and the

similar but functionally antagonist *TFL* genes in flower induction has been also postulated in other perennial species different from poplar, based on expression and transgenic studies (Kotoda and Wada, 2005; Jones et al., 2011; Ziv et al., 2014; Bai et al., 2017; Reig et al., 2017). Similarly, orthologs of *Arabidopsis* flowering genes *LEAFY* (*LFY*) and *APETALA-1* (*AP1)* perform a function related to flowering transition in perennial species. *LFY*-like genes from trees are preferentially expressed during flower induction and accelerate flowering when ectopically expressed in *Arabidopsis*, however no evidences of their flowering promoting effect have been observed when overexpressed in poplar (Rottmann et al., 2000). On the contrary, RNAi of *PtLFY* induces sterility and delays bud break in poplar (Klocko et al., 2016). On the other hand, a dominant negative mutation of *AP1* from *Arabidopsis* modifies the regulation of flowering related genes in poplar (Chen et al., 2015), and overexpression of *AP1*-like gene from *Salix integra* induces early flowering in haploid poplar (Yang et al., 2018). In addition to homologs of known flowering genes, miRNAs and hormone signaling pathways have been proposed to integrate developmental and environmental cues affecting flower induction (Xing et al., 2015; Guo et al., 2017).

The reproductive development in perennials is closely associated with phenology. Following flower induction, reproductive organs differentiate and continue growing until a given developmental stage is reached before the dormancy period. In peach and apricot, dormant anthers are arrested in the form of sporogenous tissue (Julian et al., 2011; Ríos et al., 2013). Then, after dormancy release, pollen mother cells undergo meiosis followed by pollen development and maturation, and ovaries start to form ovules (Luna et al., 1990; Julian et al., 2011) correlated with the up-regulation of genes associated to microsporogenesis (Ríos et al., 2013). The harmful effect of cold and other environmental stresses on microsporogenesis, leading to ploidy alterations in male gametes and sterility (De Storme and Geelen, 2014), suggests that dormancy arrest in a pre-meiosis stage may serve to ensure a proper production of male gametes under more favorable environmental conditions. However, there are flower structures showing certain metabolic activity during dormancy; it has been shown that starch accumulates

during dormancy in ovary primordia of *Prunus avium*, reaching the maximum at chilling fulfillment (Fadón et al., 2018). It is thought that this starch is important for correct flower development and in the reproductive process.

3. Dormancy in the context of climate change: economical importance

The tree growth annual cycle is an ordered series of phases in which the interaction with environment is essential for a properly progress through the different events. Photoperiod and temperature are major regulators of growth cycle in temperate fruit trees. In particular, low temperature affects both, dormancy establishment and dormancy release, and is considered one of the main limiting factors to extent fruit trees to warmer areas. Nowadays global warming is increasing temperatures in many major growing regions (Luedeling et al., 2011) and although warmer spring temperatures could have some advantages advancing growth resumption, warmer winter temperatures could have devastating consequences. For example, if we grow a tree species under too warm environmental conditions, buds will hardly achieve the required amount of cold for proper dormancy release, inducing irregular and defective flowering (Fig. 3B). The opposite climatic condition would not improve the scenario. Although chilling requirements would be properly fulfilled, a short period of higher temperatures during the cold season could lead to an early blooming increasingly freezing risk in the open flowers (Fig. 3C) (Ríos et al., 2016).

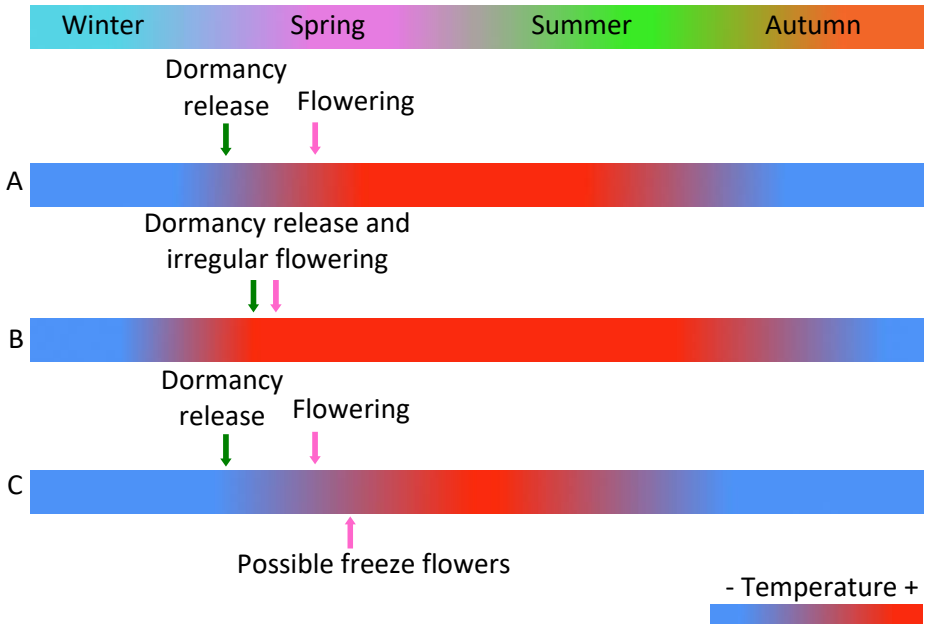


Figure 3. Dormancy events under different climatic conditions. (Based on Ríos et al., 2016)

These upcoming climatic changes will directly impact on the production of fruit crops, causing large economic losses. Some possible solutions include adapting existing cultivars to other areas, obtaining new cultivars with lower chilling requirements and developing tools to cope with insufficient chilling. But, to implement all these advancements, it becomes urgent to acquire a fundamental understanding of bud dormancy responses.

The aim of this thesis is to study the dormancy period from a molecular point of view, focusing on the characterization of three genes that were previously identified for their differential expression during peach dormancy (Leida et al., 2010) and highlighting the relationship between the three main processes during dormancy process: dormancy regulation, stress tolerance and flowering pathways. These studies will open the possibility to identify mechanisms and target genes that control dormancy process and provide useful tools for adaptation to climate change.

OBJECTIVES

The overall aim of this thesis is the study of the molecular regulation of the different processes that take place inside a reproductive bud of peach through genomic and molecular approaches. This has been divided into the following three specific objectives:

-Study of a SAP (STRESS ASSOCIATED PROTEIN) differentially expressed in reproductive buds. We will analyze *PpSAP1* gene expression pattern and its role in the dormancy process.

-Study of a sorbitol-6-phosphate dehydrogenase , an enzyme that participates in sorbitol synthesis. We will perform a biochemical and molecular analysis of *PpeS6PDH* gene, with special focus on the chromatin dependent regulation of the gene.

-Characterization of *PpeDAM6*, one of the main regulators of dormancy process. We will search for upstream and downstream factors of *PpeDAM6* regulatory pathway.

CHAPTER 1:

Dual regulation of water retention and cell growth by a stress-associated protein (SAP) gene in *Prunus*

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Abstract

We have identified a gene (*PpSAP1*) of *Prunus persica* coding for a stress-associated protein (SAP) containing Zn-finger domains A20 and AN1. SAPs have been described as regulators of the abiotic stress response in plant species, emerging as potential candidates for improvement of stress tolerance in plants. *PpSAP1* was highly expressed in leaves and dormant buds, being down-regulated before bud dormancy release. *PpSAP1* expression was moderately induced by water stresses and heat in buds. In addition, it was found that PpSAP1 strongly interacts with polyubiquitin proteins in the yeast two-hybrid system. The overexpression of *PpSAP1* in transgenic plum plants led to alterations in leaf shape and an increase of water retention under drought stress. Moreover, we established that leaf morphological alterations were concomitant with a reduced cell size and down-regulation of genes involved in cell growth, such as *GROWTH-REGULATING FACTOR* (*GRF*)1-like, *TONOPLAST INTRINSIC PROTEIN* (*TIP*)-like, and *TARGET OF RAPAMYCIN* (*TOR*)-like. Especially, the inverse expression pattern of *PpSAP1* and *TOR*-like in transgenic plum and peach buds suggests a role of PpSAP1 in cell expansion through the regulation of TOR pathway.

Introduction

Perennial plants in temperate climates have to cope with seasonal fluctuations in temperature. Particularly, during the winter period they deal with the deleterious effects of cold and water stresses by stopping growth and protecting their dormant meristems into specialized buds.

We have previously characterized transcriptomic changes associated with dormancy release in reproductive buds of peach (*Prunus persica* [L.] Batsch) (Leida et al., 2010, 2012a). A gene coding for a protein with AN1 and A20 Zn-finger domains has been consistently found to be up-regulated in dormant buds in these studies. Interestingly, gene expression down-regulation occurs concomitantly with dormancy release in genotypes with different chilling requirements, and thus gene expression regulation seems to associate with the developmental stage of buds under apparently variable environmental circumstances (Leida et al., 2010). This Zn-finger protein belongs to a family of plant regulators known as stress-associated proteins (SAP), with known homologs in animals (Vij and Tyagi, 2008; Giri et al., 2013).

SAP genes have been related to the abiotic stress response in plants. Most commonly, *SAP* genes have been found to be up-regulated under a combination of stressing conditions, including high temperature (Kim et al., 2015), chilling (Xuan et al., 2011), osmotic stress and salinity (Kang et al., 2011), water deficit (Sharma et al., 2015), and heavy metals (Dixit and Dhankher, 2011), among others. In addition, when overexpressed in transgenic plants, SAP genes confer tolerance to abiotic stresses (Mukhopadhyay et al., 2004; Kanneganti and Gupta, 2008; Hozain et al., 2012; Dansana et al., 2014).

In spite of the numerous studies devoted to *SAP* genes in plants, little is known about their molecular function. AtSAP5 from *Arabidopsis* binds different linkage-specific polyubiquitin chains but not monoubiquitin (Choi et al., 2012) and shows E3 ubiquitin ligase activity (Kang et al., 2011). The tumor suppressor c-myc binding protein (MBP-1) has been identified as an ubiquitination substrate of

AtSAP5, which is thus targeted for ubiquitin-dependent proteasome degradation (Kang et al., 2013). Regarding stress tolerance, the related OsSAP1 and OsSAP11 from rice interact with the receptor-like kinase OsRLCK253, which in turn confers tolerance to salt and water deficit stress in transgenic *Arabidopsis* plants (Giri et al., 2011). Recently, OsSAP1 has been found to interact with an aminotransferase (OsAMTR1) and a pathogenesis-related protein (OsSCP) involved in salt and water stress tolerance pathways (Kothari et al., 2016). Moreover, a conformational change in response to redox conditions has been observed in AtSAP12 from *Arabidopsis*, which could thus behave as a sensor and transmitter of redox imbalances triggered by different stresses (Ströher et al., 2009).

We have characterized *PpSAP1* gene expression in different tissues and environmental conditions, and have performed a yeast two-hybrid screening for the identification of putative protein interactors. In order to get deeper insight into SAP function we overexpressed *PpSAP1* in transgenic plum (*Prunus domestica* cv. Claudia Verde), leading to intriguing evidences about a dual role of *PpSAP1* in stress and developmental issues.

Results

Identification of a Zn-finger gene developmentally regulated in flower buds of peach

In previous transcriptomic studies in our group we have identified a Zn-finger protein gene expressed in dormant flower buds of peach, which is down-regulated concomitantly with developmental processes leading to bud dormancy release (Leida et al., 2010, 2012b). Formerly known as unigene PpB19 (Leida et al., 2010), the International Peach Genome Initiative (Verde et al., 2013) assigned to this gene model the systematic names ppa012373m (v1.0) and Prupe.2G010400 (v2.1). When analyzing the tissue-dependent expression of ppa012373m we found higher values in reproductive and vegetative buds, embryos and leaves; whereas the different flower and fruit tissues showed lower expression levels (Fig.

4A). As stated in previous reports, its expression decreased along flower bud development in 'Big Top' cultivar (Fig. 4B). Interestingly, ppa012373m expression reached its lowest level in January and February samples, previous to bud dormancy release date which was experimentally estimated between February and March sampling dates. Thus, ppa012373m expression in 'Big Top' confirmed previous data about its developmental down-regulation in buds, even if it was not tightly associated with bud dormancy release events.

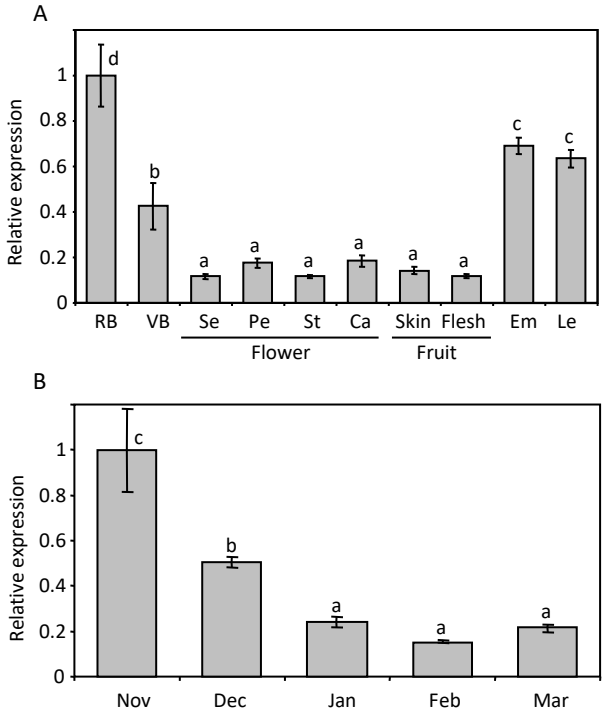

Figure 4. Relative expression of *PpSAP1* in peach by qRT-PCR. (A) Different plant tissues were tested, including reproductive bud (RB), vegetative bud (VB), sepal (Se), petal (Pe), stamen (St), carpel (Ca), fruit skin, fruit flesh, embryo (Em) and leaf (Le). (B) Reproductive buds were collected at different developmental stages, from November to March. In March samples bud dormancy was already released. An expression value of one is assigned to the first sample. Data are means from two biological samples with three technical replicates each, with error

bars representing standard deviation. Different letters (a–d) indicate significant difference between samples with a confidence level of 95%.

The ppa012373m deduced protein contained two consecutive Zn-finger domains named A20 and AN1 (Fig. 5A,B), which are found together in many stress-associated proteins (SAP) from different plant species. SAP proteins from peach, *Arabidopsis* and rice showing this particular arrangement of A20 and AN1 domains were compared by a phylogenetic analysis. The protein encoded by ppa012373m clustered jointly with two additional peach proteins, *Arabidopsis* AtSAP2, and rice OsSAP4 and OsSAP8, into a group of highly related sequences (Fig. 5C). In virtue of such phylogenetic closeness, from now on we will use the name *PpSAP1* to designate ppa012373m gene.

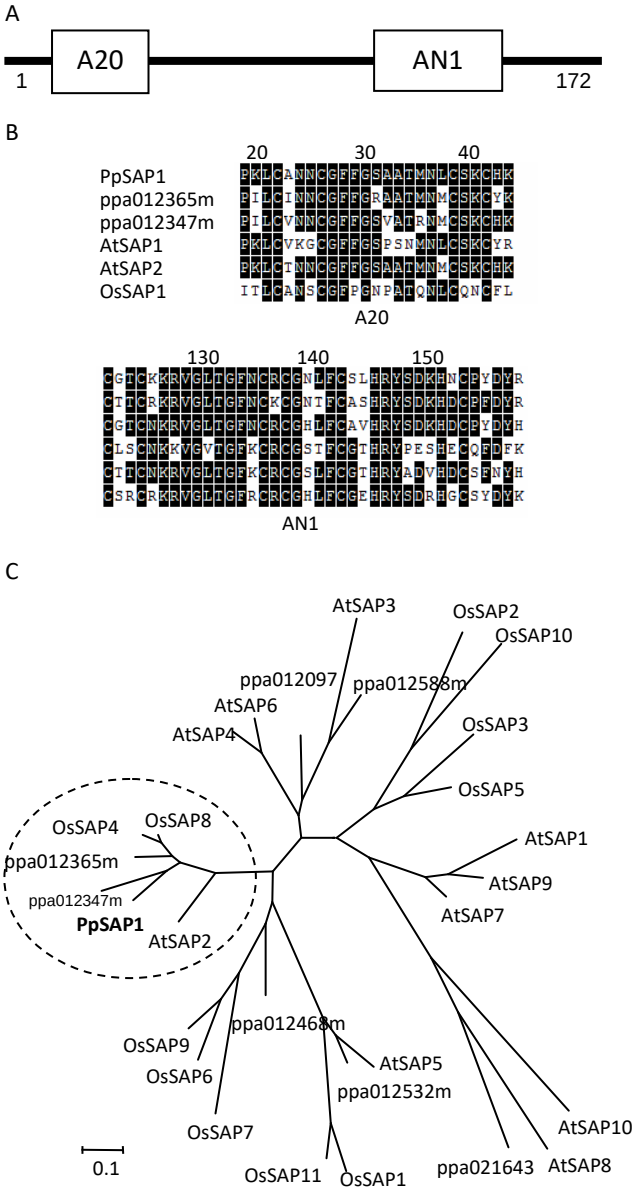

Figure 5. PpSAP1 is a stress-associated protein (SAP). (A) Schematic representation of A20 and AN1 domains in PpSAP1 protein. (B) Alignment of A20 and AN1 domains from PpSAP1 and other SAP-like proteins of peach, *Arabidopsis* and rice. (C) phylogenetic tree of SAP proteins from *Arabidopsis*, rice and peach. The tree was constructed using the Maximum Likelihood

method and bootstrapped with 1000 replicates. The scale bar indicates the branch length that corresponds to the number of substitutions per amino acid position.

PpSAP1 expression is modulated by abiotic stresses

Often, the expression of *SAP* genes from different species has been found to be induced by environmental cues, mostly abiotic stresses. In order to check the response of *PpSAP1* to abiotic stresses, flowers buds of peach were exposed to temperature and water stresses during one and three days treatments. *PpSAP1* expression was down-regulated by chilling (4°C) and up-regulated by heating (37°C) in both dormant and dormancy-released buds, although dormant buds required a longer period of three days to reach a significant difference (Fig. 6A). Water stress induced by desiccation and salinity treatments (NaCl 200 mM) also up-regulated *PpSAP1* expression in non-dormant buds in two different cultivars (Fig. 6B).

On the contrary, different experiments performed in detached leaves and leaf discs did not provide conclusive evidences about an effect of abiotic stresses on *PpSAP1* expression in plant tissues other than buds; made under conditions of desiccation, low temperature and NaCl and abscisic acid incubation that indeed induced strongly the expression of a *LATE EMBRYOGENESIS ABUNDANT* (*LEA*)-like gene (Supplementary Fig. S1).

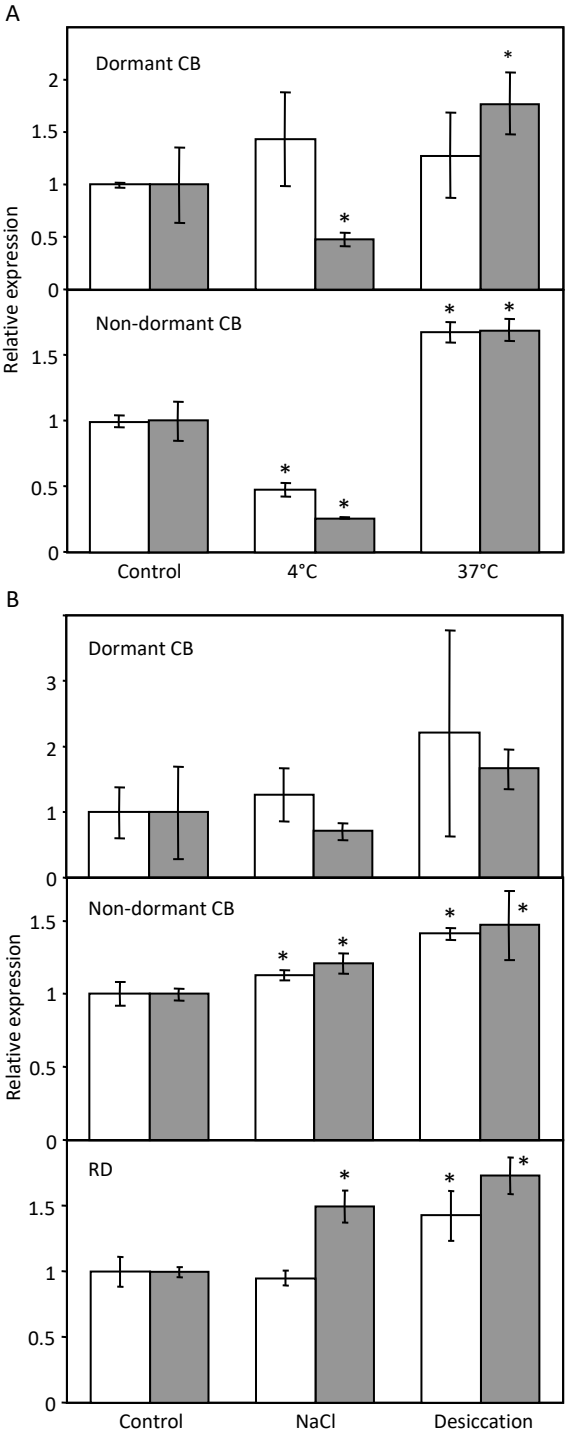
A
Dormant CB
Non-dormant CB
Relative expression
Control
4°C
37°C
B
Dormant CB
Non-dormant CB
RD
Relative expression
Control
NaCl
Desiccation

Figure 6. Effect of abiotic stresses on *PpSAP1* expression in peach buds. Treatments at 4°C and 37°C (A), and NaCl 200 mM and desiccation (B) were performed during one (white bars) and three days (grey bars). Dormant and non-dormant reproductive buds from cultivar 'Crimson Baby' (CB) and non-dormant buds from 'Rose Diamond' (RD) were employed. An expression value of one is assigned to the control. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%.

PpSAP1 binds to ubiquitin-like proteins

In order to get deeper insight into *PpSAP1* function we performed a yeast-two hybrid screening for the identification of PpSAP1 protein partners and/or targets. *PpSAP1* was cloned into pGBKT7 plasmid as a fusion with the DNA binding domain of GAL4. This construct was combined by yeast mating with a cDNA library from flower buds of peach into pGADT7 vector expressing the activation domain of GAL4. We obtained 304 positive colonies that after discarding repeated inserts and false positives were reduced to four independent genes (ppa005503m, ppa009116m, ppa005507m and ppa007117m) coding for polyubiquitin peptides (Fig. 7). This result supported the functional closeness of PpSAP1 to other SAP proteins from plants and animals. The sequence fragments of the positive clones are shown in Supplementary Fig. S2.

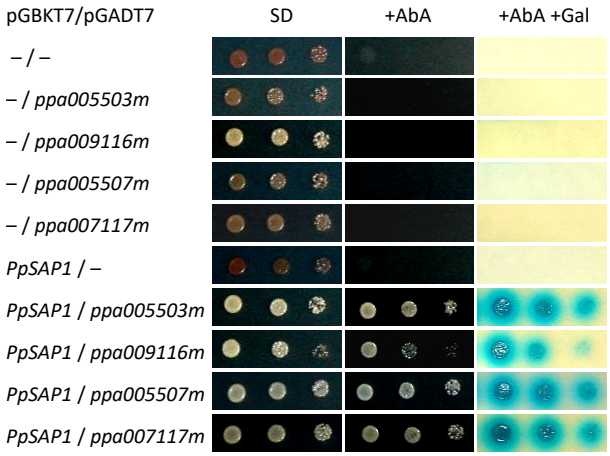

Figure 7. Two-hybrid system analysis of protein interaction. Different combinations of DNA-binding domain (pGBKT7) and activation domain (pGADT7) fused with PpSAP1, ppa005503m, ppa009116m, ppa005507m and ppa007117m, and control plasmids (-) are shown. Yeast strains were grown on a minimal medium (SD), a growth selective medium containing Aureobasidin A (+AbA) and a chromogenic medium containing Aureobasidin A and X-α-Gal (+AbA +Gal).

The constitutive expression of *PpSAP1* affects water loss under hydric stress

PpSAP1 gene was cloned into the binary vector pROK2 for its constitutive expression in plum driven by the 35S promoter. The plum model offered some advantages over other species for gene transformation, including its taxonomical proximity to peach (*Prunus persica*), their common woody perennial habit, similar developmental and physiological issues, and the availability of reliable methods for gene transformation and regeneration (Petri et al., 2008b). The expression of transgenic *PpSAP1* was assayed in shoots regenerated *in vitro* from six independent plum lines. The six lines expressed *PpSAP1* at varying levels (Fig. 8A). We selected lines #1, #5 and #6 for subsequent analyses. Southern analyses of these lines with two different restriction enzymes revealed the presence of multiple inserts with different integration patterns, confirming their independent origin (Fig. 8B). Once acclimatized, the expression of *PpSAP1*, a plum *SAP1*-like gene and both genes combined was evaluated in these three transgenic lines and the control 'Claudia Verde' (CV) using specific and common primer pairs (Supplementary Table S1). Leaves from the lines #1 and #6 accumulated more *PpSAP1* transcript than #5, even though its expression level was very high in the three lines and contributed to most of the combined expression of *PpSAP1* plus plum *SAP1*-like (Fig. 8C). On the other hand, the expression of the plum *SAP1*-like ortholog was reduced in the transgenic plants, suggesting the intervention of gene silencing mechanisms. Althoug any *SAP* from plum was previously characterized, the sequence similarities between *Prunus persica* and *Prunus domestica* allowed to detect the putative ortholog of *PpSAP1* in plum, being its expression profile

along plum bud development in concordance with peach bud expression pattern. (data not show)

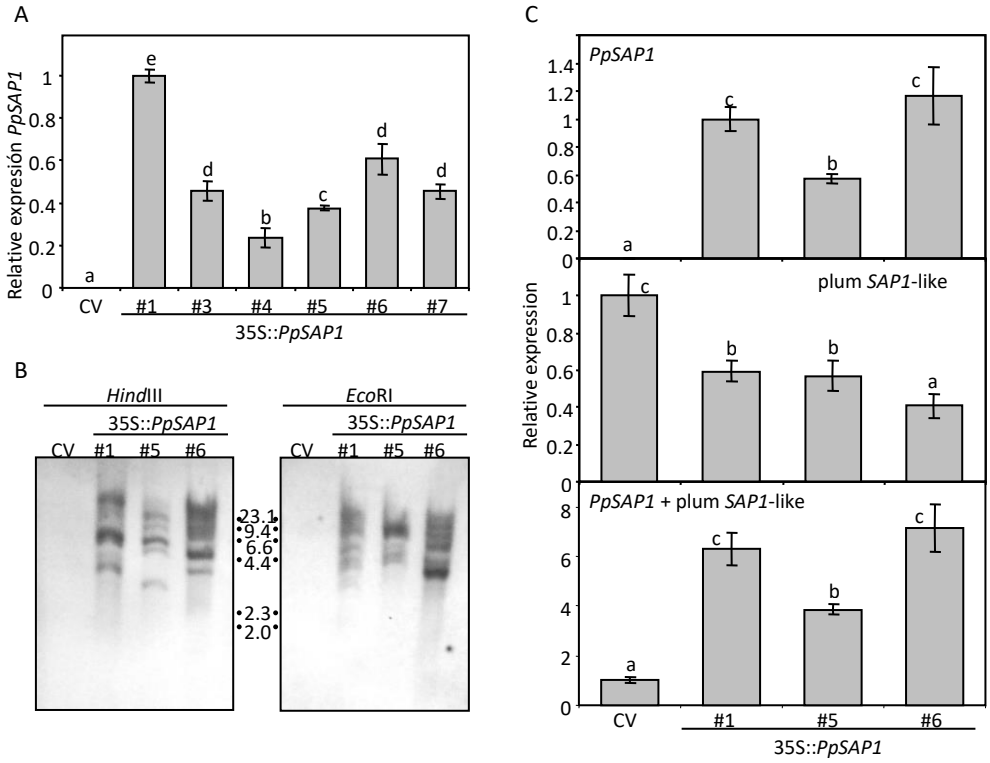

Figure 8. Overexpression of *PpSAP1* in transgenic plum. (A) Heterologous expression of *PpSAP1* in six independent transgenic lines of plum (35S::*PpSAP1* #1, #3, #4, #5, # 6, #7) and the control 'Claudia Verde' (CV). (B) Southern analysis with restriction enzymes *Hind*III and *Eco*RI of CV and transgenic lines #1, #5, # 6 , showing the position of molecular weight markers (kb).(C) The relative expression of *PpSAP1*, plum *SAP1*-like and both genes (*PpSAP1* + plum *SAP1*-like) is shown for three transgenic lines, by using specific primers. An expression value of one is assigned to the CV or the transgenic line #1. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Different letters (a–e) indicate significant difference between samples with a confidence level of 95%.

SAP genes are well known factors of tolerance to abiotic stresses when expressed in heterologous systems. The ability of *PpSAP1* to confer tolerance to abiotic stresses was assessed in overexpressing lines #1, #5 and #6. In a water loss experiment performed in detached leaves, *PpSAP1* overexpressing lines retained higher content of water than CV during the first hours of desiccation (Fig. 9A). In order to determine if such observation was due to differences in the leaf area, we calculated the specific water loss in the range of time in which water loss was lineal. Specific water loss per unit of time and leaf area was also significantly lower in transgenic lines (Fig. 9B), which confirms that differences in relative water content (RWC) were not caused by the distinct size of control and transgenic leaves.

A drought experiment was also performed in whole plants. *PpSAP1* overexpressing lines also retained a higher amount of water after seven days of stress, even though differences were significant in lines #5 and #6 exclusively (Fig. 9C, Supplementary Fig. S3). Additional salinity (NaCl) and heat stress experiments performed in acclimatized and *in vitro* plants did not support significant differences between CV and transgenic lines, thus *PpSAP1* contribution to stress tolerance was limited to drought stress.

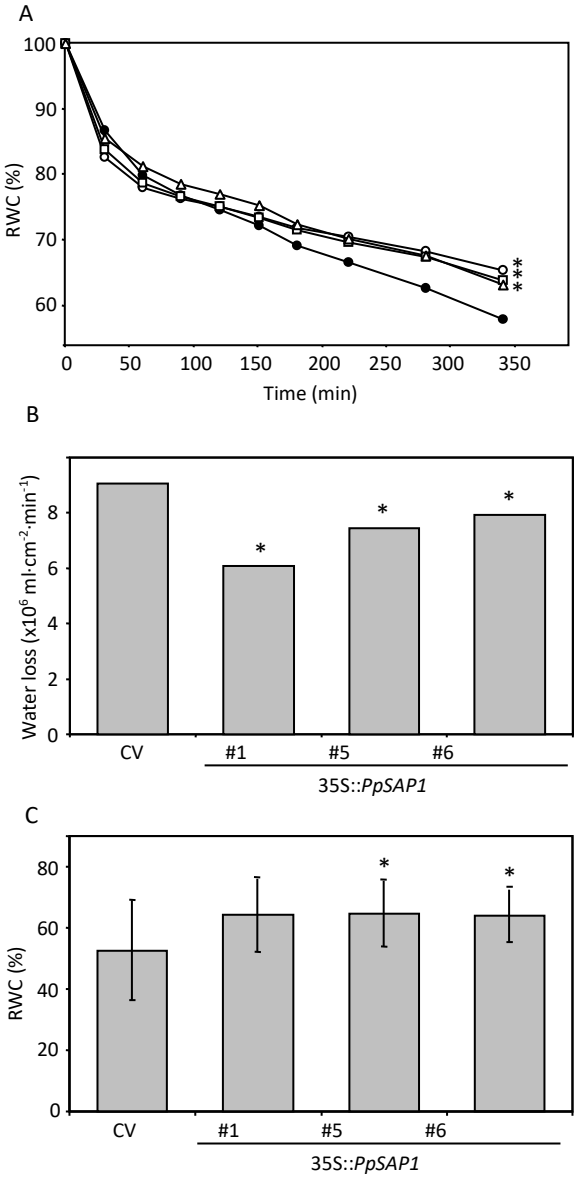

Figure 9. Analysis of water retention in plum overexpressing *PpSAP1* under drought stress. (A) The relative water content (RWC) of leaves detached from control 'Claudia Verde' (CV, black circles), and 35S::*PpSAP1* lines #1 (white circles), #5 (white squares) and #6 (white triangles) was calculated at different times along the desiccation process; Data are means from seven plants per genotype, and two leaves per plant. (B) The specific water loss was calculated as the

volume of water evaporated per cm^2 of leaf area and minute, during the time in which evaporation was constant with time in the experiment shown in a. (C) The RWC of whole plants under drought stress for seven days is shown. Data are means from twelve different plants per genotype. Error bars represent standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%.

Anatomical and cellular effects of *PpSAP1* expression in transgenic plum

The overexpression of *PpSAP1* caused evident effects on leaf morphology and plant growth in the transgenic plums under study: leaves of lines #1, #5 and #6 were smaller and with smoother (less undulate) margins, leading to plants with less dense canopy (Fig. 10A). *PpSAP1* overexpressing plums had a plant height similar to the control, but produced a higher average number of leaves (Fig. 10B). In addition transgenic leaves were shorter, narrower, smaller and lighter, and were different in shape. They had a higher length/width ratio and an acute leaf base angle (Fig. 10B), which caused a change in leaf shape from ovate (control) to elliptical (Fig. 10C).

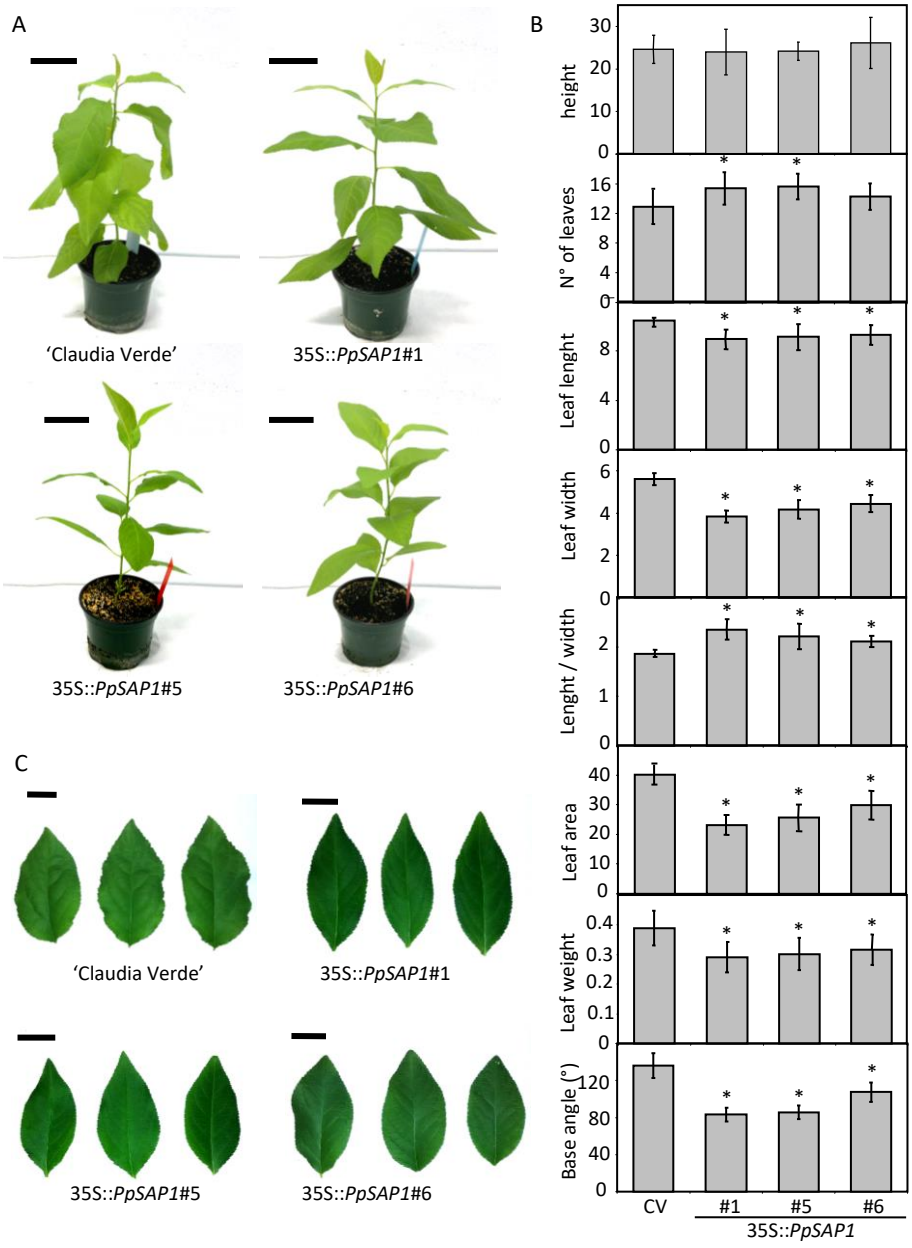

Figure 10. Plant anatomy and leaf morphology in plum overexpressing *PpSAP1*. (A)Two month old plants of control 'Claudia Verde' (CV) and transgenic lines 35S::*PpSAP1* #1, #5 and #6 are shown. Scale bar, 5 cm. (B) Different whole plant and leaf shape parameters of two month old

plants are shown. Data are means from twelve different plants, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%. (C) Photographic images of detached leaves are shown. Scale bar, 2.5 cm.

Epidermic cells were observed microscopically and their dimensions measured (Fig. 11). Differences in leaf size were associated with the presence of smaller cells in the adaxial and abaxial epidermis of *PpSAP1* overexpressing lines, whereas the calculated number of cells per leaf was not thoroughly reduced in those lines (Table 1). The total number of stomata was similar in control and *PpSAP1* plants, thus leading to an increased density of stomata in the smaller leaves of lines #1, #5 and #6 (Table 1).

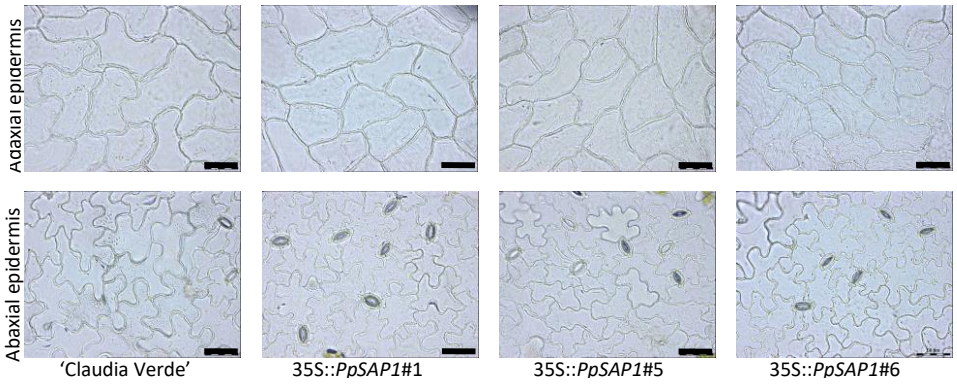

Figure 11. Microscopic photographs of epidermic cells in *PpSAP1* overexpressing lines. The adaxial and abaxial epidermis of control 'Claudia Verde' (CV) and 35S::*PpSAP1* lines #1, #5 and #6 is shown. Scale bar, 50 µm.

Table 1. Cell size and number in leaves of transgenic plum overexpressing *PpSAP1*.

	Adaxial epidermis		Abaxial epidermis		Stomata	
	Cell area (μm^2)	Cell number per leaf ($x10^4$)	Cell area (μm^2)	Cell number per leaf ($x10^4$)	Density (mm^{-2})	Number per leaf ($x10^4$)
'Claudia Verde'	4730 ± 1390	65.4 ± 16.3	3020 ± 1020	102.0 ± 21.5	65.0 ± 13.1	19.3 ± 4.0
35S::*PpSAP*#1	3890 ± 1050 **	52.9 ± 8.0 *	2550 ± 860 **	81.4 ± 17.3 *	83.4 ± 14.3 **	17.2 ± 4.4
35S::*PpSAP*#5	4040 ± 1090 **	53.6 ± 8.1 *	2560 ± 1050 **	84.6 ± 12.1	79.1 ± 16.6 *	16.8 ± 3.6
35S::*PpSAP*#6	3190 ± 1010 **	76.7 ± 15.0 *	2190 ± 860 **	111.0 ± 25.7	87.0 ± 12.8 **	20.7 ± 4.8

The significant difference with respect to 'Claudia Verde' is labelled, with a confidence level of 95 % (*) and 99% (**).

Genes related to cell growth are down-regulated in plum plants overexpressing *PpSAP1*

A series of plum candidate genes to mediate the phenotypical features observed in *PpSAP1* overexpressing plants were selected for quantitative real-time PCR (qRT-PCR) analysis. Thus, several rice and *Arabidopsis* genes showing down- or up-regulated expression in water-deficit stress tolerant plants overexpressing different *SAP* genes (Dansana et al., 2014; Giri et al., 2011; Kang et al., 2011) were compared with the peach genome by similarity searches (Supplementary Table S2). We found putative orthologs in peach of nine of these genes by reciprocal BLASTP analysis. In addition, four genes related to drought and stress response identified as differentially regulated in peach buds (Leida et al., 2012b) were selected for expression analysis (Supplementary Table S2). We designed specific primers for gene expression analysis based on peach sequences. Subsequently, PCR products amplified with such primers using plum cDNA as template were sequenced to confirm that qRT-PCR signals were in fact proceeding from plum putative orthologs of those genes. Among others, we analyzed the expression of late embryogenesis abundant (LEA)-like (ppa008651m), AWPM-19-like (ppa012188m), dehydrin (Prupe.7G161100), ABI5 binding protein

(ppa006974m), histone H1-3 (ppa011941m), galactinol synthase 2 (ppa008294m), NaCl-inducible calcium-binding protein (ppa012594m), and responsive to desiccation (RD) 29B (ppa001989m). However, none of them showed an altered pattern of expression in *PpSAP1* overexpressing lines (Supplementary Fig. S4). Thus, transcriptional targets of *PpSAP1* could be different from targets described for *SAP* genes from *Arabidopsis* and rice, or alternatively the observed effect on water retention could rely on the regulation of protein stability or activity instead of transcriptional regulation.

In parallel, a similar approach to identify putative orthologs in plum by successive reciprocal BLASTP analysis in peach and sequencing of plum amplicons was applied to several *Arabidopsis* genes involved in leaf morphology and cell growth (Supplementary Table S2).

Three of those genes were found to be down-regulated by qRT-PCR in plants overexpressing *PpSAP1* (Fig. 12), while other candidate genes did not show a significant variation (Supplementary Fig. S4). *TONOPLAST INTRINSIC PROTEIN* (*TIP*)-like, and *GROWTH-REGULATING FACTOR* (*GRF*)1-like are putative orthologs of genes regulated by the overexpression of *OsiSAP1* in rice (Dansana et al., 2014), whereas *TARGET OF RAPAMYCIN* (*TOR*)-like is related to a cell growth gene described in *Arabidopsis* and other species (Kurepa et al., 2009).

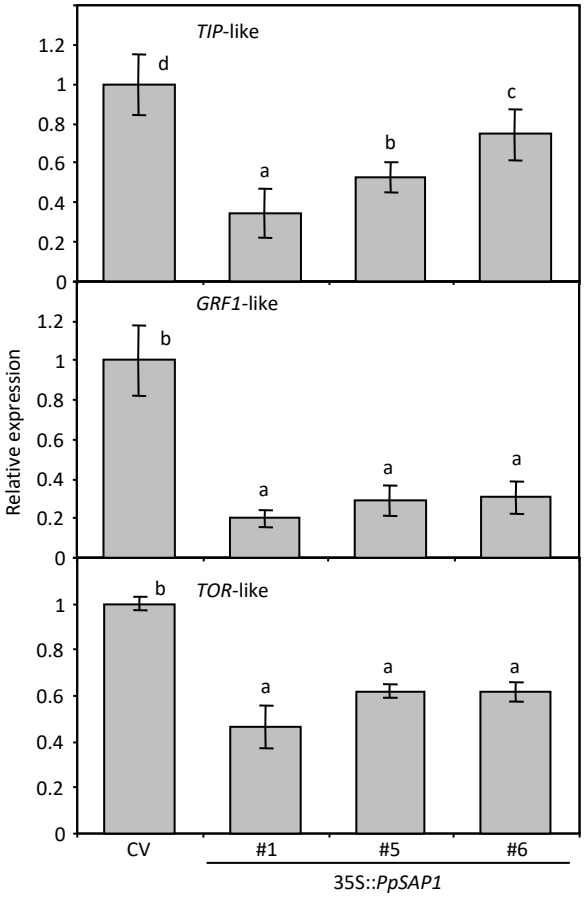

Figure 12. Genes differentially expressed in *PpSAP1* overexpressing lines. The relative expression of *TIP*-like, *GRF1*-like and *TOR*-like genes in Claudia Verde (CV) and 35S::*PpSAP1* lines #1, #5 and #6 is shown. An expression value of one is assigned to the CV. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Different letters (a–d) indicate significant difference between samples with a confidence level of 95%.

These results prompted us to examine the expression of *TIP*-like and *TOR*-like genes in the species (peach) and tissue (flower bud) in which *PpSAP1* was first identified. Interestingly, the decrease in *PpSAP1* expression along seasonal bud development correlated well with a quantitatively similar increase in *TOR*-like

expression in two independent experiments using two distinct cultivars (Fig. 1). However, the expression of *TIP*-like gene was essentially constant along bud development until it burst in samples collected on February in 'Springlady' and March in 'Big Top' (Fig. 13). Based on previous physiological measurements (Leida et al., 2010; Leida et al., 2012a), such burst of *TIP*-like expression occurred in dormancy-released buds, while dormant buds only showed a basal level of expression.

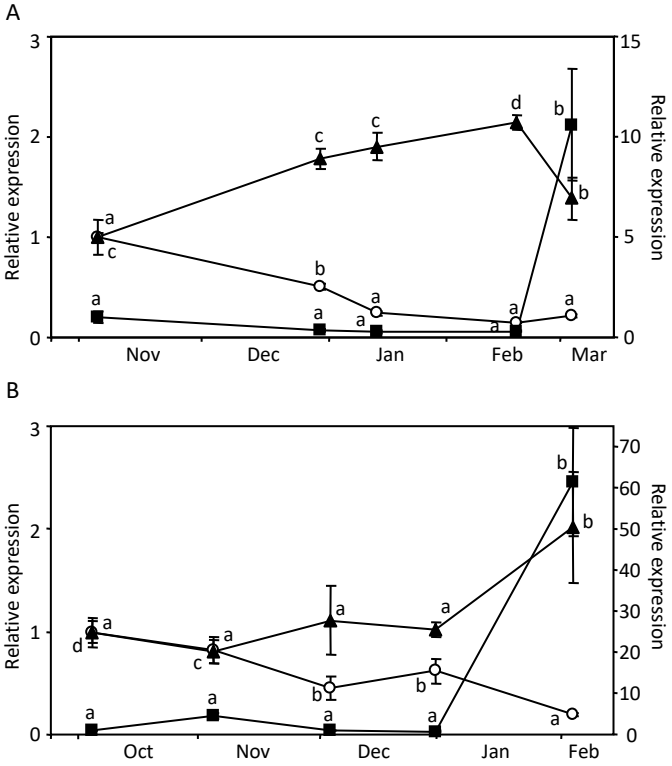

Figure 13. Expression of *TOR*-like and *TIP*-like genes in reproductive buds of peach. The relative expression of *PpSAP1* (white circles, left y-axis), *TOR*-like (black triangles, left y-axis) and *TIP*-like genes (black squares, right y-axis) was measured along bud development in cultivars (A) 'Big Top' (BT) and (B) 'Springlady' (SL). Dormancy was released in March (BT) and February samples (SL). An expression value of one is assigned to the first sample. Data are means from

two biological samples with three technical replicates each, with error bars representing standard deviation. Different letters (a–d) indicate significant difference between samples for each gene, at a confidence level of 95%.

Discussion

Classification of PpSAP1 as a stress-associated protein has taken into account the presence of A20 and AN1 Zn-finger domains and its phylogenetic closeness to described SAP proteins from rice and *Arabidopsis* (Vij and Tyagi, 2006; Jin et al., 2007), but also molecular and functional issues. *PpSAP1* gene expression was slightly but significantly affected by abiotic stresses such as cold, heat, drought and salinity, in flower buds of peach. In addition, heterologous expression of *PpSAP1* improved retention of water under drought stress in transgenic plum, which resembles increased tolerance to different abiotic stresses conferred by overexpression of *SAP* genes in other species (Mukhopadhyay et al., 2004; Kanneganti and Gupta, 2008; Dixit and Dhankher, 2011; Xuan et al., 2011; Hozain et al., 2012).

Relatively little is known about the molecular mechanism of SAP proteins, but some animal and plant counterparts have been postulated to regulate protein stability and regulation by related ubiquitination pathways. In animals, A20 protein performs deubiquitinase and E3 ubiquitin ligase activities to regulate nuclear factor κB signalling (Heyninck and Beyaert, 2005), and ZNF216 plays a role on muscle atrophy through the ubiquitin-proteasome system (Hishiya et al., 2006). In plants, both *Arabidopsis* AtSAP5 and rice OsiSAP7 regulate abscisic acid (ABA) signalling and show E3 ubiquitin ligase activity *in vitro* (Kang et al., 2011, 2013; Sharma et al., 2015). In summary, sequence analysis, expression profile and functional characterization of PpSAP1 contributed to categorize it into the SAP group of ubiquitin-binding regulators.

PpSAP1 is preferentially expressed in peach organs and tissues undergoing dormancy such as bud and embryo, but also in adult leaves. Its expression decreases along flower bud development and embryo cold stratification (Leida et

al., 2012b), which could point to a role of *PpSAP1* in dormancy setting-up and maintenance. *PpSAP1* expression is not strictly linked to the dormancy status of buds, as illustrates the early drop of *PpSAP1* expression depicted in Fig. 4B; however a role of *PpSAP1* in dormancy regulation and meristem growth resumption can not be rule out, as discussed below.

Under overexpression, *PpSAP1* exerts a low but significant effect on water retention in stressed leaves and plants, which supports a potential role of *PpSAP1* in drought tolerance in vegetative tissues and plant organs experiencing developmental dormancy, such as buds and embryos. This becomes particularly meaningful in buds of temperate perennial plants, which have to cope with seasonal environmental constraints such as low temperature and drought stress. Unexpectedly, *PpSAP1* overexpressing plants had an additional morphological phenotype affecting the size and form of leaves. Leaves were smaller and narrower, with an acute leaf base angle, leading to an elliptical shape instead of the most habitual ovate one. Thus, as a consequence of *PpSAP1* overexpression plum leaves became somehow more similar to peach leaves. This smaller leaf size was concomitant with and most likely a result of decreased cell size. The rice *SAP* genes *OsDOG* and *ZPF185* were previously described as suppressors of cell growth by a gibberellin-mediated mechanism (Liu et al., 2011; Zhang et al., 2016). However, contrarily to *PpSAP1*, *ZPF185* expression increased sensitivity to abiotic stresses, suggesting that *SAP* roles on stress and developmental processes are unexpectedly diverse.

Observed water retention and anatomical phenotypes were similar in overexpressing lines #1, #5 and #6, in spite of their different *PpSAP1* expression level (Fig. 8). This could be explained by a saturating effect of *PpSAP1* accumulation on those measurements; or alternatively the effective amount of PpSAP1 protein could be similar in the three transgenic lines, regardless of their distinct *PpSAP1* transcript expression values.

The expression of putative orthologs of genes affected by the overexpression of *SAP*-like genes in rice and *Arabidopsis* (Dansana et al., 2014; Giri et al., 2011; Kang et al., 2011), in addition to several stress-related genes differentially

regulated in peach buds were investigated in *PpSAP1* transgenic lines. *GRF1*-like genes were down-regulated in both transgenic rice and plum as a consequence of *OsiSAP1* (Dansana et al., 2014) and *PpSAP1* expression, respectively. GRF transcription factors are important regulators of plant growth and development affecting the response to abiotic stresses and leaf morphology and size, among other processes (Omidbakhshfard et al., 2015). Interestingly, a triple insertional mutant of *AtGRF1-AtGRF3* showed smaller leaves due to a decrease in cell size (Kim et al., 2003), resembling the phenotype observed in *PpSAP1* overexpressing lines. These data present *GRF1*-like as a putative transcriptional target of PpSAP1 regulatory pathway with presumable impact on the stress response and cell growth effects described in *PpSAP1* transgenic plants.

On the other hand, *TIP*-like orthologs were differentially regulated in *OsiSAP1*-expressing rice (up-regulated) and *PpSAP1*-expressing plum (down-regulated), which points towards diverging roles and mechanisms of related members of the *SAP* family. TIP aquaporins are involved in water permeability and transport of small molecules across the tonoplast membrane, impinging on stress responses and cell turgor-driven growth (Maurel et al., 2015; Afzal et al., 2016). In fact, γ-TIP expression in *Arabidopsis* correlates with cell enlargement (Ludevid et al., 1992), and is increased by gibberellins (Phillips and Huttly, 1994).

PpSAP1 overexpression in plum also reduced the expression of *TOR*-like gene, a key regulator of cell growth and metabolism in eukaryotic species in response to nutrient and stress related cues (Rexin et al., 2015). *TOR* was essential for embryo development in *Arabidopsis* (Menand et al., 2002), and inhibition of TOR function with rapamycin impaired plant growth and development. Most relevantly to this study, suppression of TOR signalling reduced cell elongation in the hypocotyl and led to smaller leaves due to decreased cell size (Ren et al., 2012).

PpSAP1 and *TOR*-like showed opposite expression profiles not only in *PpSAP1* overexpressing lines, but also in flower buds of peach along development (Fig. 13). These data strongly support *TOR*-like repression by PpSAP1 activity or by PpSAP1 downstream effectors (Fig. 14). However, *TIP*-like expression did not correlate with *PpSAP1* accumulation in buds; *TIP*-like expression only peaked in

late bud samples, precisely after dormancy release, which suggests that an additional unknown factor may link dormancy release with *TIP*-like expression. The transport of water and other molecules into the tonoplast performed by TIP-like aquaporins would contribute then to increase the cell turgor required for cell expansion and growth, leading to bud-break after the integration of different environmental and intrinsic signals (Fig. 14).

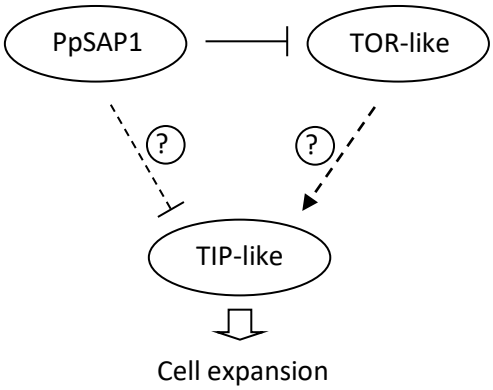

Figure 14. Proposed model of transcriptional interactions between *PpSAP1*, *TOR*-like and *TIP*-like. Transcriptional activation is labelled with an arrow. Transcriptional repression is labelled with a T-shaped line.

Methods

Plant material

The peach plants required for this study were grown at the Instituto Valenciano de Investigaciones Agrarias (IVIA) located in Moncada (Spain). For tissue-dependent gene expression analysis, reproductive and vegetative buds (collected on 12 November 2009), leaves (6 November 2012), embryos, flower parts (26 March 2010) and fruit tissues (29 Juny 2010) were obtained from cv. 'Big Top'. Reproductive buds of peach were obtained from 'Springlady' and 'Big Top' cultivars. Collection dates and dormancy status of these buds have been

described in detail previously (Leida et al., 2010, 2012b). The effect of stresses on gene expression in peach was studied in dormant buds (collected on 3 November 2015) and dormancy-released buds (25 January 2016) of cv. 'Crimson Baby', and non-dormant buds of cv. 'Rose Diamond' collected on 2 February 2013. For gene expression analysis of peach leaves under drought stress, leaves gathered from three different trees of cv. 'Red Candem' were collected on 27 April 2015. Finally, for leaf discs assays, leaves from five different trees of cv. 'Big Top' were collected on 9 June 2015. The culture chamber was maintained at 24°C with 12h:12h light (3 klx):dark photoperiod.

Stress treatments

To analyze *PpSAP1* expression in flower buds under stress conditions, six budsticks from three different trees per time and treatment were collected. Budsticks were placed in glass tubes with 25 ml of water. Temperature incubations were made at 37°C, 25°C (control) and 4°C in the dark. For salt-stress treatment budsticks were watered with 200 mM NaCl solution, and desiccation stress was carried out in the absence of water. Routinely the base of the budsticks was cut and the solution replaced with fresh one. Flower buds were gathered at 24 h and 72 h.

Stress experiments were also performed on leaf material. For desiccation stress, leaves from three different trees were collected and placed into glasses with the petiole in contact with water (control) or without water (stressed samples), for one, three and seven days. For temperature, salt and ABA treatments, leaf discs were used as described previously (Trotel et al., 1996). Discs (1 cm diameter) were immersed in a solution containing 5 mM HEPES, 1.5 mM $CaCl_2$, and 10 mM KCl during 4 h with gently shaking. After incubation, discs were transferred to fresh solution at 37°C, 25°C (control) and 4°C in the dark for temperature stress. For salt and ABA incubations, discs were transferred and submerged in fresh solutions with 250 mM NaCl and 50 µM ABA, respectively. Ten discs per treatment were collected at 4 h and 24 h. As a control, the expression of a *LEA*-like gene was monitored in leaf samples.

Isolation of RNA and qRT-PCR

Total RNA from peach material was isolated using the RNeasy Plant Mini Kit (Qiagen). Polyvinylpyrrolidone (PVP-40) 1 % (w/v) was added to the kit extraction buffer before use. RNA from transgenic plum material was isolated using a rapid cetyltrimethylammonium bromide (CTAB)-based procedure (Gambino et al., 2008). In both cases, contaminant genomic DNA was removed with the RNase-Free DNase Set (Qiagen) according to manufacturer's instructions. Total RNA (500 ng) was reverse transcribed with PrimeScript RT reagent kit (Takara Bio) in a final volume of 10 µl. Two µl of a 20X-diluted first-strand cDNA was used for PCR in a total volume of 20 µl. Quantitative RT-PCR was performed on a StepOnePlus Real-Time PCR System (Life Technologies), utilizing SYBR premix Ex Taq (Tli RNaseH plus) (Takara Bio). The PCR protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Specificity of the amplification was evaluated by the presence of a single peak in the dissociation curve after PCR and by size estimation of the amplified product by electrophoresis.

Actin-like, AGL26-like, SAND-like and tubulin-like transcripts were used as optional reference genes. Determination of the most stable housekeeping genes was performed using Bestkeeper (Pfall et al., 2004), NormFinder (Andersen et al., 2004) and ΔCt (Silver et al., 2006). For each group of samples (tissues, bud development and stresses), the genes with better stability value following these three methods were selected as reference genes (Supplementary Table S3). SAND-like was selected as the most stable gene for stress assays and, along with actin-like, for expression analysis of reproductive buds of peach. For tissue expression analysis, actin-like and tubulin-like were the most suitable reference genes. Finally, we used actin-like and AGL26-like for expression experiments in the transgenic plum lines. When two reference genes were required for the analysis, the normalization factor was calculated by the geometric mean of the values of both genes. Relative expression was measured using a relative standard curve. Results were the average of two or three independent biological replicates, with

2–3 technical replicates each. The primers used in this study are listed in Supplementary Table S1.

Cloning of *PpSAP1* and plasmid construction

For cloning of *PpSAP1* into the yeast two-hybrid plasmid pGBKT7, the whole coding region of *PpSAP1* was PCR-amplified from cDNA obtained from dormant buds of peach, using the primers listed in Supplementary Table S1 under the following PCR conditions: 5 min at 95°C, followed by 5 cycles of 30 s at 95°C, 30 s at 57°C and 1 min at 68°C, then 30 cycles of 30 s at 95°C, 30 s at 69°C and 1 min at 68°C, and finally 10 min at 68°C. The PCR product was digested with *Eco*RI and *Bam*HI enzymes and cloned between the *Eco*RI/*Bam*HI restriction sites of pGBKT7 vector (Clontech-Takara Bio).

In order to clone *PpSAP1* into the pROK2 plasmid for constitutive expression of the gene in transgenic plum, *PpSAP1* was amplified using pGBKT7-*PpSAP1* as DNA template with primers listed in Supplementary Table S1. The PCR protocol consisted of 5 min at 94°C, followed by 3 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, then 22 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C, and a final step of 5 min at 72°C. The PCR product was digested with *Xba*I and *Bam*HI enzymes and cloned between the *Xba*I/*Bam*HI sites of pROK2 plasmid (Baulacombe et al., 1986).

Phylogenetic analysis of PpSAP1 protein

Sequences similar to *Arabidopsis thaliana* SAP proteins were obtained from *Prunus persica* genome database (https://phytozome.jgi.doe.gov/) through BLASTN search (default parameters, BLOSUM62 comparison matrix), and checked for the presence of both A20 and AN1 domains using the Simple Modular Architecture Research Tool (Schultz et al., 1998) (SMART; http://smart.embl-heidelberg.de/). Predicted protein sequences were aligned together with PpSAP1 and SAP proteins described in *Arabidopsis* and rice (Vij et al., 2006) using Clustal Omega (Sievers et al., 2011) (http://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was elaborated using Maximum Likelihood method

(Bootstrapped with 1000 replicates). Both alignment and phylogeny were carried out in MEGA version 6 (Tamura et al., 2013).

Analysis of protein interaction by yeast two-hybrid system

The pGBKT7-*PpSAP1* plasmid expressing a fusion of PpSAP1 protein with the Gal4 DNA-binding domain (pGBKT7) was introduced into the *Saccharomyces cerevisiae* strain Y2HGold following the yeast transformation procedure and solutions included within the Matchmaker Gold Yeast Two-Hybrid System (Clontech-Takara Bio). The pGBKT7-*PpSAP1* plasmid did not activate autonomously the protein interaction reporters in minimal medium supplemented with the antibiotic Aureobasidin A (AbA) and the chromogenic substrate X-α-Gal at the recommended concentrations (Clontech-Takara Bio).

A yeast two-hybrid library was performed in pGADT7-Rec vector expressing a fusion with the Gal4 activation domain, following the Make Your Own "Mate & Plate™" Library System (Clontech-Takara Bio). Briefly, one µg of total RNA obtained by pooling RNA from dormant and dormancy-released flower buds of peach ('Big Top') was reverse transcribed, and cDNA was cloned into pGADT7-Rec by *in vivo* recombination in the yeast strain Y187, following the manufacturer's instructions. The library contained about 1.5 x 10^6 independent clones. After mating of Y2HGold strain harbouring pGBKT7-*PpSAP1* with the Y187 library, approximately 3.5 x 10^7 clones were screened. Two-hybrid interactions were tested in minimal medium without histidine and adenine, and supplemented with AbA (125 ng/ml) and X-α-Gal (40 µg/ml). The inserts contained into positive colonies were amplified using the Matchmaker Insert Check PCR Mix 2 (Clontech-Takara Bio), and digested with *Alu*I and *Rsa*I restriction enzymes for the identification of repeated clones with identical restriction patterns. Independent clones were rescued from yeast using the Easy Yeast Plasmid Isolation Kit (Clontech-Takara Bio), transformed into *Escherichia coli* and sequenced. The protein interaction was confirmed by subsequent transformation of Y2HGold containing pGBKT7-*PpSAP1* with positive clones.

Genetic transformation of plum

Agrobacterium tumefaciens preparation and transgenic plant regeneration of plum (*Prunus domestica* cv. Claudia Verde) were performed according to a previous report (Petri et al., 2008b). The *Agrobacterium tumefaciens* strain LBA4404, carrying the binary vector pROK2-*PpSAP1* was used. The construction contained the neomycin phosphotransferase gene (*npt*II) for aminoglycoside antibiotic selection of the transgenic plants. For co-cultivation, a 10 ml overnight culture of *Agrobacterium tumefaciens* with an optical density (OD) at 600 nm of 0.2-1.0 was centrifuged at 5,000xg for 10 min and resuspended in 50 ml of bacterial resuspension medium consisting of MS salts, 2% (w/v) sucrose and 100 µM acetosyringone. This culture was shaken (175 rpm) at 25°C for 5 h before use. For plant explant preparation, the endocarp was removed with a nutcracker, and the seeds were surface-sterilized for 30 min using 1% sodium hypochlorite solution containing 0.02% of Tween-20 and rinsed three times with sterile distilled water. Disinfected seeds were soaked in sterile distilled water overnight at room temperature and the seed coats were removed with a scalpel. The radicle and the epicotyl were discarded, and the hypocotyl was sliced into several cross sections (less than 1 mm), which were used for co-transformation.

After 3 days of slice co-culture on shoot regenerating medium (SRM: ¾ MS based medium with 7.5 µM thidiazuron (TDZ), 0.25 µM indole butyric acid (IBA), 9.05 µM 2,4-D and 100 µM acetosyringone), the hypocotyl slices were transferred to SRM selective medium without 2,4-D and acetosyringone, and containing timentin (600 mg/l) and kanamycin (80 mg/l) for 8 weeks. Regenerated shoots were transferred to the shoot growing medium (SGM), in which TDZ was replaced with 1.0 µM 6-benzylaminopurine (BAP).

Plum shoots were maintained by sub-culturing at 4-week intervals on the selective SGM at 23°C under cool white fluorescent tubes (1.5 klx) and a 16-h photoperiod. When shoots reached 2-3 cm long they were separated from the cluster and transferred to rooting media (RM) (Gonzalez-Padilla et al., 2003) supplemented with kanamycin (40 mg/l). In 3-4 weeks roots started appearing and after 1-3 more weeks, shoots were ready for acclimatization. *In vitro* plants

were removed from culture pots and transplanted into pots containing sterilized topsoil sand (4:1) mixture. Plants were covered with transparent plastic pots and progressively removed as plants hardened-off. Control plants were subjected to the same *in vitro* techniques that transformed plants.

Southern analysis

About 20 µg of *Hind*III- and *Eco*RI-digested genomic DNA samples were separated on 1 % (w/v) agarose gels and transferred to positively charged nylon membranes (Roche Diagnostics Corporation) by capillary blotting. A 696-bp PCR fragment of *npt*II gene was labelled with digoxigenin (DIG) using the PCR DIG labeling mix (Roche Diagnostics Corporation) and the specific primers previously used (Petri et al., 2008). Prehybridization and hybridization of blots to the labelled probe were performed at 42°C. The blots were then washed twice at 23°C in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) plus 0.1% (w/v) sodium dodecyl sulfate (SDS) for 15 min, and twice at 65°C in 0.5x SSC, 0.1% SDS for 15 min. Hybridizing bands were visualized with anti-DIG antibody-alkaline phosphatase and CDPStar (Roche Diagnostics Corporation) on X-ray films.

Evaluation of water loss and drought tolerance

The water loss under drought conditions was evaluated in detached leaves and whole plants. In the first experiment, we used leaves from plum plants six months after acclimatization. Two leaves from the medium part of the plant were detached from the control 'Claudia Verde' and the transgenic lines #1, #5 and #6, with seven independent plants each genotype. Leaves were dried out on trays at the growth culture chamber conditions. Leaf weight was measured at 30-60 min intervals, for a total time of 340 min.

To determine drought tolerance in transgenic plum plants, two months acclimatized plants were exposed to dehydration stress by stopping watering. After seven days of stress, the fresh weight, weight after rehydration and dry weight of all the plant leaves were measured. The experiment was made with the three different transgenic lines, with twelve plants each line.

The relative water content (RWC) was measured by using the following formula:

RWC (%) = 100 * (FW-DW) / (TW-DW)

where FW is fresh weight, DW is dry weight and TW is turgid weight (the initial leaf weight in the experiment of detached leaves, and the leaf weight after rehydration in the whole plant experiment).

Morphological and cell size measurements

Morphological measurements were made to wild type and the three transgenic lines (twelve plants each line) two months after acclimatization. To determine leaf area and base angle, photographed leaves were analyzed using ImageJ (version 1.49v, Wayne Rasband,).

For cell size measurements, two medium leaves of each plant were collected. After harvest, thin sections of epidermis leaf were excised from the middle part of the leaf, immersed in water, observed using a Leica CTR Mic microscope, and analyzed using ImageJ. Final measurements are the average of 300 cells from ten plants of each line.

Similarity searches

In order to identify putative orthologs of rice and *Arabidopsis* genes in peach we performed a reciprocal BLASTP analysis. First we made a BLASTP similarity search (https://phytozome.jgi.doe.gov/) on peach database using selected proteins from *Arabidopsis* and rice as queries. The best hit in peach was subsequently compared by BLASTP with *Arabidopsis* or rice databases, and those genes obtained reciprocally by both searches were considered as putative orthologs.

Statistical analysis

Statistical analyses were performed using the Statgraphics XVI.I package (Statpoint Technologies). Previously, data were evaluated for homoscedasticity and normality in order to select parametric or non-parametric tests. The means of two samples were compared using a Student t-test and comparisons of multiple samples were evaluated by the parametric Fishers's least significant difference (LSD) test and non-parametric Klustal-Wallis test, with a confidence level of 95% or 99%. Significantly different samples were labelled with asterisks or different letters.

CHAPTER 2:

Chromatin-associated regulation of sorbitol synthesis in flower buds of peach

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Abstract

Perennial plants have evolved an adaptive mechanism involving protection of meristems within specialized structures named buds in order to survive low temperatures and water deprivation during winter. A seasonal period of dormancy further improves tolerance of buds to environmental stresses through specific mechanisms poorly known at the molecular level. We have shown that peach *PpeS6PDH* gene is down-regulated in flower buds after dormancy release, concomitantly with changes in the methylation level at specific lysine residues of histone H3 (H3K27 and H3K4) in the chromatin around the translation start site of the gene. PpeS6PDH encodes a NADPH-dependent sorbitol-6-phosphate dehydrogenase, the key enzyme for biosynthesis of sorbitol. Consistently, sorbitol accumulates in dormant buds showing higher *PpeS6PDH* expression. Moreover, *PpeS6PDH* gene expression is affected by cold and water deficit stress. Particularly, its expression is up-regulated by low temperature in buds and leaves, whereas desiccation treatment induces *PpeS6PDH* in buds and represses the gene in leaves. These data support the involvement of chromatin modification mechanisms in the transcriptional regulation of *PpeS6PDH* expression and sorbitol accumulation in flower buds of peach. In addition to its role as a major translocatable photosynthate in Rosaceae species, sorbitol is a widespread compatible solute and cryoprotectant, which suggests its participation in tolerance to environmental stresses in flower buds of peach.

Introduction

Dormancy ensures the survival of vegetative and reproductive meristems in a quiescent state, which is released after the fulfilment of bud-intrinsic requirements of chilling, resembling the vernalization process described in *Arabidopsis thaliana* and cereals (Chouard, 1960; Horvath et al., 2003). Buds undergoing dormancy are more tolerant to low and freezing temperatures and to desiccation, in virtue of physiological and molecular mechanisms that are insufficiently known, and that could partially overlap with those implicated in bud dormancy regulation (Fennell, 2014; Wisniewski et al., 2015).

Among other, several epigenetic mechanisms involving genomic DNA methylation (Santamaría et al., 2009; Kumar et al., 2016a), histone modifications (Horvath et al., 2010; Leida et al., 2012b; Ríos et al., 2014; Saito et al., 2015) and small RNAs production (Bai et al., 2016; Niu et al., 2016) have been postulated to mediate dormancy-dependent regulation of gene expression. Foremost targets of those regulatory pathways are *DORMANCY-ASSOCIATED MADS-box* (*DAM*) genes, identified as key transcriptional factors modulating bud dormancy in leafy spurge and stone-fruit tree species (Bielenberg et al., 2008; Horvath et al., 2010; Niu et al., 2016). Four from six tandemly arrayed *DAM* genes in peach are specifically modified by trimethylation of histone H3 in lysine 27 residue (H3K27me3) at specific genomic regions (de la Fuente et al., 2015). In spite of such burst of dormancy literature, supporting evidence on the participation of similar epigenetic pathways in the regulation of stress-related genes along bud development is still lacking.

Sorbitol is the primary photosynthetic product and the major phloem-translocated form of carbon in the Rosaceae (Webb and Burley, 1962; Bieleski, 1969). This sugar alcohol, or polyol, has been proposed to perform a protective role against stresses, acting as cryoprotectant, osmolyte and compatible solute under freezing, osmotic and water stress, respectively (Bieleski, 1982; Loescher, 1987; Escobar-Gutiérrez and Gaudillère, 1996). Drought stress increases sorbitol accumulation in peach, although its participation in osmotic adjustment is a

matter of controversy (Escobar-Gutiérrez et al., 1998; Bianco et al., 2000). Sorbitol is produced in source tissues (photosynthetic leaves) via reduction of glucose-6-phosphate to sorbitol-6-phosphate by NADPH-dependent sorbitol-6-phosphate dehydrogenase (S6PDH), and the subsequent dephosphorylation of sorbitol-6-phosphate by a specific phosphatase, whereas sorbitol utilization occurs in sink tissues (Grant and ap Rees, 1981; Loescher, 1982). Since the first cloning of *S6PDH* gene from apple (Kanayama et al., 1992), many *S6PDH* have been identified based on sequence similarity to this gene, however few of them have been characterized at the enzymatic level. The overexpression of *S6PDH* from apple in transgenic tobacco increases sorbitol content and induces necrotic lesions in some cases (Tao et al., 1995; Sheveleva et al., 1998), whereas *S6PDH* silencing in apple reduces sorbitol accumulation and alters carbon partitioning (Teo et al., 2006). Moreover, the expression of *S6PDH* is up-regulated by abscisic acid, low temperature and NaCl treatments in apple (Kanayama et al., 2006). Interestingly, expression of *S6PDH* from apple in a *Saccharomyces cerevisiae* mutant deficient in synthesis of the osmolyte glycerol partially restores the tolerance to high NaCl concentrations (Shen et al., 1999). Also, overproduction of sorbitol by the expression of apple *S6PDH* confers NaCl tolerance in transgenic Japanese persimmon (Gao et al., 2001).

Very recently, an ortholog of *S6PDH* has been cloned in peach, and its encoded protein (PpeAld6PRase) has been purified and extensively characterized at the enzymatic level (Hartman et al., 2017). In this work we refer to the gene encoding PpeAld6PRase as *PpeS6PDH*, following nomenclature suggestions of the Genome Database for Rosaceae (https://www.rosaceae.org/gene_class_listing). *PpeS6PDH* was unexpectedly expressed in dormant flower buds, a sink tissue, in a developmentally regulated manner. We have postulated its participation in the environmental and developmental dependent synthesis of sorbitol in buds.

Results

Characterization of S6PDH protein in peach

In previous transcriptomic studies we have identified some expressed sequence tags (GenBank accessions GR410685 and JK006377) corresponding to a S6PDH-like gene with differential expression along the development of flower buds of peach (Leida et al., 2010, 2012b). The International Peach Genome Initiative (Verde et al., 2013) named this gene ppa009007m (v1.0) and Prupe.8G083400 (v2.1). In this study we will refer to ppa009007m gene as *PpeS6PDH*, according to the standard gene nomenclature in the Rosaceae (Jung et al., 2015) and suggestions of the Genome Database for Rosaceae (https://www.rosaceae.org/gene_class_listing).

The PCR-amplified coding DNA sequence of *PpeS6PDH* was identical to ppa009007m and to the mRNA coding for an Ald6PRase enzyme recently characterized (Hartman et al., 2017). The 310 amino acids long PpeS6PDH protein shared 76 % identity with NADPH-dependent sorbitol-6-phosphate dehydrogenase from *Malus domestica* and 62% identity with NADPH-dependent sorbitol-6-phosphate dehydrogenase from *Oryza sativa*, both enzymes well characterized. The phylogenetic analysis showed that PpeS6PDH is very close to S6PDH-like proteins from the *Prunus* genus and to other well characterized S6PDH proteins from the Rosaceae family (*Malus domestica* and *Pyrus pyrifolia*) (Fig. 15; Hartman et al., 2017).

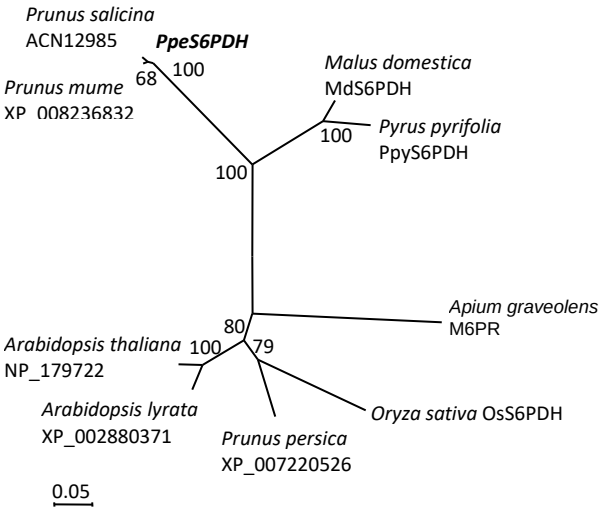

Figure 15. Phylogenetic tree of S6PDH and related proteins. The tree was constructed using the Maximum Likelihood method and bootstrapped with 1000 replicates. The scale bar indicates the branch length that corresponds to the number of substitutions per amino acid position

The gene has been recently cloned with an N-terminal His-tag fusion into pET19b vector, and the recombinant protein purified and characterized at the enzymatic level, showing a NADPH-dependent reductase activity on glucose-6-phosphate to produce sorbitol-6-phosphate (Hartman et al., 2017). We independently amplified *PpeS6PDH* gene and cloned it into pET302/NT-His vector inserting a 6xHis tag at the N-terminal end of the protein and pET303/CT-His leading to a C-terminal 6xHis tag. The SDS-PAGE analysis of the purified recombinant protein showed a band between 29 kDa and 47 kDa markers, which was in concordance with the expected molecular mass of 36.7 kDa (Supplementary Fig. S5). In a specific enzymatic assay, purified His-PpeS6PDH reduced glucose-6-phosphate to sorbitol-6-phosphate using NADPH as electron donor with a specific activity of 2.95 U/mg (Table 2), slightly higher than the activity described for related S6PDHs fused to N-terminal His tags from rice and apple (Figueroa and Iglesias, 2010; Yadav and Prasad, 2014), and very similar to

the Vmax parameter found by Hartman et al. (2017) in their particular His-PpeS6PDH preparations. Reduction of mannose-6-phosphate occurred at a much lower specific activity of 0.07 U/mg. We could not detect the enzymatic activity of PpeS6PDH with a His tag at the C-terminus, as similarly observed in apple S6PDH (Figueroa and Iglesias, 2010). Taken together, these results confirmed that PpeS6PDH is able to perform sorbitol-6-phosphate dehydrogenase activity *in vitro* and that short fusions at the C terminus abolish PpeS6PDH activity.

Table 2. Enzymatic activity of recombinant PpeS6PDH

	Substrate	Activity (mU)	Protein (µg)	Specific activity (U/mg)
His-PpeS6PDH	Glucose-6-phosphate	8.85 ± 0.81	3.0	2.95 ± 0.27
His-PpeS6PDH	Mannose-6-phosphate	0.25 ± 0.09	3.0	0.07 ± 0.03
PpeS6PDH-His	Glucose-6-phosphate	ND	1.5	-

ND, not detected

PpeS6PDH expression is developmentally regulated in buds

To properly characterize *PpeS6PDH* at the molecular level, its relative expression was studied in different peach tissues. *PpeS6PDH* showed higher expression in leaves and flower buds, being slightly lower in petals and sepals (Fig. 16). *PpeS6PDH* expression was also appreciable in other flower organs (stamens and carpels), but insignificant in fruit (skin and flesh) and embryo samples.

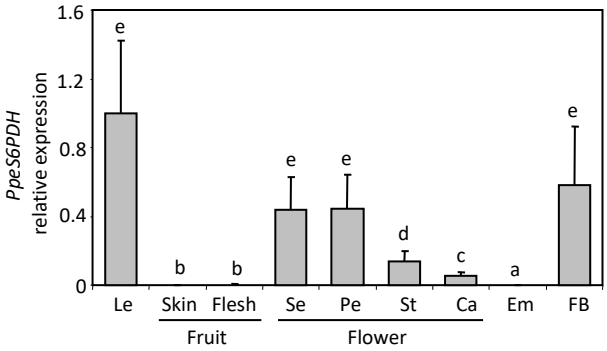

Figure 16 Relative expression of *PpeS6PDH* by qRT-PCR. Samples included different plant tissues and organs: leaf (Le), fruit skin, fruit flesh, sepal (Se), petal (Pe), stamen (St), carpel (Ca), embryo (Em) and flower bud (FB). Tubulin-like and actin-like genes were used as reference genes. An expression value of one is assigned to the leaf sample. Data are means from two biological samples with three technical replicates each, with error bars representing standard deviation.

We estimated the expression profile of *PpeS6PDH* along bud development in two cultivars with different chilling requirements for bud dormancy. Results confirmed the down-regulation of *PpeS6PDH* in flower buds after dormancy release (Fig. 17), as revealed by previous transcriptomic studies (Leida et al., 2010, 2012b). Interestingly, *PpeS6PDH* expression peaked at different times in dormant samples of 'Red Candem' and 'Big Top' cultivars, in concordance with their different chilling requirements. The early cultivar 'Red Candem' showed maximal expression on December 1 (RC2 sample), while the medium cultivar 'Big Top' reached maximal levels of expression on January 12 (BT3 sample). The expression decreased drastically in dormancy released buds, on December 29 (RC4) and March 2 (BT5) in 'Red Candem' and 'Big Top' cultivars, respectively (Fig. 17).
Interestingly, the 'Big Top' sample collected on December 29 (BT2) increased *PpeS6PDH* expression with respect to the sample collected on November 3 (BT1), contrarily to the strong reduction observed in 'Red Candem' in the same period of time. These data argue for a dormancy-dependent regulation of *PpeS6PDH* expression in flower buds, acting independently of putative environmental effects.

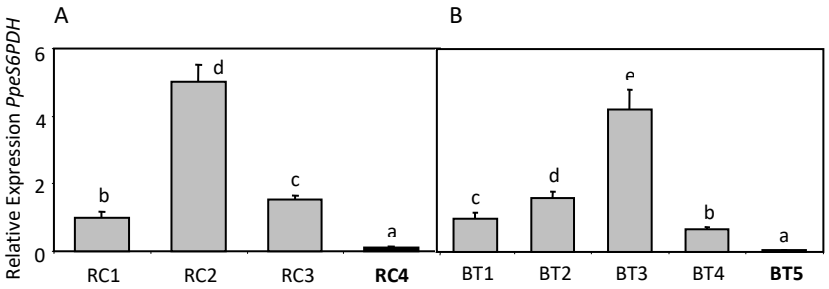

Figure 17 Relative expression of *PpeS6PDH* during flower bud development. Bud samples from the cultivars 'Red Candem' (**A**) and 'Big Top' (B) were collected in autumn and winter (2009/2010) at different dates: November 3 (RC1 and BT1), December 1 (RC2), December 15 (RC3), December 29 (RC4 and BT2), January 12 (BT3), February 16 (BT4) and March 2 (BT5). In RC4 and BT5 samples (dark bars in the graph) dormancy was already released. SAND-like and actin-like genes were used as reference genes. An expression value of one is assigned to the first sample. Data are means from two biological samples with three technical replicates each, with error bars representing standard deviation. Different letters (a–e) indicate significant difference between samples with a confidence level of 95%.

PpeS6PDH expression is regulated at the chromatin level

We performed ChIP analysis in order to identify chromatin modifications at *PpeS6PDH* during dormancy release. A previous genome-wide study of H3K27me3 enrichment in buds was used to identify this specific modification in *PpeS6PDH* locus (de la Fuente et al., 2015). We found that H3K27me3 was significantly enriched on the translation start region of *PpeS6PDH* locus in ND sample, obtained from dormancy released buds (Fig. 18A). H3K27me3 modification has been found associated with silencing of gene expression in peach and other plant and animal species, in close agreement with the repression of *PpeS6PDH* expression in dormancy released buds (Fig. 17). H3K27 trimethylated region in *PpeS6PDH* contained a repetitive GAGA motif (marked with asterisks in Fig. 18A), which has been found associated with H3K27me3 stretches in peach and *Arabidopsis* (Deng et al., 2013; de la Fuente et al., 2015). In order to confirm these data, we amplified by quantitative real-time PCR a fragment contained into the

H3K27 trimethylated region in H3K27me3- and H3K4me3-immunoprecipitated samples along bud development. A concomitant increase in H3K27me3 and decrease in H3K4me3 enrichment was observed in BT5 sample containing buds after dormancy release (Fig. 18B,C). Thus, differential H3K27me3 in the region around the ATG of *PpeS6PDH* was confirmed by quantitative PCR, and this modification was paralleled by a slight decrease in trimethylation of H3K4, a modification usually associated with transcriptional activation.

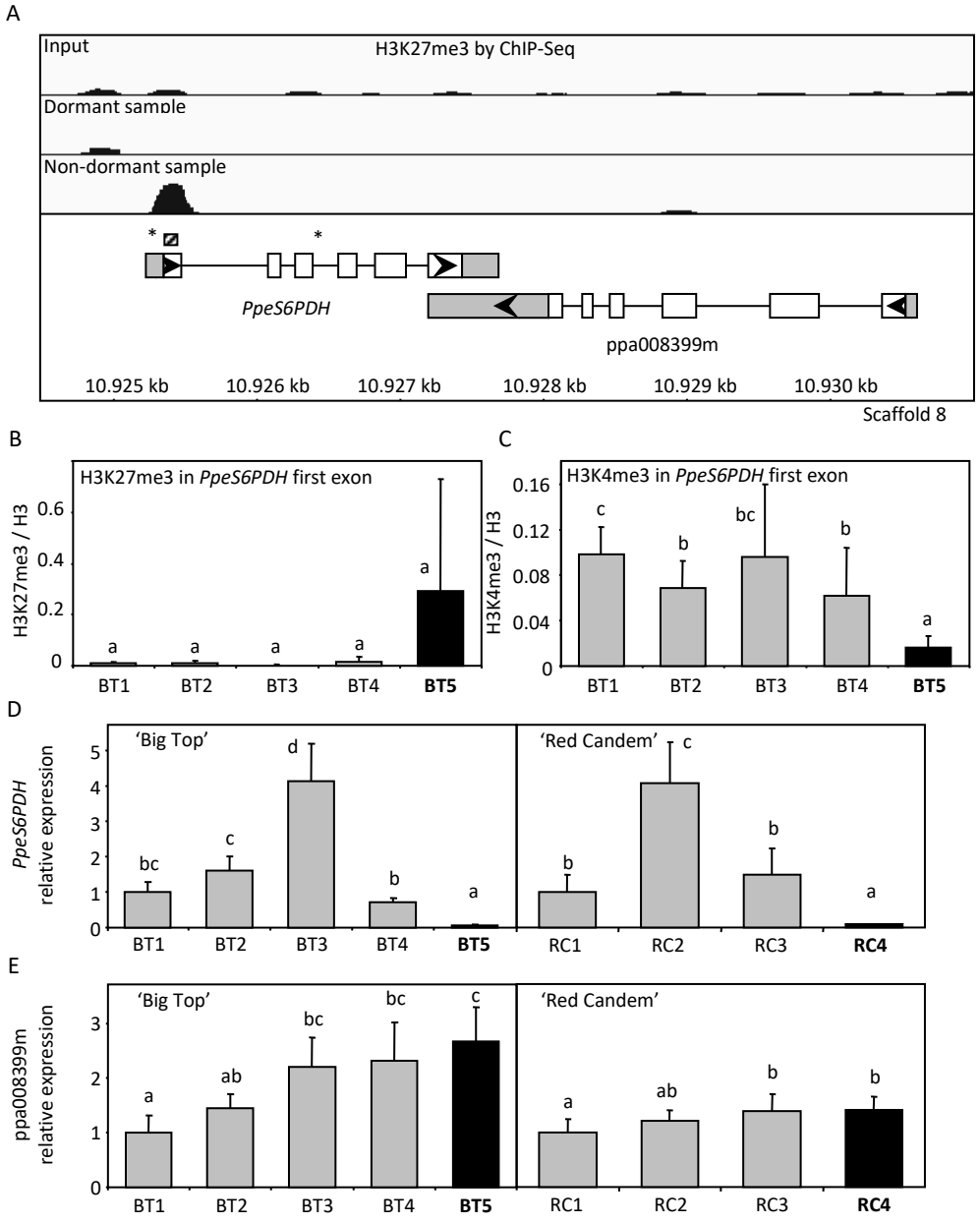

Figure 18. Chromatin immunoprecipitation (ChIP) analysis of *PpeS6PDH* gene during bud development. (A) H3K27me3 enrichment in *PpeS6PDH* and the adjacent gene ppa008399m by ChIP-Seq analysis in dormant (BT1) and non-dormant (BT5) buds of 'Big Top', compared with

input sample. The differentially methylated region is labelled with a striped rectangle. Predicted *PpeS6PDH* and ppa008399m transcripts are shown (peach genome v1.0) with their respective coding sequences (white rectangles) and untranslated 5' and 3' regions (grey rectangles). Repeated GAGA elements are labelled with asterisks. The chromatin H3K27me3 (B) and H3K4me3 (C) modifications around the translation start site of *PpeS6PDH* (labelled with a filled square in panel a) have been analyzed by qRT-PCR of immunoprecipitated samples at different bud development stages. Filled and empty bars correspond to two independent ChIP experiments. The relative expression level of ppa008399m in bud samples of cultivars 'Red Candem' (D) and 'Big Top' (E) is shown. SAND-like and actin-like genes were used as reference genes. An expression value of one is assigned to the first sample. Expression data are means from two biological samples with three technical replicates each, with error bars representing standard deviation. Bud sample code used in Fig. 17 also applies to this figure. Different letters (a–d) indicate significant difference between samples with a confidence level of 95%.

The gene model ppa008399m has been proposed to overlap *PpeS6PDH* in their common 3'-UTRs and the last exon of *PpeS6PDH* (Fig. 18A). In order to verify if ppa008399m and *PpeS6PDH* show concerted regulation hypothetically mediated by a double strand RNA intermediate, we measured ppa008399m gene expression along development of flower buds of 'Red Candem' and 'Big Top' (Fig. 18D,E). The expression of ppa008399m increased slightly during bud development, but transcript accumulation did not correlate inversely with *PpeS6PDH* expression (Fig. 17). Consequently we did not obtain evidences of co-regulated expression.

***PpeS6PDH* shows cold-inducible expression**

The response of *PpeS6PDH* expression to abiotic stresses was assayed in buds and leaves, since certain previous studies propose a protective role of sorbitol and S6PDH against environmental stresses.

Firstly, flower buds were exposed to temperature and water stresses during one and three days treatments. *PpeS6PDH* expression was highly up-regulated after desiccation and cold (4°C) stresses in both dormant and non-dormant buds (Fig. 19A,B). In addition, transcript accumulation slightly increased with the

duration of the treatment in both cases. On the other hand, *PpeS6PDH* expression showed a complex behaviour under heat treatment (37°C), being down-regulated in dormant buds and up-regulated in dormancy released buds (Fig. 19B). Finally, we could notice a decreased *PpeS6PDH* expression following saline stress in dormant buds (Fig. 19A).

In leaf discs, cold exposure induced *PpeS6PDH* expression at a level similar to buds (Fig. 19E). However, desiccation caused a drastic down-regulation of *PpeS6PDH* (Fig. 19D), in contrast to the opposite behaviour observed in buds. Neither saline nor heat treatments affected significantly *PpeS6PDH* expression in leaves (Fig. 19C,E).

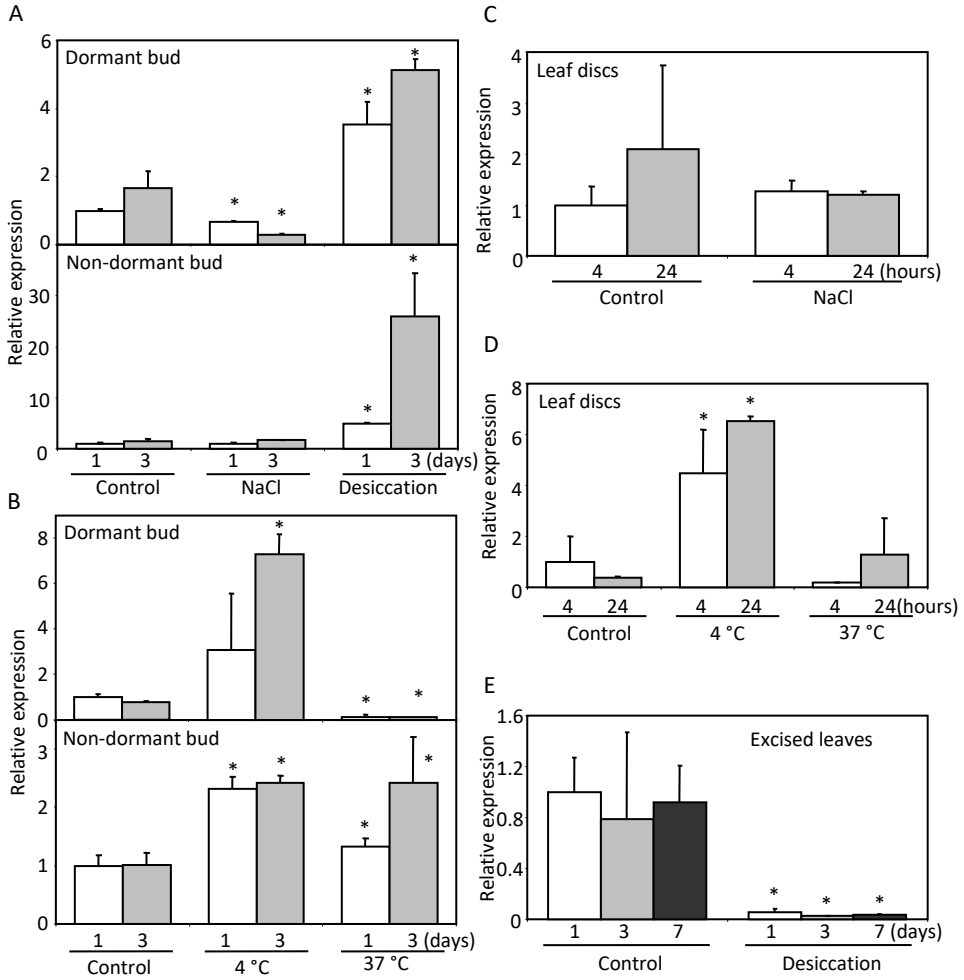

Figure 19. Effect of abiotic stresses on *PpeS6PDH* expression. Dormant and non-dormant flower buds of peach were treated with 200 mM NaCl and desiccated (A), and incubated at 4°C and 37°C (B) for 24 h (white bars) and 72 h (grey bars). Leaf discs were also treated with 200 mM NaCl (C), and incubated at 4°C and 37°C (E) for 4 h (white bars) and 24 h (grey bars). (D) Excised leaves were desiccated for one (white bars), three (grey bars) and seven days (black bars). SAND-like gene was used as reference. An expression value of one is assigned to the first sample. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. An asterisk indicates significant difference with the untreated control at a confidence level of 95%.

Sorbitol content increases along bud development

The content of some sugars (sucrose, glucose and fructose) and the sugar alcohol sorbitol was determined at different stages of bud development. Glucose and fructose contents were not altered in the assay. In contrast, sucrose and sorbitol amounts increased along bud development until the sample previous to dormancy release. After dormancy release their content remained stable or decreased slightly (Fig. 20). The increase in sorbitol level was concordant with changes in *PpeS6PDH* expression during the first stages of bud development (Fig. 17). After that point, the transcriptional repression of *PpeS6PDH* explained the interrupted accumulation of sorbitol.

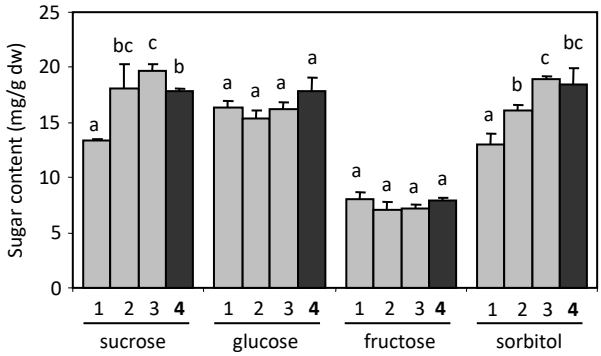

Figure 20. Sugar and sorbitol accumulation in buds. Sucrose, glucose, fructose and sorbitol were measured in flower bud samples RC1 (1), RC2 (2), RC3 (3) and RC4 (4) of 'Red Candem'. Sample code is explained in Fig. 17. Dormancy has been released in RC4 (black bars). Different letters (a–c) indicate significant difference between samples with a confidence level of 95%.

Discussion

A recent study by Hartman et al. (2017) has shown by phylogenetic and enzymatic analysis that peach *PpeS6PDH* encodes a NADPH-dependent aldose-6-phosphate reductase with specificity for the reduction of glucose-6-phosphate to

sorbitol-6-phosphate (also referred as S6PDH). The enzyme was inhibited by several hexose-phosphates, orthophosphate and oxidizing agents, offering alternative pathways for enzyme regulation. In our study, we have obtained similar activity values of the His-PpeS6PDH recombinant protein, and have confirmed the inhibitory effect of C-terminal His fusions on S6PDH activity, as also observed in apple S6PDH (Figueroa and Iglesias, 2010).

In many Rosaceae, S6PDH enzymes are involved in sorbitol synthesis in source tissues (photosynthetic leaves). Subsequently, sorbitol is translocated to sink tissues and converted to fructose by sorbitol dehydrogenase (SDH) enzymes (Loescher, 1987). Thus *S6PDH* genes are expected to be mainly expressed in fully developed leaves, where photosynthesis takes place. However we have found that *PpeS6PDH* is highly expressed in dormant flower buds, a sink tissue, in line with sorbitol accumulation data (Fig. 17; Fig. 20). These results indicate an active biosynthesis of sorbitol in flower buds mediated by PpeS6PDH. In pear, as a response to artificial chilling exposure, sorbitol, sucrose and hexoses accumulated in flower and vegetative buds concomitantly with starch hydrolysis, suggesting the utilization of starch reserves to synthesize soluble sugars and sorbitol during bud dormancy (Hussain et al., 2015). In another study, sucrose and stachyose/raffinose carbohydrates accumulated in vegetative buds of peach instead of sorbitol, but no data about flower buds were presented (Marquat et al., 1999). A high increase in sorbitol content was observed in xylem sap of Japanese pear in late December, around bud dormancy release date, which prompted the authors to postulate a role of soluble sugars and sorbitol in flower bud dormancy regulation (Ito et al., 2012). An independent increase of sorbitol and carbohydrates occurred in xylem sap under 0°C treatment, suggesting a role of sugar accumulation in acquisition of freezing tolerance (Ito et al., 2013). Similarly, sorbitol and sucrose accumulation observed in this study could perform a protective role against water deficit and low temperature stresses in flower buds. In that case, *PpeS6PDH* could exert a key regulatory role in seasonal tolerance of buds to abiotic stresses through sorbitol production.

Gene expression analyses provide further insight into *PpeS6PDH* function.

PpeS6PDH is up-regulated in dormant buds and subsequently repressed in dormancy-released samples, while a fragment including the translation start codon and a GAGA motif stretch undergoes H3K27me3 chromatin modification (Fig. 18). This modification, associated with gene expression silencing at specific loci, has been also proposed to mediate stable silencing of several *DAM* genes, leading to bud dormancy release after the accomplishment of chilling requirements (Leida et al., 2012b; de la Fuente et al., 2015). Interestingly, GAGA motifs have been found enriched in H3K27me3 modified and FIE-binding regions in *Arabidopsis* (Deng et al., 2013), suggesting its participation in chromatin regulatory circuits.

Expression analysis of the gene adjacent to *PpeS6PDH* (ppa008399m) served to conclude that H3K27me3-associated silencing affected locally to *PpeS6PDH*, instead of being sprayed to near genes. Moreover, expression profiles of *PpeS6PDH* and ppa008399m were not complementary along bud dormancy and release, suggesting that both genes are not co-regulated by a double strand RNA intermediate, in spite of the expected overlapping of their transcripts (Fig. 18).

Up-regulation of *PpeS6PDH* expression by low temperature treatments in buds and leaves (Fig. 19B,E) confirmed its participation in the chilling response. Induction of *S6PDH* expression by low temperature has been also observed in apple leaves (Kanayama et al., 2006; Liang et al., 2012). Interestingly, high temperature and desiccation treatments produced antagonistic effects in different samples. Particularly, desiccation induced *PpeS6PDH* expression in buds, whereas *PpeS6PDH* was strongly down-regulated in leaves (Fig. 19A,D). These differences reveal tissue-specific mechanisms of regulation that could respond to distinct source/sink roles. Different degrees of drought stress also caused a reduction in S6PDH enzymatic activity in leaves of peach, whereas SDH enzymatic activity in shoot tips (a sink tissue) decreased (Bianco et al., 2000).

In our opinion, developmental and environmental issues affecting *PpeS6PDH* expression, in addition to sorbitol accumulation data, suggests a role of this gene in protection against abiotic stresses, particularly chilling and desiccation, in flower buds of peach. Moreover, down-regulation of *PpeS6PDH* in dormancy-

released buds involves a chromatin modification mechanism similar to *DAM6* gene, suggesting the participation of common regulatory factors in *PpeS6PDH* and bud dormancy regulation.

Materials and methods

Plant material and stress treatments

Peach (*Prunus persica* [L.] Batsch) plants that were employed in this study were grown at Instituto Valenciano de Investigaciones Agrarias (IVIA) located in Moncada (Spain). Flower buds were harvested and evaluated for the dormancy status as described previously (Leida et al., 2012b). Samples required for tissue gene expression analysis (cv. 'Big Top') were obtained from flower buds (collected on January 12, 2010), leaves (November 6, 2012), embryos, flower parts (March 26, 2010) and fruit tissues (June 29, 2010).

For expression analysis in buds under stress conditions, dormant buds (November 3, 2015) and dormancy-released buds (January 25, 2016) of cv. 'Crimson Baby' were collected from three different trees. Six budsticks for each treatment were placed in glass tubes with 25 ml of water at 25°C (control) during 24 h and 72 h. Temperature stress incubations were made at 37°C and 4°C, salinity stress was made by adding 200 mM NaCl, and desiccation stress was performed without water. Routinely the base of budsticks was cut and the solution replaced with fresh one after two days incubation.

A stress analysis was carried out on leaf discs as described previously (Trotel et al., 1996). Ten discs of 1 cm of diameter per treatment were excised from five different trees of cv. 'Big Top' (June 9, 2015) and were incubated in 5 mM HEPES, 1.5 mM $CaCl_2$, and 10 mM KCl solution at 25°C (control). After 4h incubation, discs were transferred to fresh solution with 250 mM NaCl for salt stress treatment, or incubated at 37°C or 4°C for temperature stress. Discs were collected at 4 h and 24 h.

For the desiccation assay on leaves, adult leaves from three different trees of cv. 'Red Candem' (April 27, 2015) were placed into glasses with the petiole in

contact with water (control) or without water (stressed samples), for one, three and seven days.

Cloning of *PpeS6PDH* in pET-derived vectors

The *PpeS6PDH* gene was cloned into the expression vectors pET302/NT-His and pET303/CT-His (Invitrogen), which facilitates the purification of the recombinant protein. For that, *PpeS6PDH* was amplified using cDNA from peach flower buds collected on January 12 of 2010. The Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used under the following PCR conditions: 2 min at 94°C, 5 cycles of 30 s at 94°C, 30 s at 58°C, and 1min at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C, and a final step of 5 min at 72°C. All the primers used in this study are listed in Supplementary Table S4. The PCR product was purified with High Pure PCR Product Purification Kit (Roche) and digested with enzymes *Xho*I and *Bam*HI (Roche) to have an N-terminal His tag or with *Xho*I and *Xba*I (Roche) for a C-terminal His tag. The purified product and corresponding vectors were ligated with T4 DNA ligase (Roche) and cloned into *Escherichia coli* BL21 (DE3) (Novagen). The nucleotide sequence of the inserted gene was confirmed by sequencing.

Phylogenetic analysis of PpeS6PDH protein

For the phylogenetic analysis, PpeS6PDH, S6PDH of *Malus domestica* (Kanayama et al., 1992)*, Pyrus Pyrifolia* (Liu et al., 2013)*, Oryza sativa* (Yadav and Prasad, 2014) and other S6PDH-like proteins were used. In addition, mannose-6-phosphate reductase (M6PR) of *Apium graveolens* (Everard et al., 1997) and M6PR-like proteins were also included. M6PRs and S6PDHs belong to the same superfamily of aldo-keto reductases (Hyndman et al., 2003; Yadav and Prasad, 2014) and keep high similarity (Bortiri et al., 2002). Sequence alignments were performed by ClustalW (Larkin et al., 2007), and Gblocks (Talavera and Castresana, 2007) was used to remove wrong aligned regions. To build a phylogenetic tree, MEGA7 (Kumar et al., 2016b) was used with Maximum Likelihood method and tested using a Bootstrap with 1000 replicates.

Expression and purification of recombinant protein

In order to express the His-tagged PpeS6PDH, 50mL of LB medium supplemented with antibiotic was inoculated with 1/20 of an overnight culture of transformed *E. coli* BL21 (DE3) cells and incubated at 37°C in an orbital shaker until the OD_{600}~0.6. Then the expression of recombinant protein was induced by adding 1mM isopropyl-β-D-thiogalactoside (IPTG; Roche) and incubated for 2h at 37°C. Induced BL21 (DE3) cells were harvested by centrifugation at 10,000 x g during 10 min at 4°C. The enzyme was extracted using Bugbuster Plus Lysonase Kit (Novagen) and purified with PureProteome Nickel Magnetic Beads (Novagen), according to manufacture's instructions in both cases. Protein concentration was then measured with the Protein Quantification Kit-Rapid (Fluka) using Bovine Serum Albumin as a standard. Protein size was determined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on 15 % resolving gel and 3.5 % stacking gel (Laemmli, 1970). Protein bands were stained using Coomassie brilliant blue R-250.

Enzymatic activity assay

The enzymatic activity of PpeS6PDH was calculated as described previously (Yadav and Prasad, 2014) with minor modifications. The assay solution contained 100 mM Tris-HCl (pH 8), 200 mM NADPH, 50 mM of substrate and 2.5 µg/mL of recombinant protein in a final volume of 0.8 mL. The decrease of absorbance at 340 nm was measured. Glucose-6-phosphate and mannose-6-phosphate were used as substrates and the assay was repeated three times for each condition.

Expression analysis by real-time quantitative PCR

For *PpeS6PDH* gene expression analysis, total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and purified with the RNase-Free DNase Set (Qiagen), according to manufacturer's instructions with minor modifications. Polyvinylpyrrolidone (PVP-40) 1 % (w/v) was added to the kit extraction buffer before use. Then, RNA (500 ng) was reverse transcribed with PrimeScript RT

reagent kit (Takara Bio) and cDNA was analyzed by quantitative real-time PCR on a StepOnePlus Real-Time PCR System (Life Technologies), utilizing SYBR premix Ex Taq (Tli RNaseH plus) (Takara Bio). Cycling conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Specificity of the amplification was evaluated by the presence of a single peak in the dissociation curve after PCR and by size estimation of the amplified product in agarose gel. Relative expression was measured using a relative standard curve. Bestkeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004) and ΔCt (Silver et al., 2006) methods were used in a previous study in order to determine the most stable housekeeping genes (Lloret et al., 2017). According to this study, actin-like and tubulin-like genes were used as references for tissue-dependent expression, actin-like and SAND-like for bud development samples, and SAND-like for stress treatments (Supplementary Table S4). When two reference genes were required for the analysis, the normalization factor was calculated by the geometric mean of the values of both genes. Results were the average of two or three independent biological replicates with 2–3 technical replicates.

Chromatin immunoprecipitation (ChIP) assays

The chromatin immunoprecipitation method and 'Big Top' samples collected along bud development have been described previously (Leida et al., 2012b). Those preceding immunoprecipitated samples were employed in real-time quantitative PCR assays using primers listed in Supplementary Table S4 and following PCR conditions shown above. The enrichment in H3K27me3 and H3K4me3 modifications present in *PpeS6PDH* gene were made relative to H3 values. Results were the average of two independent biological replicates with three technical replicates.

Measurement of sugars and sorbitol content in buds

Soluble sugars and sorbitol were analyzed as previously described (Eshghi et al., 2007) with minor modifications. Ground and dried buds of cv 'Red Candem' (100 mg) were mixed with 5 mL of petrolium ether (40-60°) and centrifuged at

1,109 x g for 10 min at 4°C. Then, petrolium ether containing lipids, chlorophyll and other contaminants was removed and 4 mL of ethanol 80 % and 0.1 mL of mannitol (60 mg/mL) were added to the pellet. Mannitol was used as an internal control. The samples were incubated during 20 min at 65°C. After centrifugation for 5 min at 1,109 x g at 4°C, the supernatant was recovered and the remaining plant material was then re-extracted with ethanol twice. All supernatants were mixed and dried again. For dried residue purification, 4 mL of H_2O MiliQ and 20 µg of activated charcoal were added and then, after another centrifugation, the supernatant was recovered and filtered through a nylon membrane (0.45 µm). Finally, the samples were diluted and injected in a HPLC (High Performance Liquid Cromatography Spectra System) with CarboSep COREGEL-87 (Transgenomic) and a refractive index detector. The mobile phase (ultrapure water) was supplied at a flow rate of 0.6 mL/min. Standard solutions containing glucose, fructose, sorbitol, sucrose and mannitol at different concentrations were injected into the column and their peaks were used to construct calibration curves for each compound. The concentration of individual sugars and sugar alcohols in each tissue sample was then calculated using peak areas and the calibration curves. Results were the average of two independent replicates assayed twice.

Statistical analysis

Statistical analyses were performed using the Statgraphics XVI.I package 324 (Statpoint Technologies). The means of two samples were compared using non-parametric Man-Whitney U test and comparisons of multiple samples were evaluated by non-parametric Klustal-Wallis test with a confidence level of 95 %. Significant differences between samples were labelled with asterisks or different letters.

CHAPTER 3:

Regulatory Circuits Involving Bud Dormancy Factor *PpeDAM6*

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Abstract

DORMANCY-ASSOCIATED MADS-BOX genes (*DAM*) recently emerged as potential regulators of the dormancy cycle in tree species and specifically, *PpeDAM6,* has been proposed to act as a major repressor of bud dormancy release in peach. *PpeDAM6* is transcriptionally modulated by environmental cues, mainly by low temperature, and by epigenetic modifications. Here, through a yeast one hybrid screening, we identified three BASIC PENTACYSTEINE PROTEIN in peach (*PpeBPCs*) that directly interact to two GAGA motif present in an H3K27me3 enriched region of *PpeDAM6* gene. In addition to gene regulation studies, we have also tried to elucidate *PpeDAM6* biological functions through its ectopic expression in transgenic model plants. Overexpression in *Arabidopsis* resulted in a range of abnormal flowering phenotypes arising from protein-protein interactions with flowering regulatory genes. On the other hand, overexpression in plum plants impair growth resulted in dwarf plants with shorter internodes. We established that these pleiotropic defects were concurrent with an altered hormone homeostasis due to a modulation of genes involved in JA, CK and GA pathways. Therefore, we hypothesized that *PpeDAM6* works as a master regulator of peach dormancy, acting as a growth repressor but also promoting stress tolerance response and repressing flowering, most probably by means of hormone homeostasis modulation.

Introduction

Dormancy facilitates survival of growing tissues under the low and freezing temperatures of autumn and winter by interrupting cell division and growth, and activating general and specific defense mechanisms.

Plant hormones play a key role mediating the regulation of the dormancy cycle (Liu and Sherif, 2019). The ectopic expression of the dominant negative allele *abi1-1* of *ABSCISIC ACID INSENSITIVE 1* (*ABI1*) gene in hybrid aspen, leading to a reduced ABA response, impairs dormancy induction and plasmodesmata closure under short photoperiod conditions, stressing the relevance of ABA and cell-cell communication in daylength-dependent induction of bud dormancy (Tylewicz et al., 2018). The overexpression of a *SHORT VEGETATIVE PHASE-LIKE* (*SVL*) gene restores photoperiodic dormancy in *abi1-1* plants, whereas *SVL* silencing plants behave similarly to *abi1-1*. Interestingly, following short photoperiod treatment *SVL* up-regulates *CALLOSE SYNTHASE 1* (*CALS1*) involved in plasmodesmata closure, and a GA2 oxidase gene involved in GA catabolism, indicating a sequence of regulatory events implicating ABA and GA responses in bud dormancy induction (Singh et al., 2019). Consistently with these data, GA application increases bud break and cell permeability in hybrid aspen by inducing 1,3-β-glucanase genes that break down callose in order to open plasmodesmata sphincters (Rinne et al., 2011); and poplar transgenic plants with modified GA metabolism and signaling show altered bud set, bud break and flowering (Zawaski et al., 2011). GA applications also accelerate bud break and dormancy release in Japanese apricot (*Prunus mume*) and other fruit crops (Zhuang et al., 2013).

In Rosaceae tree species and other perennial plants, *DORMANCY-ASSOCIATED MADS-BOX* (*DAM*) genes, phylogenetically related to the *Arabidopsis thaliana* flowering factor *SHORT VEGETATIVE PHASE* (*SVP*), act as key regulators of bud dormancy maintenance and release (Falavigna et al., 2019). Thus, the ectopic expression of *DAM1* gene from leafy spurge (*Euphorbia esula*) delays flowering and decreases the expression of the flowering gene *FLOWERING LOCUS T* (*FT*) in *Arabidopsis thaliana* (Horvath et al., 2010). Moreover, *PmDAM6* gene from

Japanese apricot induces early growth cessation and terminal bud set when overexpressed in transgenic poplar (Sasaki et al., 2011) and apple (Yamane et al., 2019). This phenotype has been found associated with increased ABA and decreased cytokinin contents in terminal buds of *PmDAM6* apple plants (Yamane et al., 2019). Finally, apple plants overexpressing *MdoDAMb* and *MdoSVPa* genes show delayed bud break (Wu et al., 2017).

DAM-like expression has been found closely associated with the dormancy status of buds in several species (Horvath et al., 2008; Li et al., 2009; Ubi et al., 2010; Sasaki et al., 2011; Falavigna et al., 2014), but few specific elements of molecular pathways integrating environmental and developmental inputs on *DAM*-like expression have been currently identified. Among them, the C-REPEAT BINDING FACTOR (CBF)-like proteins are cold response signal factors involved in cold acclimation processes that are able to bind *PmDAM6* promoter of Japanese apricot in the yeast one hybrid system (Zhao et al., 2018b), and activate the promoters of pear *PpDAM1* and *PpMADS13-1* in transient reporter assays (Saito et al., 2015; Niu et al., 2016). In addition, the overexpression of peach *PpCBF1* in apple increases the expression of *MdoDAM1* and *MdoDAM3* genes in bud tissue (Wisniewski et al., 2015). These studies provide a mechanism for *DAM*-like activation under low temperature conditions leading to dormancy induction and maintenance, but do not account for *DAM*-like down-regulation concurrent with the completion of genotype-specific chilling requirements and bud dormancy release.

A succession of epigenetic events have been found associated with *DAM*-like repression and dormancy release in different species (Ríos et al., 2014; Conde et al., 2019), resembling *FLOWERING LOCUS C* (*FLC*) regulation by vernalization in *Arabidopsis thaliana* (Horvath, 2009; Hemming and Trevaskis, 2011). In peach, in addition to H3K4me3 decrease and H3 deacetylation around the translation start site of *PpeDAM6*, a larger region of the gene becomes enriched in H3K27me3 modification concomitantly with gene down-regulation (Leida et al., 2012b). A similar decrease in dormancy-dependent H3K4me3 enrichment has been observed in several promoter, exonic and intronic regions of *PpMADS13-1* gene

from Japanese pear, in addition to a lower load of the histone variant H2A.Z (Saito et al., 2015). In parallel to these changes in chromatin structure and modifications, the global level of DNA methylation, commonly associated with transcriptional repression, fluctuates along bud dormancy and development in chestnut (Santamaría et al., 2009), poplar (Conde et al., 2017a), apple (Kumar et al., 2016a), sweet cherry (Rothkegel et al., 2017) and almond (Prudencio et al., 2018).Besides the effect of environmental and developmental cues on *DAM*-like gene expression, the search of downward transcriptional targets has fostered knowledge on *DAM*-like function in bud dormancy regulation. *DAM*-like genes have been postulated to exert such dormancy promoting function through the transcriptional down-regulation of orthologs of the flowering factor *FT* and the regulation of hormone biosynthesis enzymes. With respect to hormone modulation, pear *PpDAM1* binds and up-regulates in transient expression assays the expression of *PpNCED3* gene, coding for a 9-cis-epoxycarotenoid dioxygenase involved in ABA biosynthesis, in close agreement with changes in ABA content across flower bud development (Tuan et al., 2017). Similarly, hybrid aspen SVL binds and activates *NCED3* gene expression, but also up-regulates the expression of the putative ABA receptors *RCAR1*/*PYL1* and *RCAR2*/*PYL2*, and represses *GA20ox1* and *GA20ox2* genes involved in the biosynthesis of active GAs (Singh et al., 2018). These data strongly support a role of *DAM*-like genes in modulation of hormone levels in developing buds for dormancy regulation.

In this study we have identified an ortholog of *BASIC PENTACYSTEINE* (*BPC*) gene coding for a transcription factor binding GA repeat sequences within a *PpeDAM6* region enriched in H3K27me3 in dormancy-released buds of peach (de la Fuente et al., 2015), and have postulated its participation in developmental *PpeDAM6* repression. We have also studied *PpeDAM6* regulatory circuits by expressing ectopically the gene in *Arabidopsis thaliana* and plum (*Prunus domestica*) transgenic plants, supporting a role of *PpeDAM6* gene in growth regulation mediated by plant hormone responses.

Results

Dormancy-dependent expression of *PpeDAM6* gene in peach

In previous transcriptomic and genetic studies *PpeDAM6* gene has been proposed as a major regulator of dormancy release in peach (Leida et al., 2010; Yamane et al., 2011; Jimenez et al., 2010). We analyzed *PpeDAM6* expression profile along bud development in two cultivars with different chilling requirement for dormancy release ('Red Candem' with low and 'Crimson Baby' with medium requirements). As expected, *PpeDAM6* was down-regulated during the dormancy process in these cultivars, with the lowest expression value at the last measured date (Fig. 21A). The profile of *PpeDAM6* down-regulation was quite linear along bud development, with an abrupt reduction in gene expression in concordance with the specific dormancy release dates of 'Red Candem' and 'Crimson Baby' cultivars. Thus, *PpeDAM6* expression correlated with dormancy release events and seemed to rely exclusively on chilling requirements, independently of other environmental cues. For a further characterization of *PpeDAM6* expression pattern, we performed a tissue-dependent expression analysis. *PpeDAM6* was highly expressed in leaf, flower and vegetative buds and noticeably less in embryo, whereas its expression was practically imperceptible in fruit and flower parts (Fig. 21B). The fact that *PpeDAM6* was appreciably expressed in tissues that display growth arrest and dormancy mechanisms evidences a patent relationship between the gene and dormancy processes.

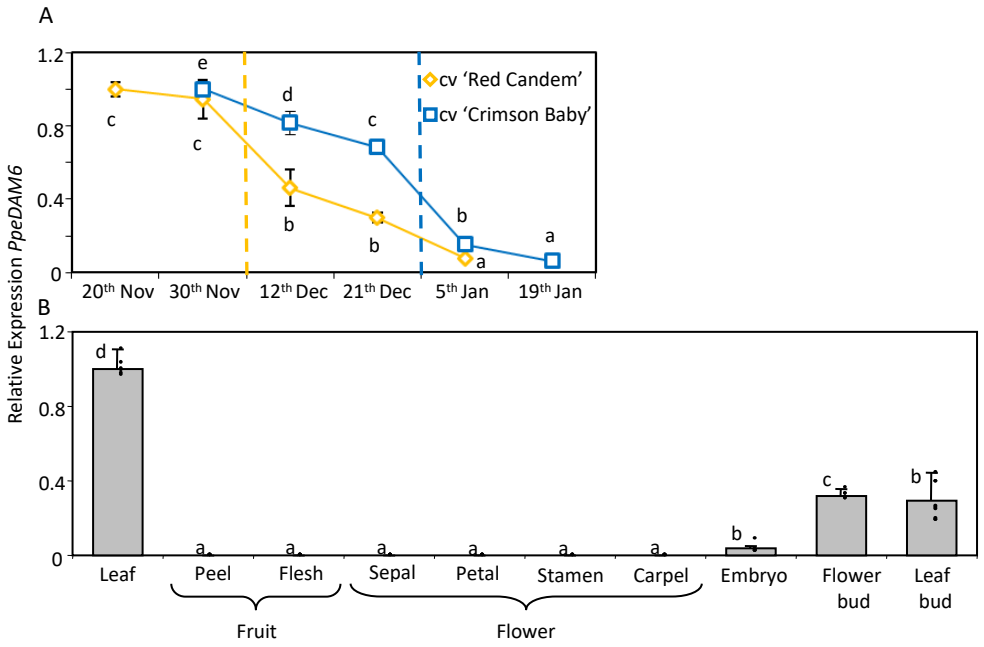

Figure 21. Relative expression of *PpeDAM6* in peach by real-time RT-PCR. (A) Bud samples from the cultivars 'Red Candem' (yellow line) and 'Crimson Baby' (blue line) were tested. Dash lines represent dormancy release. SAND-like gene was used as reference gene. an expression value of one is assigned to the first sample. (B) Different plant tissues were tested. Tubulin-like and actin-like genes were used as reference genes. An expression value of one is assigned to the leaf sample. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Different letters (a–e) indicate significant difference between samples with a confidence level of 95%.

BPC family proteins bind a regulatory intronic region of *PpeDAM6* gene

Previous studies indicated that a genomic region of *PpeDAM6* was highly enriched in the repressive histone mark H3K27me3 concomitantly with dormancy release (Leida et al., 2012b). This region spans about 1.1 kb containing the first intron, the translation start site and part of the large second intron of the gene (de la Fuente et al., 2015) (Fig. 22A). In order to identify putative regulatory factors of H3K27me3 modification binding specifically to this region we performed

a yeast one-hybrid (Y1H) approach. The H3K27me3 enriched region was divided in two fragments of 558 bp (named Reg1) and 575 bp (named Reg2) that were used independently as Y1H baits against a cDNA expression library made from mixed dormant and dormancy-released flower bud samples (Fig. 22B). We screened a total of 10^6 yeast transformants with pABAi-Reg1 construction and $5x10^5$ transformants with pABAi-Reg2 bait. No positive candidates were obtained in the screening of Reg1, whereas two positive clones bound Reg2 fragment containing the start of the second intron of *PpeDAM6* (Fig. 22C). The positives clones corresponded respectively to partial sequences of the transcripts Prupe.1G338500 and Prupe.1G369400. In a BLASTP analysis against *Prunus persica v2.1* genome database (Verde et al., 2017), we detected one more gen in peach genome with high similarity to our positive sequences, Prupe.8G082900. The deduced proteins of all these genes contain a GAGA binding domain, which has been previously described in the BASIC PENTACYSTEINE PROTEIN (BPC) / BARLEY B RECOMBINANT (BBR) family protein. Thus, from now on we will use the names *PpeBPC1* to designate gene Prupe.1G338500, *PpeBPC2* to Prupe.1G369400 and *PpeBPC3* to Prupe.8G082900. Since these genes showed alternatively spliced transcripts in databases, we selected for subsequent analysis the transcripts which were obtained in the Y1H analysis.

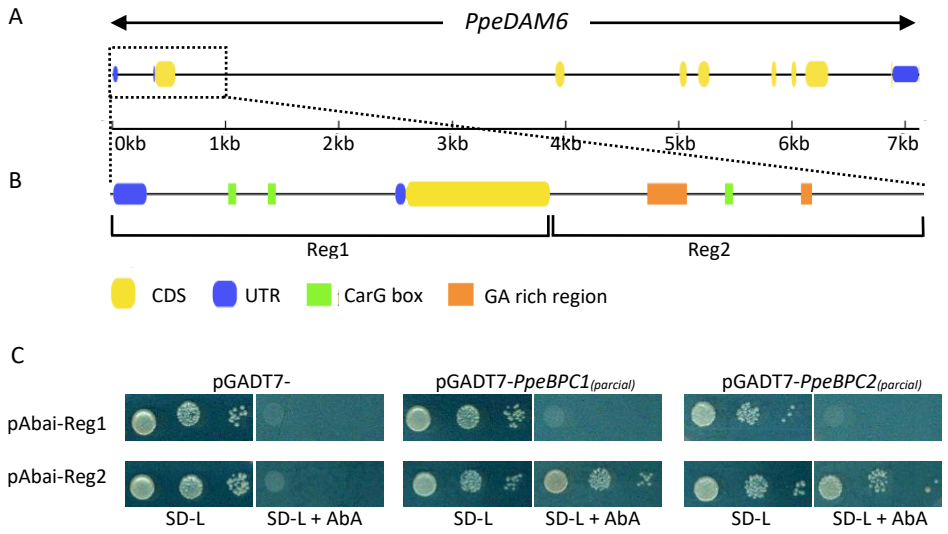

Figure 22. PpeBPCs bind to an intronic regulatory region of *PpeDAM6*. (A) Genomic structure of *PpeDAM6* with the respective coding sequence (yellow rectangles) and untranslated 5' and 3' regions (blue rectangles). (B) The designed baits for Y1H experiment. The first part of the gene was split into two parts of 500pb each one. Potential binding sites like CarG boxes and GAGA motifs are marked by green and orange blocks, respectively. (C) The results of Y1H screening. Different combinations of pABAi vectors with the regulatory regions and prey vectors (pGADT7), transformed with the positive screening clones and control plasmids (-), are shown. Yeast strains were grown on a minimal medium (SD) and a growth selective medium containing 200 µM of Aureobasidin A (+AbA).

In order to highlight their clustering into BPC family, a phylogenetic tree was constructed using protein sequences of previously characterized *BPC* genes from *Arabidopsis thaliana*, *Hordeum vulgare* (Santi et al., 2003), *Populus trichocarpa* and *Vitis vinifera* (Theune et al., 2019). As shown in Fig. 23A, BPCs proteins were classified into three groups (I, II and III), which was consistent with previous studies where BPC proteins were discriminated based on their divergent N-terminal part (Meister et al., 2004). PpeBPC1 belonged to group I, while PpeBPC2 and PpeBPC3 were part of group II. Within group II, PpeBPC2 clustered with

AtBPC6, PtBBR/BPC6 and VvBBR/BPC6, suggesting that PpeBPC2 may share structural or functional resemblances with BPC6-like proteins. None of peach BPC proteins was located in group III.

The analysis of *PpeBPC* gene expression during bud dormancy showed a slight increase along flower bud development in two cultivars with different chilling requirements, although it was not strictly associated with dormancy release dates (Fig. 23B).

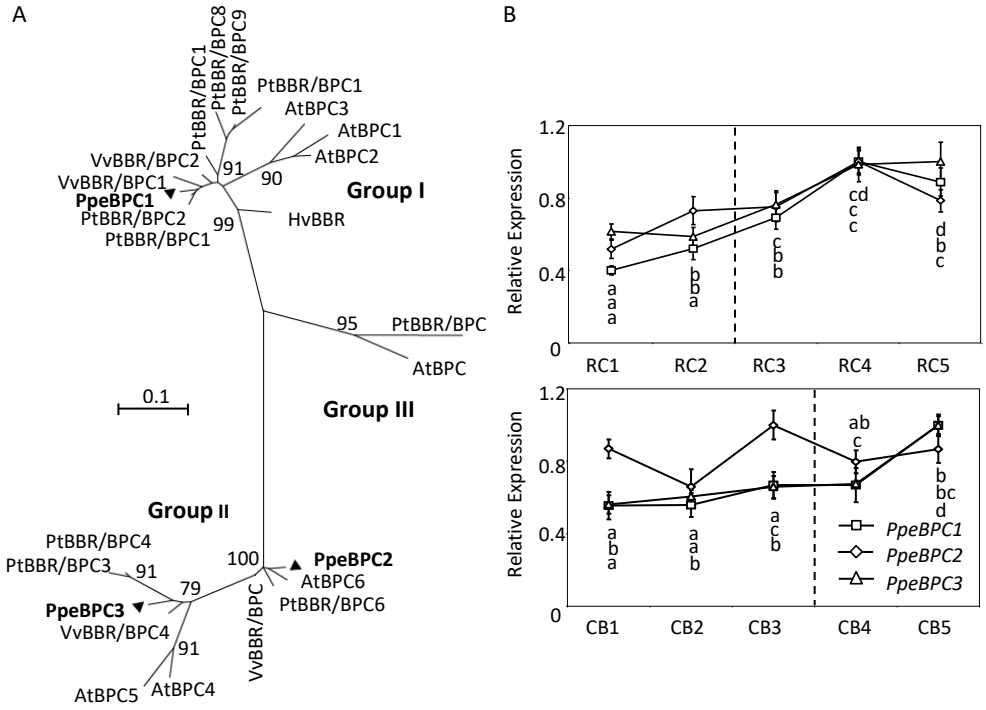

Figure 23. Characterization of *PpeBPCs* from peach. (A) Phylogenetic tree of BPC proteins from *Arabidopsis, Hordeum vulgare, Populus trichocarpa, Vitis vinifera* and *Prunus persica.* The tree was constructed using the Maximum Likelihood method and bootstrapped with 1000 replicates. The scale bar indicates the branch length that corresponds to the number of substitutions per amino acid position. (B) The relative expression of *PpeBPC1* (white squares), *PpeBPC2* (white rhomb) and *PpeBPC3* (white triangle) measured along bud development. Bud

samples from the cultivars 'Red Candem' (rc) and 'Crimson Baby' (cb) were collected in autumn and winter (2015/2016) at different dates: November 20 (RC1), November 30 (RC2 and CB1), December 14 (RC3 and CB2), December 21 (RC4 and CB3), January 4 (RC5 and CB4) and January 19 (CB5)collected in autumn and winter (2015/2016) at different dates: November 20 (RC1), November 30 (RC2 and CB1), December 14 (RC3 and CB2), December 21 (RC4 and CB3), January 4 (RC5 and CB4) and January 19 (CB5). A dash line represents dormancy release event. SAND-like genes was used as reference gene. An expression value of one is assigned to the highest value in each gene. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Different letters (a–d) indicate significant difference between samples for each gene, at a confidence level of 95%.

PpeBPC1 represses *PpeDAM6* by binding to GAGA motifs in Reg2 region

BPC is a plant-specific transcription factor family characterized by the ability to bind gene promoter sequences at GA-repeat stretches (GAGA motif). Two GAGA motifs are present in Reg2 sequence, containing respectively 25 (GA1) and 7 (GA2) GA repeats (Fig. 22A). To determine the DNA-binding specificity of peach *BPC* factors by Y1H, we used yeast strains containing seven different reporter constructs with serial deletions in the Reg2 fragment (Fig 24A). As shown in Fig. 24B, *PpeBPC1*, *PpeBPC2* and *PpeBPC3* were only able to activate reporter constructs containing one or both GAGA motifs, indicating that their interaction with *PpeDAM6* regulatory region is exclusively mediated by these motifs.

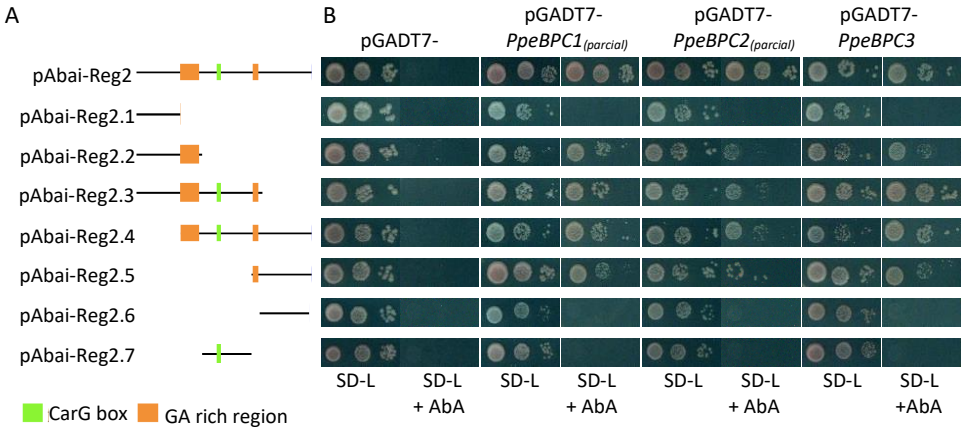

Figure 24. PpeBPCs bind to GA motif. (A) The designed baits in Y1H. The positive bait Reg2 was split in seven different fragments. Potential binding sites like CarG boxes and GAGA motif are marked by green and orange blocks, respectively. (B) The results of Y1H experiment. Different combinations of pABAi vectors, cloned with the seven different regulatory fragments, and prey vectors (pGADT7), fused with *PpeBPC1*, *PpeBPC2* and *PpBPC3* and control plasmids (-), are shown. Yeast strains were grown on a minimal medium (SD) and a growth selective medium containing 200 µM of Aureobasidin A (+AbA).

In order to clarify the role of *PpeBPC* genes in *PpeDAM6* gene expression regulation, a dual luciferase transient expression assay was performed in *Nicotiana Benthamiana* leaves. We used the complete sequences of *PpeBPCs* to construct the effector vectors. For constructing the reporter vectors with the luciferase gene (LUC) we cloned a *PpeDAM6* genomic fragment including promoter (1 kb), 5' untranslated region (5'-UTR), the translation start site and full first and second introns (Fig 25A). Three different versions of this vector contained none (Pro.1-LUC), one (Pro.2-LUC) or two GAGA motifs (Pro.3-LUC) were used. A second reporter expressing the renilla luciferase gene (REN) under 35S promoter was employed as an internal reference. Dual luciferase assay results indicated that *PpeBPC1* was able to reduced LUC/REN ratio when co-infiltrated with Pro.3-LUC vector showing the native intronic structure of *PpeDAM6* with both GAGA motifs (Fig. 25B). These results confirmed that GAGA motifs are necessary for the interaction between PpeBPC1 protein and *PpeDAM6* regulatory region, and that PpeBPC1 acts as a transcriptional repressor of *PpeDAM6*.

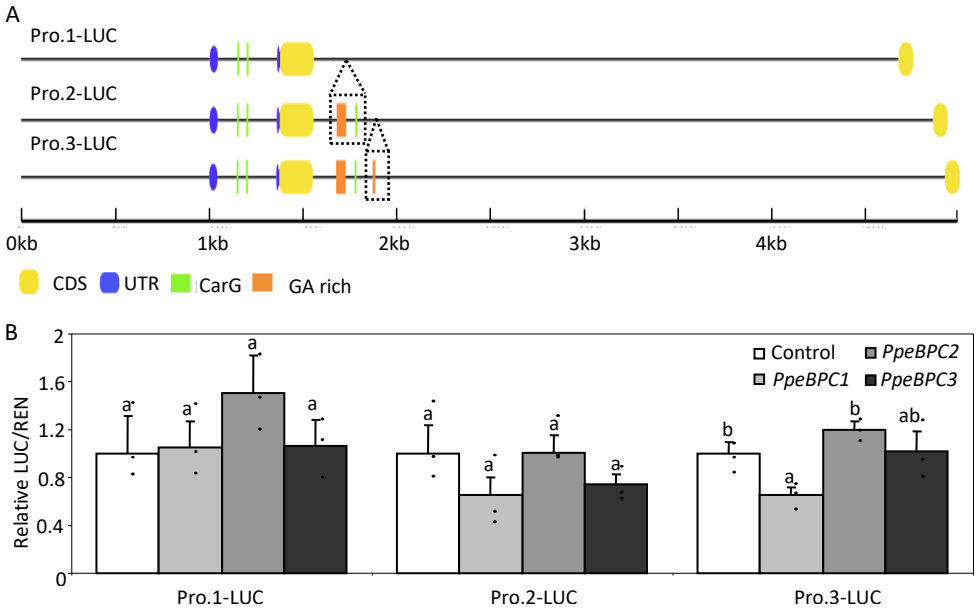

Figure 25. PpeBPC1 represses *PpeDAM6* expression in a transient expression assay. (A) The different reporter vector constructions for the dual luciferase assay. Genomic fragment including promoter (1 kb), 5' untranslated region (5'-UTR) (blue rectangles) and first and second exons (yellow rectangles) (about 5Kb) was represented. Potential binding sites like CarG boxes and GAGA motif are marked by green and orange blocks, respectively. In the different reporter constructions different motif was removed. (B) The relative LUC/REN ratio measured in the different combination of reporter vectors (-LUC), cloned with Pro.1, Pro.2 and Pro.3, and the effectors vectors, fused with control plasmid (white bar), *PpeBPC1* (light grey bar), *PpeBPC2* (dark grey bar) and *PpeBPC3* (black bar). In each combination, the value for reporter construction with empty pGreenII62sk plasmid (control, white bar) was set to 1. Data are means of four biological replicates with error bars representing standard deviation. Different letters (a–b) indicate significant difference between samples for each reporter construction, at a confidence level of 95%.

In order to explore the molecular mechanisms underlying *PpeBPC*s mediated repression, we performed a yeast two-hybrid assay (Y2H) to test the physical interaction of PpeBPC proteins with peach orthologs of known interactors of BPC

proteins from other species (Simonini et al., 2012; Hecker et al., 2015; Mu et al., 2017). In this Y2H assay, PpeBPC proteins interacted with other PpeBPCs, but neither peach orthologs of LIKE HETEROCHROMATIN PROTEIN1 (LHP1), SWINGER (SWN) nor SEUSS showed interaction with PpeBPCs (Fig. 26).

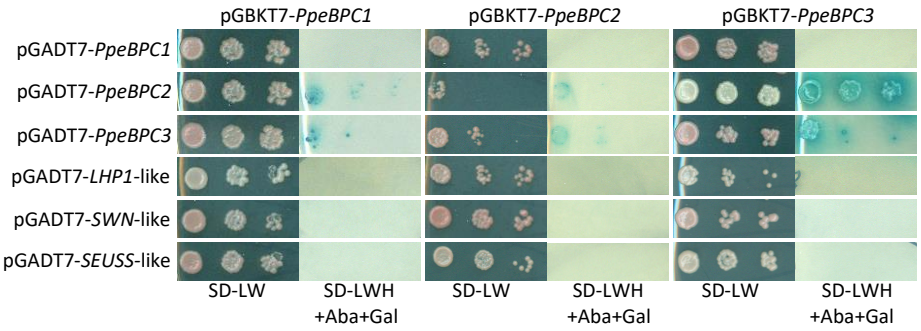

Figure 26. PpeBPCs form homodimers and heterodimers between themselves. Yeast two-hybrid analysis of the protein interactions between different combinations of bait vectors (pGBKT7), fused with *PpeBPC1, PpeBPC2, PpeBPC3,* and prey vectors (pGADT7), cloned with *PpeBPC1, PpeBPC2, PpeBPC3, LHP1*-like, *SWN*-like and *SEUSS*-like. Yeast strains were grown on a minimal medium (SD-LW) and a chromogenic medium containing Aureobasidin A and X-α-Gal (+AbA +Gal).

Protein interactions link PpeDAM6 with flowering

MADS-box domain proteins like PpeDAM6 have been reported to form dimers with other MADS-box transcriptional factors to modulate gene expression (de Folter et al., 2005). A Y2H screening assay was performed in order to identify putative partners of PpeDAM6.

Firstly, a full-length cDNA of *PpeDAM6* fused to the DNA binding domain of GAL4 to construct pGBKT7-*PpeDAM6* vector was shown to autoactivate Y2H reporters. Then, we made a second plasmid containing a partial *PpeDAM6* clone with a deletion in the acidic C-terminal end (pGBKT7-*PpeDAM6*$^{1\text{-}537}$) that showed no autoactivation response and hence was suitable for Y2H screening.

Approximately 10^6 transformants were screened, and 91 colonies growing in minimal medium (SD-LWH supplemented with aureobasidin A (AbA) 125 ng/mL and X-α-gal 40 µg/mL) were selected as positive clones. After discarding repeated transcripts, 15 independent clones were retro-transformed and finally 11 independent positive clones were identified (Fig. 27, Supplementary Table S5). Searches in the peach genome database confirmed that most of these clones encoded putative transcription factors similar to proteins characterized in model plants such as *Arabidopsis thaliana*. Among them, we found *TRANSPARENT TESTA2*-like (*TT2*-like), *SHATTERPROOF1*-like (*SHP1*-like), *PISTILLATA*-like (*PI*-like), *AGAMOUS*-like (*AG*-like), *BASIC LEUCINE-ZIPPER6*-like (*bZIP6*-like), *SUPRESSOR OF CONSTANS1*-like (*SOC1*-like), *FRUITFUL*-like (*FUL*-like), *MYB*-like, *ARABIDOPSIS THALIANA ACTIVATING FACTOR1*-like (*ATAF1*-like) and *SEPALLATA2*-like (*SEP2*-like). Although most of them presented strong interactions, *bZIP6*-like and *ATAF1*-like showed a weaker binding with PpeDAM6 protein (Fig. 27). Some of these genes function as flowering regulators, suggesting that a specific interaction between PpeDAM6 and these genes could be involved in controlling flowering pathways. We also conducted an expression analysis of these genes along bud development. Although *SOC1*-like was down-regulated during dormancy release, most of them showed a slight up-regulation when dormancy was released (Supplementary Fig. S6).

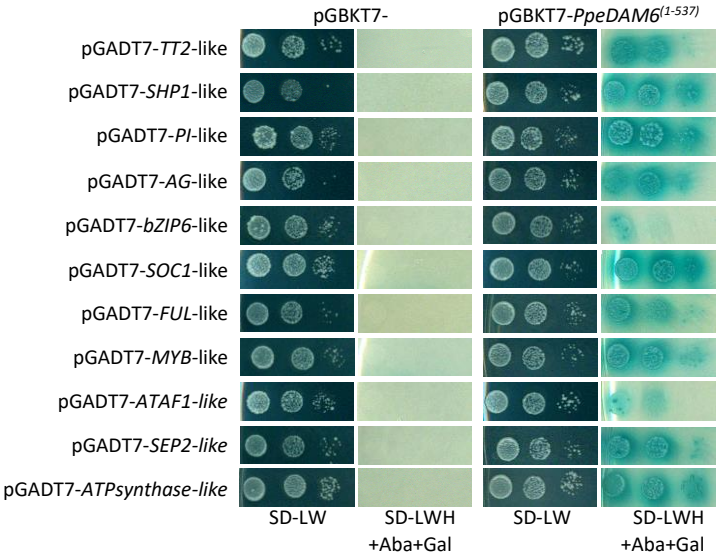

Figure 27. Protein-protein interaction of PpeDAM6. Yeast two-hybrid analysis of the protein interactions between different combinations of bait vectors (pGBKT7), transformed with *PpeDAM6*$^{1\text{-}537}$ and control plasmids (-), and prey vectors (pGADT7), cloned with positive screening clones. Yeast strains were grown on a minimal medium (SD-LW and a chromogenic medium containing Aureobasidin A and X-α-Gal (+AbA +Gal).

PpeDAM6 overexpression affects flower development in *Arabidopsis*

To investigate the function of *PpeDAM6*, it was overexpressed fused to c-myc epitope either in N-terminal or in C-terminal position under the control of the 35S promoter in *Arabidopsis thaliana*. At least, 20 independent transgenic lines were obtained for each construction, showing qualitatively similar results. The transgenic lines compared with the wild-type Columbia displayed morphological abnormalities in floral structures at different degrees (Fig. 28), which resembled floral defects of 35S::At*SVP* transgenic plants (Masiero et al., 2004). The presence of the transgen and PpeDAM6 protein was evaluated by PCR reaction and western-blot analysis. Although all the kanamycin-selected plants had the transgene, PpeDAM6 protein was variably detected according to the severity of observed phenotypic features (Table 3). Thus, PpeDAM6 protein was not detected

in transgenic plants showing wild type phenotype. Transgenic plants expressing moderately the protein showed mild defects and developed abnormal flowers with vegetative traits, enriched with trichomes and malformed siliques with no or few viable seeds. Some of them showed leafy sepals and normal petals (e.g. 35S::*PpeDAM6* #15) and other leafy sepals and petals (e.g. 35S::*PpeDAM6* #9) (Fig. 28A,B). On the other hand, plants expressing high levels of PpeDAM6 protein (e.g. 35S::*PpeDAM6* #7) had the most severe phenotype, with inflorescences instead of flowers that often developed on the tip a new aberrant inflorescence without siliques (Fig. 28A,B). As we said before, most of these abnormal plants were sterile with the exception of two lines that had few viable seeds. We measured the flowering time in genotype 35S::*PpeDAM6* #15, with no significant differences compared to wild-type (Supplementary Table. S6).

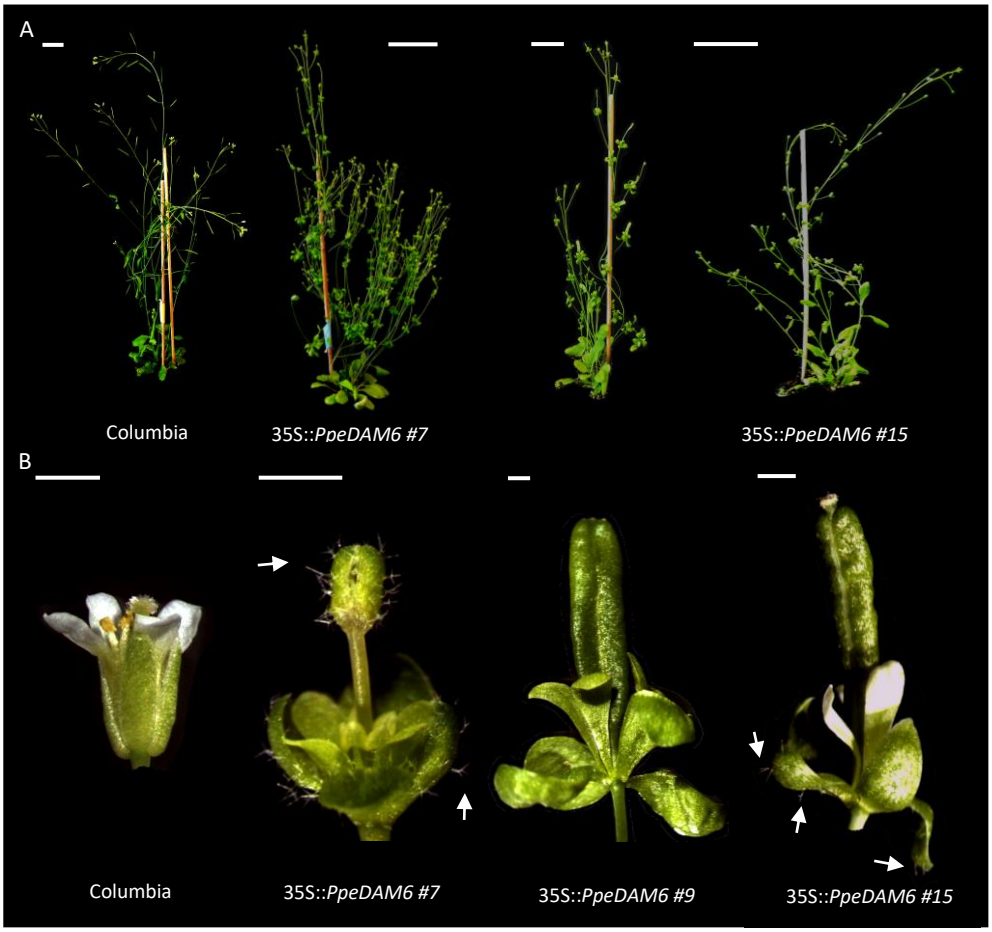

Figure 28. The effect of ectopic expression of *PpeDAM6* gene in *Arabidopsis.* (A) Plant phenotype of Wild-type (Columbia) and 35S::PpeDAM6 lines #7, #9 and #15 is shown. Scale bars represent 5cm. (B) Flower phenotype of Wild-type (Columbia) and 35S::PpeDAM6 lines #7, #9 and #15 is shown. Scale bars represent 1mm. A white arrow marks the presence of trichomes.

Table 3. Summary of 35S::*PpeDAM6* overexpressing *Arabidopsis* lines indicating their phenotype features, the presence of seeds, the presence of transgen by PCR analysis and the protein level by western-blot.

Genotype	Line	Phenotype features	Seeds	PCR	Western
Columbia		WT	Yes	-	nd
		WT	Yes	-	nd
		WT	Yes	-	nd
35S::*c-myc-DAM6*	1	WT	Yes	+	nd
	2	WT	Yes	+	nd
	3	WT	Yes	+	nd
	4	WT, some leafy sepals	Yes	+	nd
	5	WT	Yes	+	+
	6	Leafy sepals, leafy petals, no siliques	Sterile	+	++
	7	Leafy sepals, abnormal siliques	Sterile	+	++
	8	WT	Yes	+	nd
	9	Leafy sepals, leafy petals, abnormal siliques	Sterile	+	+
	10	Leafy sepals, leafy petals, abnormal siliques	Sterile	+	++
	11	Leafy sepals, leafy petals, no siliques	Sterile	+	+++
	12	WT	Yes	+	nd
	13	WT	Yes	+	nd
	14	Leafy sepals, abnormal siliques	Few	+	nd
	15	Leafy sepals, leafy petals, no siliques	Sterile	+	+++
	16	Leafy sepals, leafy petals, abnormal siliques	Sterile	+	++
	17	WT	Yes	+	nd
35S::*DAM6-c-myc*	1	WT	Yes	+	nd
	2	WT	Yes	+	nd
	3	WT	Yes	+	nd
	4	WT, some leafy sepals	Yes	+	nd
	5	WT	Yes	+	nd
	6	Leafy sepals, leafy petals, no siliques	Sterile	+	++
	7	Leafy sepals, leafy petals, no siliques	Sterile	+	++
	8	Leafy sepals, leafy petals, abnormal siliques	Sterile	+	++
	9	WT	Yes	+	nd
	10	WT	Sterile	+	nd
	11	WT, some leafy sepals	Yes	+	nd
	12	WT	Yes	+	nd
	13	WT	Yes	+	nd
	14	WT	Yes	+	nd
	15	Leafy sepals, abnormal siliques	Few	+	+
	16	WT, some leafy sepals, abnormal siliques	Yes	+	++
	17	WT	Yes	+	nd
	18	WT	Yes	+	nd

(+) represents presence of transgen in PCR column and signal intensity of the band in western column (more (+) indicated stronger signals)
nd, not detected

***PpeDAM6* overexpression impairs growth in plum**

To explore the consequences of overexpressing *PpeDAM6* in a woody plant, we transformed plum (*Prunus domestica* cv. 'Claudia Verde', 'CV') with the constitutive expression vector used for *Arabidopsis* transformation which had *PpeDAM6* fused to c-myc epitope in its N-terminal end. As nowadays we still do not have efficient protocols to transform peach, plum offered some advantages over other species, mainly its taxonomical proximity to peach and their similar developmental and physiological issues (Petri et al., 2008b). After transformation, three independent plum lines that expressed *PpeDAM6* in leaves were identified by quantitative real-time RT-PCR (qRT-PCR) (Fig. 29A). In the three lines, the transgene transcripts were highly expressed and contributed to most of the combined expression of *DAM6* genes from both species (*PpeDAM6 + DAM6*-like). On the other hand, we also observed that the expression of the plum ortholog of *DAM6* was moderately reduced in the trangenic lines compared with the control 'Claudia Verde'. The presence of PpeDAM6 protein was also detected by western-blot analysis (Fig. 29B), although the results showed poor correlation between mRNA and protein expression levels, most likely due to post-transcriptional regulation. In fact, leaves from line #1 accumulated more *PpeDAM6* transcripts than lines #2 and #3 whereas the western-blot analysis showed that line #2 expressed a higher amount of protein.

Regarding plant phenotype, transgenic lines exhibited alterations in vegetative development compared with the control. Broadly, *in vitro* cultured explants presented approximately a similar phenotype to 'Claudia Verde' but once they were acclimated growth problems appeared. *PpeDAM6* transformed plants grew slower and despite the fact that they developed about the same number of leaves than control 'Claudia Verde' (Fig 29D), the internodes were much shorter due to the elongation problems (Fig 29C). Finally, in a certain moment, the vegetative apical meristem collapsed and the plant died few months after acclimation. Probably these pleiotropic defects were due to an activity reduction of the vegetative apical meristem that concluded with the meristem collapse (Fig. 29E). For subsequent analysis we selected lines #1 and #2.

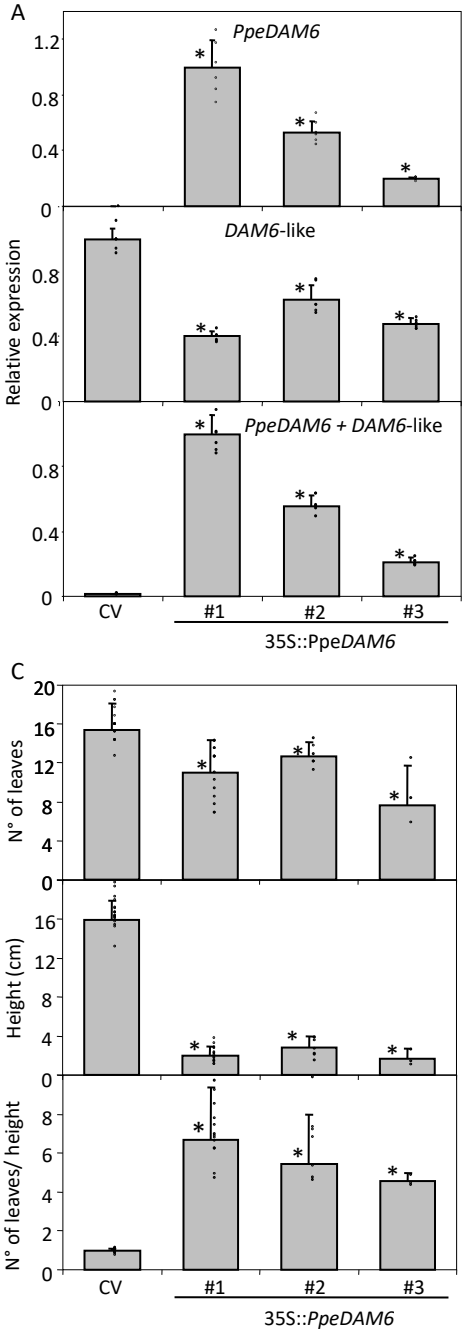
A
PpeDAM6
DAM6-like
PpeDAM6 + DAM6-like
Relative expression
CV
#1
#2
#3
35S::PpeDAM6
B
CV
#1
#2
#3
35S::PpeDAM6
C
N° of leaves
Height (cm)
N° of leaves/ height
CV
#1
#2
#3
35S::PpeDAM6
D
E
Claudia Verde
35S::PpeDAM6#1
35S::PpeDAM6#2
35S::PpeDAM6#3

Figure 29. Effect of ectopic expression of *PpeDAM6* gene in plum. (A) The relative expression of *PpeDAM6*, plum *DAM6*-like and both genes (*PpeDAM6* + plum *DAM6*-like) is shown for three transgenic lines. AGL24-like and actin-like genes were used as reference genes. An expression value of one is assigned to the highest level line. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%. (B) Protein level of *PpeDAM6* in Claudia Verde (CV) and transgenic lines 35S::*PpeDAM6* #1, #2 and #3. (C) Different whole plant parameters of three month old plants are shown. Data are means from at least three different plants per genotype, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%. (D) Phenotype of three month old plants of Claudia Verde (CV) and transgenic lines 35S::*PpeDAM6* #1, #2 and #3. Scale bar, 5 cm. (E) Photographic details of shoot apex. Scale bar, 1 cm.

Transcriptomic analysis of *PpeDAM6* overexpressing lines

To get deeper insight into the molecular mechanisms that control the stunted growth phenotype caused by the overexpression of *PpeDAM6,* we studied the global expression pattern of leaves from 3-months-old *PpeDAM6* transgenic plum lines #1 and #2 and control 'Claudia verde' by RNA-seq analysis, with three replicates per sample. High-throughput sequencing resulted in 84 million high-quality paired-end reads per replicate (Table 4). Clean reads were succesfully *de novo* assembled by Trinity and 187,901 unigenes were obtained (Table 5).

Table 4. Summary of sequencing data

Genotype	n° replicate	n° raw sequences	n° clean sequences
Claudia verde	1	84080676	80192132
	2	85379832	81638374
	3	80969978	76624540
35S::DAM6#1	1	90585386	88722278
	2	94326032	91738150
	3	84776892	81478948
35S::DAM6#2	1	107235792	104284730
	2	97445734	95165920
	3	90599952	88721900

Table 5. Summary of transcriptome assembly

Transcriptome assembly	n° unigenes	Mean length	n° unigenes >1kb
	187901	1154	75449

The overexpression of *PpeDAM6* modified the expression of around 13,000 differentially expressed unigenes (DEUs) in both transgenic lines #1 and #2 (Fig. 30A). To describe the main pathways modified in transformed plants, we analyzed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (Fig. 30B, 30C). In the scatter maps, the colour and the size of the points represent respectively the q-value and the number of DEUs enriched in the corresponding category. Eleven KEGG pathways were significantly up-regulated in both lines, whereas 14 were down-regulated, among which 'ribosome' (ko03010) and 'carbon metabolism' (map01200) accounted for the largest proportion of DEU. Apart from those mentioned, other essential pathways for plant survival and development were down-regulated in both transformed lines like 'photosynthesis-antenna pathway' (map00196), 'photosynthesis' (ko00195), 'nitrogen metabolism' (map00910) and 'carbon fixation in photosynthetic organisms' (ko00710). The analysis of KEGG pathways suggests that *PpeDAM6* overexpressing plum lines had lower cellular activity, in agreement with their dwarf phenotype.

Figure 30. Transcriptomic analysis of *PpeDAM6* plum overexpressing lines. (A) Venn diagram of up-regulated and down-regulated transcripts of 35S::*PpeDAM6* #1 and #2 compared with

control ‘Claudia Verde’. (B-C) Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) functional terms compared 35S::*PpeDAM6* #1 and #2 vs control ‘Claudia Verde’. In the scatter maps, rich factor indicated the number of enriched genes divided to number of all background genes in corresponding pathway. The smaller the q-value, the closer the colour is to red, and the size of the points represent the number of DEUs enriched in the corresponding function.

***PpeDAM6* overexpression modifies hormones synthesis and response**

KEGG enrichment analysis also revealed that ‘alpha-linolenic acid metabolism’ (map00592), that is jasmonic acid (JA) biosynthesis pathway, was significantly up-regulated in 35S::*PpeDAM6* transgenic plum whereas ‘plant hormone signal transduction’ (map04075) was down-regulated (Fig. 30B). Since phytohormones have long been known to affect bud dormancy (Liu and Sherif, 2019) we decided to identify the contributions of hormone-mediated transcriptional regulation to the transcriptome of 35S::*PpeDAM6* transgenic plants. We identified DEUs associated with various aspects of hormone homeostasis and response, mostly related to ABA, auxin, ethylene, cytokinin (CK), GA and JA hormones (Supplementary Table S7).

The transcript abundance of JA biosynthetic genes (Fig. 31A) was found enhanced in both lines of transgenic plants. From *13-LYPOXIGENASE1*-like (*LOX1*-like) to *3-KETOACYL-COA THIOLASE*-like (*KAT*2-like) all the genes that participate in the biosynthetic pathway were significantly up-regulated in transgenic lines, with the exception of *OPC-8:0 COA LIGASE* (*OPCL*) (Fig. 31B). The enhanced expression levels of the aforementioned biosynthetic genes correlated well with JA and (+)-7-*iso*-JA-Ile (JA-Ile) hormone content, but there was no difference in the content of their precursor *cis*-(+)-12-oxo-phytodienoic acid (OPDA) (Fig.31C).

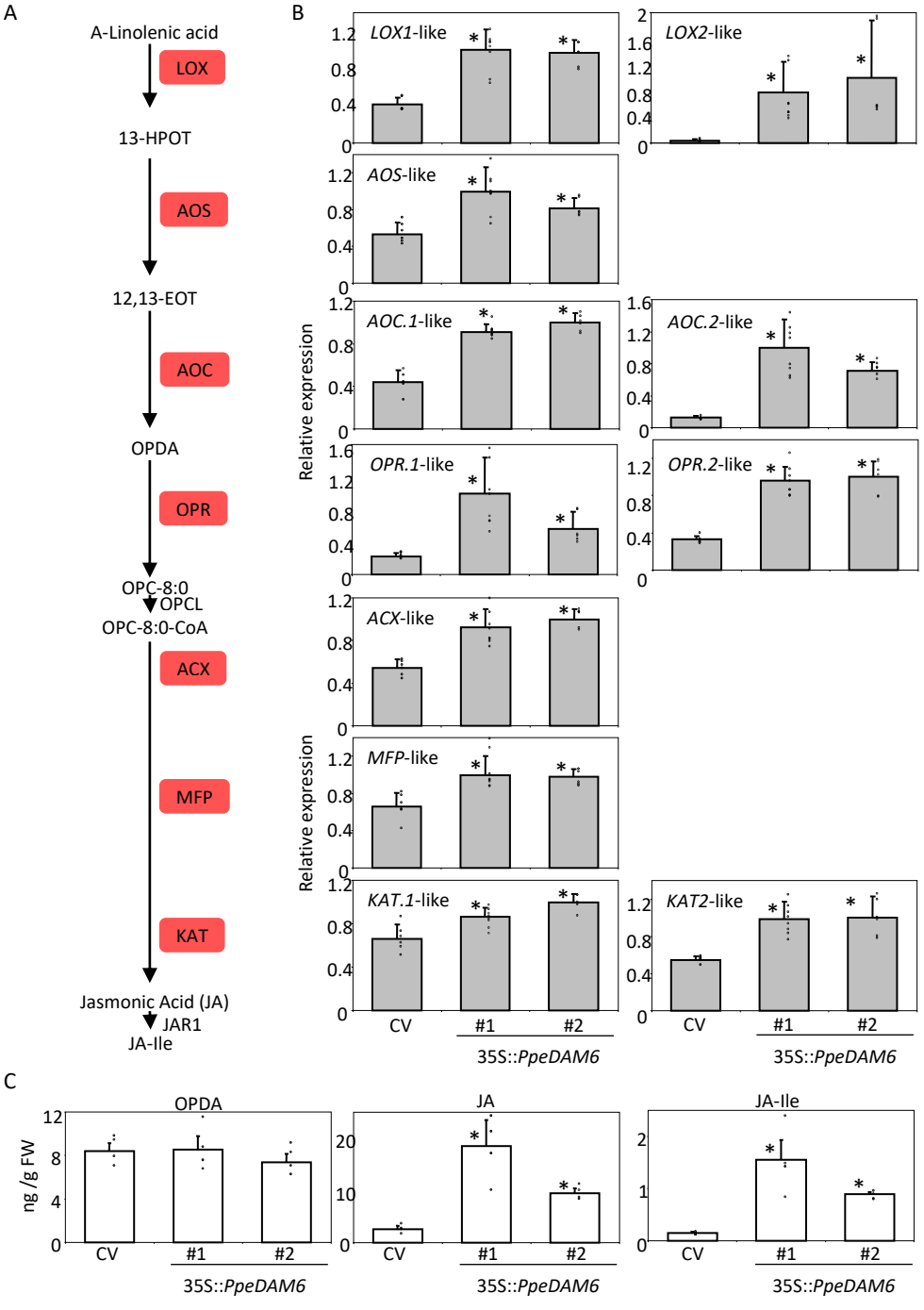
A
A-Linolenic acid
LOX
13-HPOT
AOS
12,13-EOT
AOC
OPDA
OPR
OPC-8:0
OPCL
OPC-8:0-CoA
ACX
MFP
KAT
Jasmonic Acid (JA)
JAR1
JA-Ile
B
LOX1-like
LOX2-like
AOS-like
AOC.1-like
AOC.2-like
OPR.1-like
OPR.2-like
ACX-like
MFP-like
KAT.1-like
KAT2-like
Relative expression
CV
#1
#2
35S::PpeDAM6
C
OPDA
JA
JA-Ile
ng /g FW

Figure 31. Regulation of JA in 35S::*PpeDAM6* overexpressing lines. (A) Summary of JA biosynthesis pathway (B) Relative expression levels of JA biosynthesis genes in Claudia Verde (CV) and 35S::*PpeDAM6* #1 and #2. AGL24-like and actin-like genes were used as reference genes. An expression value of one is assigned to the highest level line. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%. (C) Hormonal content of OPDA, JA and JA-Ile in Claudia Verde (CV) and 35S::*PpeDAM6* #1 and #2. Data are means from four biological samples, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%

On the other hand, the expression of *CYTOKININ DEHYDROGENASE*-like gene (*CKX*-like), which catalyzes the irreversible degradation of CKs and is thus a key regulator of CK content in plants (Fig. 32A), was higher in transgenic lines (Fig. 32B). In close agreement with these results, the content of the CK hormone isopentyl-adenine (iPA) was reduced in leaves of transformed plum plants compared with 'Claudia Verde', although only in line #2 was statistically significant (Fig. 32C).

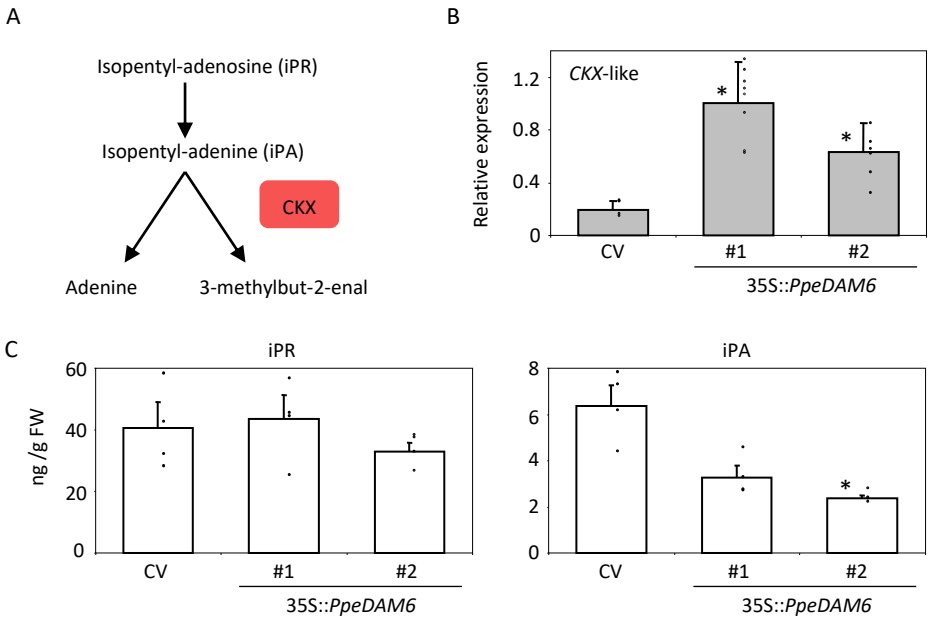

Figure 32. Regulation of CK in 35S::*PpeDAM6* overexpressing lines. (A) Summary of CK catabolism pathway. (B) Relative expression levels of CKX genes in Claudia Verde (CV) and 35S::*PpeDAM6* #1 and #2. AGL24-like and actin-like genes were used as reference genes. An expression value of one is assigned to the highest level line. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. (C) Hormonal content of iPR and iPA in Claudia Verde (CV) and 35S::*PpeDAM6* #1 and #2. Data are means from four biological samples, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%.

In addition, the expression of genes involved in GA biosynthetic, catabolic and signal transduction pathways were also identified (Fig. 33A). In the GA biosynthetic pathway *ENT-COPALYL DIPHOSPHATE SYNTHASE 1*-like (*CPS1*-like), *ENT-KAURENOIC ACID OXIDASE 2*-like (*KAO2*-like) and *GA20-OXIDASE 2*-like (*GA20OX2*-like) were down-regulated, while the GA catabolic gene *GA2-OXIDASE 8*-like (*GA2OX8*-like) was up-regulated in transgenic lines. With respect to the GA signalling pathway, we found that the GA receptor *GIBBERELLIN INSENSITIVE*

DWARF1b-like (*GID1b*-like) was up-regulated, while *GA-STIMULATED TRANSCRIPT 1*-like (*GAST1*-like) and the GA signalling repressor *DELLA1*-like were down-regulated (Fig 33B).

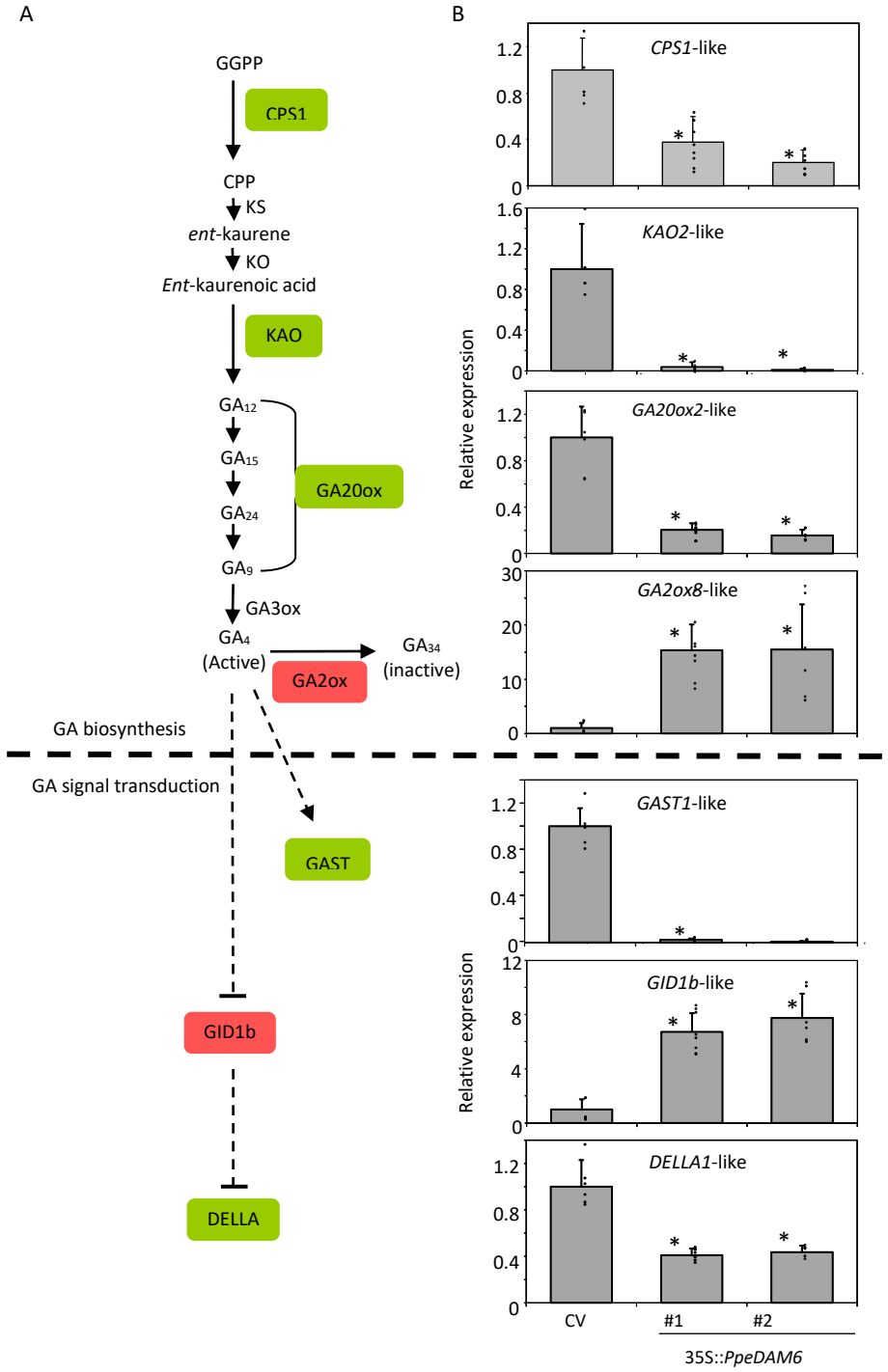
A
GGPP
CPS1
CPP
KS
ent-kaurene
KO
Ent-kaurenoic acid
KAO
GA12
GA15
GA24
GA9
GA20ox
GA3ox
GA4
(Active)
GA34
(inactive)
GA2ox
GA biosynthesis
GA signal transduction
GAST
GID1b
DELLA
B
CPS1-like
KAO2-like
GA20ox2-like
GA2ox8-like
GAST1-like
GID1b-like
DELLA1-like
Relative expression
CV
#1
#2
35S::PpeDAM6

Figure 33. Regulation of GA in 35S::*PpeDAM6* overexpressing lines. (A) Summary of GA biosynthesis and signalling pathway (B) Relative expression levels of GA biosynthesis and signalling genes in Claudia Verde (CV) and 35S::*PpeDAM6* #1 and #2. AGL24-like and actin-like genes were used as reference genes. An expression value of one is assigned to the highest level line. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%.

Despite the fact that gene expression analysis in the GA pathway suggested a reduction of bioactive GA content in transformed plum plants, we could not detect a change in GA levels (Fig. 34A). However, the exogenous application of GA significantly enhanced growth of both transgenic lines, becoming comparable with that of the control 'Claudia Verde' (Fig. 34B). Considering everything, despite we could not detect altered levels of GA in *PpeDAM6* overexpressing plum plants, the results insinuated a close relationship between the lack of GA content and the reduced growth phenotype of the transgenic plants.

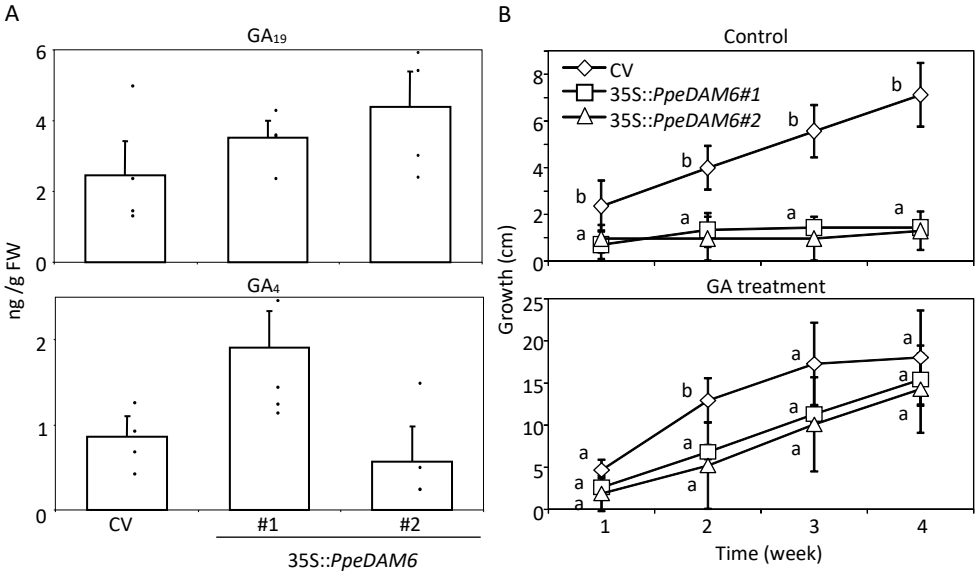

Figure 34. Suppression of growth alterations in 35S::*PpeDAM6* lines by GA application. (A) Content of GA_{19} and GA_4 in Claudia Verde (CV) and 35S::*PpeDAM6* #1 and #2. Data are means from four biological samples, with error bars representing standard deviation. An asterisk indicates significant difference with the control at a confidence level of 95%. (B) Growth of 'Claudia verde' (white rhomb) and 35s::*PpeDAM6* #1 (white square) and #2 (white triangle) under water (control) and GA treatment. Data are means from at least three different plants per genotype. Different letters (a–b) indicate significant difference between different genotypes in each week, at a confidence level of 95%.

Identification of putative transcriptional targets of *PpeDAM6*

MADS-box transcription factors like PpeDAM6 directly bind and regulate the expression of a set of target genes to coordinate different biological functions. All the identified DEUs in the transgenic lines were potential targets of *PpeDAM6* but only those that were differentially expressed during bud dormancy in peach were further considered. We analyzed the expression of DEUs related to hormone pathways along flower bud development in peach (Supplementary Fig. S6).

As shown in Fig. 35A, the expression profiles of *PpeDAM6* and *GAST1*-like were inversely correlated in cultivars 'Red Candem' and 'Crimson Baby' during bud dormancy. High expression values of *PpeDAM6* corresponded with low levels of *GAST1*-like, and vice versa. A similar expression profile was obtained in *GA20OX2*-like analysis. Interestingly, *GAST1*-like and *GA20OX2*-like genes show CarG box elements along their structural sequence, the recognition motif of MADS-box proteins, enabling a potential regulatory mechanism by *PpeDAM6* (Fig. 35B).

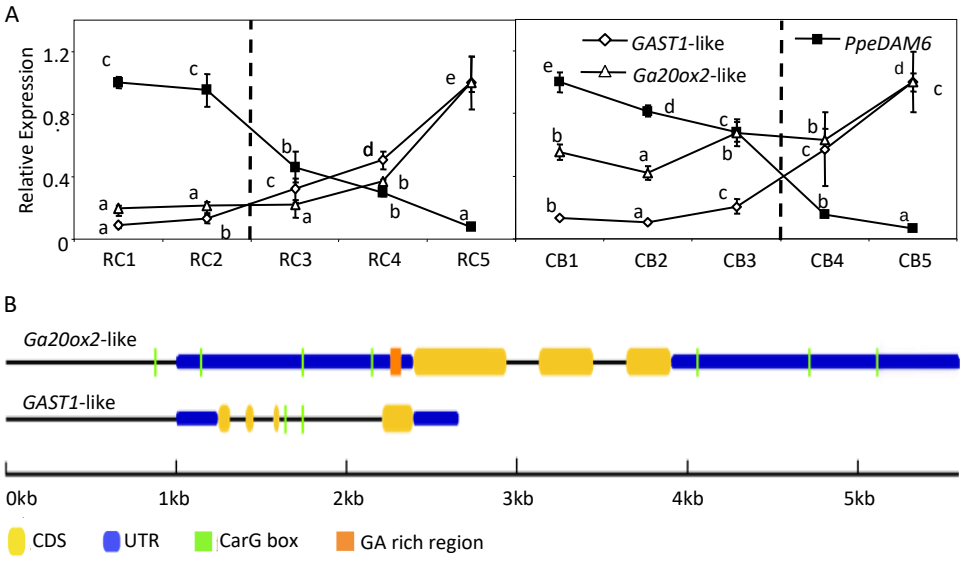

Figure 35. Relative expression of *GAST1*-like and *GA20ox2*-like in reproductive buds of peach. (A) Relative expression of *PpeDAM6* (dark square), *GAST1*-like (white rhomb) and *GA20ox2*-like genes (white triangle) was measured along bud development in cultivars 'Red Candem' (RC) and 'Crimson Baby' (CB). Bud sample code used in Fig. 23 also applies to this figure SAND-like gene was used as reference gene. Dash line represents dormancy release event. An expression value of one is assigned to the highest value per gene. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Different letters (a–e) indicate significant difference between samples for each gene, at a confidence level of 95%. (B) Genomic structure of *GA20ox2*-like and *GAST1*-like with the respective coding sequence (yellow rectangles) and untranslated 5' and 3' regions (blue rectangles). Potential binding sites like CarG boxes and GA rich region are marked by green and orange blocks, respectively.

DISCUSSION

BPC proteins bind and regulate *PpeDAM6* expression

Previous studies have reported the involvement of *PpeDAM6* in the establishment and maintenance of bud dormancy. Similarly, our results showed

an abrupt reduction in the levels of *PpeDAM6* transcripts concomitantly with dormancy release. However, the molecular mechanisms underlying *DAM*s transcriptional regulation associated with dormancy events still remains unclear. In different species, *DAM* genes have been postulated to be modulated by epigenetic mechanisms, such as the histone modifications H3K4me3 and H3K27me3. Specifically in peach, H3K27me3 modification was found associated with GAGA motifs located in all *DAM* and other genes (de la Fuente et al., 2015). The GAGA motif, consisting of tandem GA repeats, is widespread inside the regulatory genomic regions of both animals and plants and has been related to both activation and inhibition of gene transcription (Berger and Dubreucq, 2012). In plants, GAGA motifs are mainly recognized by a specific family of transcription factors called BPC/BBR, firstly characterized in barley (Santi et al., 2003) and subsequently in *Arabidopsis* (Meister et al., 2004) and cucumber (Mu et al., 2017). In this study, we postulated a novel regulatory mechanism of *PpeDAM6* gene through these GAGA motifs. We have found that PpeBPC1 represses *PpeDAM6* transcriptional activity by binding to two GAGA motifs located in an intronic region of *PpeDAM6* that becomes enriched in H3K27me3 modification concomitantly with dormancy release events. Indeed, an association between BPC binding and H3K27me3 enrichment was already observed in *Arabidopsis* (Hercker et al., 2015). BPC/BBR family has been related to transcription inhibition via induction of conformational changes in DNA structure, by interacting between themselves (Simonini et al., 2012) or recruiting repressors like *SEUSS* (Simonini et al., 2012) and components of the Polycomb Repressive Complex (PRC) such as LIKE HETEROCHROMATIN PROTEIN1 (LHP1) (Hecker et al., 2015). In a previous study, BPCs from *Arabidopsis* have been found to down-regulate *ABI4* gene by recruiting the component of the PRC2 complex SWINGER (SWN) to its promoter, mediated by the specific H3K27me3 modification (Mu et al., 2017). Contrarily to this study, we could not confirm the interaction of PpeBPCs with a peach SWN-like protein nor other putative repressors by using the Y2H. The presence of two GAGA motifs in *PpeDAM6* locus and the ability of PpeBPCs to interact with

themselves suggests that PpeBPCs effect on *PpeDAM6* expression could rely on changes in DNA structure.

Potential role of *PpeDAM6* in the flowering pathway

Since light and temperature are involved in flowering and dormancy, many reviews have hypothesized that similar mechanisms regulate both processes (Horvath, 2009; Lloret et al., 2018). In fact, *DAM*s from temperate trees are homologs of *SVP* and *AGAMOUS-LIKE 24* (*AGL24*), two main flowering regulators in *Arabidopsis* (Falavigna et al., 2018). *SVP* act as flowering repressor (Hartman et al., 2000) by direct inhibition of the floral integrator *FT* (Lee et al., 2007). Similarly, *DAM* proteins bind to CArG boxes located in promoter regions of leafy spurge *FT* gene during endodormancy (Hao et al., 2015). Consistently with this, our transgenic *Arabidopsis* plants overexpressing *PpeDAM6* presented abnormal flower development (Fig. 27), resembling 35S::*AtSVP* plants in the literature, with the exception of the late flowering phenotype. Likewise, the overexpression in *Arabidopsis* of *PavDAM1-6* and *SVP*-like genes, which are involved in the dormancy process in *Prunus avium* and *Actinidae deliciosa* respectively, resulted in similar defects in the flower phenotypes (Wang et al., 2020a; Wu et al., 2012).

Moreover, yeast two hybrid analysis confirmed the presence of three common protein interactors between PpeDAM6 and SVP: FUL (Balanza et al., 2014), SEP2 (Trigg et al., 2017) and SOC1 (de Folter et al., 2005). In addition, SOC1-like have been also shown to interact with DAM6 from apricot (Kitamura et al., 2016) and sweet cherry (Wang et al., 2020a), similarly to the interaction observed between SVP-like and SOC1-like in kiwifruit (Wu et al., 2017). SOC1 ensures that floral induction and floral development occur in the right time and space integrating different flowering signals (Lee and Lee, 2010). One of this signal is cold-dependent, in concordance with our results showing that *PpeSOC1*-like expression is down-regulated along bud development (Supplementary Fig. S6). Similarly, in recent studies in woody plants, *SOC1*-like genes have been associated with chilling requirements and dormancy duration (Trainin et al., 2013, Voogd et al., 2015). Considering these results, the interaction between PpeDAM6 and SOC1

could be central for suppressing dormancy release and controlling flowering development through cold-dependent cues. On the other hand, *Arabidopsis* FUL modulates the activity of floral regulators, and its interaction with SVP suppresses the flowering repressor effect of SVP (Balanza et al., 2014). Extrapolating this behaviour to peach bud dormancy, the interaction between PpeDAM6 and FUL could perform a repressive effect on PpeDAM6 dormancy promoting role, facilitating dormancy release and flowering. Finally, SEP2-like, together with PI-like and SHP-like, are regulators of floral identity that were also identified as PpeDAM6 interactors. In *Arabidopsis,* B, C and E floral homeotic genes maintain floral meristem identity and are directly repressed by SVP (Gregis et al., 2009). The abnormal flower of 35S::*PpeDAM6 Arabidopsis* transgenic plants could be due to the direct repression of these genes by PpeDAM6 binding or could be mediated by PpeDAM6 interaction with floral organ identity proteins or flowering regulators.

The presence of six *DAM* genes in peach and other *Prunus* species seems not to be just due to functional redundancy. Instead of that, they may have specialized roles in dormancy, flowering and growth pathways. This study suggests that their partners could add a further degree of functional specialization, providing distinct pools of transcriptional targets and different specific roles.

Is *PpeDAM6* a master growth repressor acting through the regulation of hormone pathways?

Despite *DAM* genes are commonly associated with dormancy establishment and maintenance in many woody species, further functional insight is required. To investigate the role of *PpeDAM6* in dormancy regulation, we used transgenic plums overexpressing 35S::*PpeDAM6.* These lines showed a strong stunted growth that mainly affected internode elongation, in concordance with the altered phenotypes observed in 35S::*PmDAM6* transgenic poplar (Sasaki et al., 2011) and apple (Yamane et al., 2019). We could not analyze the dormancy phenotype of these lines since transgenic plants died few months after soil

acclimation. In any case, as *PpeDAM6* gene in peach is well expressed in both, buds and leaves (Fig. 21B), we studied the effect of *PpeDAM6* overexpression in the leaves of transgenic plants. Our data suggest that *PpeDAM6* affects growth in transgenic lines most likely due to an altered hormone homeostasis, mainly by an increase of JA and a decrease of CK. In fact, *PmDAM6* was previously described as a modulator of CK and ABA content in transgenic apple plants (Yamane et al., 2019). Given the critical importance of hormones in plant developmental processes, we investigated their metabolism and response in detail.

Unexpectedly, our transgenic plants presented altered levels of JA due to an up-regulation of its biosynthesis pathway at several steps. Similarly to ABA, JAs inhibit plant reproductive development (Huang et al., 2017) and plant growth by arresting cell cycle (Wasternack and Hause, 2013). In maize, *TEOSINTE BRANCHED1* (*TB1*) gene induces bud arrest through the production of the inhibitory phytohormones JA and ABA (Dong et al., 2019). TB1 is proposed to bind *TASSELSEED* (*TS1*), which encodes a JA biosynthesis gene required for a proper male flower development (Acosta et al., 2009). A recent review indicates an additional role of JA as regulator of cold-acclimation (Liu and Sherif, 2019). In *Arabidopsis*, JA modulates freezing tolerance by up-regulating the COR pathway (Hu et al., 2013). In fact, Horvath et al. (2008) detected a high number of JA regulated genes during dormancy and proposed that JA perception through paradormancy might be needed to prepare plants for winter. This study suggested that JA promoted an accumulation of storage proteins that were needed for buds to survive the dormant state and renew their growth when growth-conducive conditions return.

On the other hand, transgenic plants also showed lower CK content. CKs generally affect cell division, cell differentiation and stress tolerance among other processes, and are particularly important in modulating meristem activity and morphogenesis (Liu and Sherif, 2019). CKs are required during development of the shoot, in particular in the shoot apex. CK levels increase in buds during dormancy release in grapes (Noriega and Pérez et al., 2017) whereas in *Rosa hybrida* and Japanese pear, ABA and CK may act antagonistically in the regulation of bud break

(Corot et al., 2017; Yamane et al., 2019). CK degradation is achieved by down-regulation of CK biosynthesis or catabolism activation. We analyzed the expression level of different genes involved in CK biosynthesis and catabolism. Although no significant differences were found in the expression of biosynthesis genes, *PdoCKX*-like expression was markedly increased in transgenic plants compared with controls. Werner et al. (2003) showed that overexpression of *CKX* genes reduces the size of the shoot apical meristem (SAM) affecting cell proliferation and differentiation and even stops its activity completely in strong *CKX* overexpressing lines. On the contrary, the meristems of *ckx* mutants showed a larger stem cell niche where the cells are maintained in undifferentiated state (Bartrina et al., 2011). These studies are in close agreement with the stunted growth observed in the transgenic plants which even reached meristem collapse. On the other hand, *AtCKX1* led to CK-deficient developmental phenotypes and improved drought stress tolerance in transgenic apple and tobacco plants (Macková et al., 2013; Liao et al., 2017). In addition, *MsCKX* gene expression was significantly up-regulated under salt stress and ABA treatment, suggesting that *MsCKX* may play a role as positive regulator in the salt stress response and participate in ABA signalling pathway in alfalfa (Li et al., 2019).

Finally, GAs are also considered key regulators of bud dormancy. GA content decreases at the dormancy induction stage and increases during dormancy release in *Prunus mume* (Wen et al., 2016), *Pyrus Pyrifolia* (Ito et al., 2019), *Prunus avium* (Duan et al., 2004) and *Vitis Vinifera* (Zheng et al., 2018) among others species, supporting a role in dormancy release and growth resumption. These changes in GA level have been mainly correlated with *GA20OX*, *GA3OX* and *GA2OX* gene expression variation (Yue et al., 2018; Zheng et al., 2018). In sweet cherry, exogenous treatments with GA4 have been proposed to release dormancy of flower buds through the regulation of H_2O_2 content, coincidently with changes in the antioxidant defence system (Cai et al., 2019). In *Populus*, dormancy release was associated with a restoration of plasmodesmata channels by GA4-induced β-1,3-glucanase expression (Rinne et al., 2011). Interestingly, GA and ABA are reciprocally regulating each other content (Liu and Sherif, 2019). Thus, in tea plant

GA treatments repress the expression of ABA biosynthetic and catabolic genes (Yue et al., 2017). Inversely, mutation in the ABA pathway showed that this hormone is involved in the suppression of GA biosynthesis in *Arabidopsis* seeds (Seo et al., 2006). In our study, unexpectedly, GA levels were statistically similar comparing transgenic lines to 'Claudia Verde', although GA biosynthesis, catabolism and signalling genes were markedly different. Despite this contradiction, the suppression of the growing phenotype of transgenic plants after exogenous GA treatment suggests that 35S::*PpeDAM6* plants have a deficiency in GA content compared with the control. In the present study, *GA20OX2*-like and *GAST*-like genes were differentially expressed in transgenic plants but also during bud development in peach, supporting them as putative direct targets of *PpeDAM6*. In *Arabidopsis, SVP* was proposed to repress flowering through the down-regulation of *GA20OX2* and consequently GA biosynthesis (Andres et al., 2014), suggesting that similar mechanisms involving *PpeDAM6* gene could operate in peach dormant buds. In close agreement with our data, *PpyGAST1* and *PpyGA20OX2* gene expression increased during dormancy release in *pear* (Yang et al., 2019). The GAST family is widely distributed among plant species and plays central roles in multiple aspects of plant growth and development although their functions have not been completely elucidated. Members of this family have been related to flowering time control in *Arabidopsis* and Petunia (Qu et al., 2016, Nissan et al., 2004). Interestingly, the *GAST*-like GA-inducible genes *GASA4* and *GASA6* were also up-regulated by auxin and CK and down-regulated by ABA, JA and SA in *Arabidopsis* (Qu et al., 2016). In fact, *GASA6* plays a role as an integrator of GA and ABA signalling, resulting in the regulation of seed germination through the promotion of cell elongation (Zhong et al., 2015).

In the light of our data *PpeDAM6* seems to contribute to winter growth arrest and dormancy establishment through the modification of hormone contents and response. Both, down-regulation of CK and up-regulation of JA may potentially protect the dormant bud against abiotic stresses. Once chilling requirements are fulfilled, *PpeDAM6* is repressed, leading to an increase of *PpeGA20OX2*-like transcript and GA hormone levels. This GA rise may in turn modulate ABA content,

contributing thus to dormancy release. On the other side, an increase in CK may promote cell division and differentiation in the meristem, favouring growth resumption. In conclusion, *PpeDAM6* works as a master regulator of peach dormancy, acting as a growth repressor but also promoting stress tolerance response and repressing flowering, most probably by means of hormone homeostasis modulation.

METHODS

Plant material

Peach trees (*Prunus persica* (L.) Batsch) required in this study were grown at Instituto Valenciano de Investigaciones Agrarias located in Moncada (Spain). For reproductive bud development expression analysis, two peach cultivars requiring different chilling requeriments (CR) were collected during autumn-winter 2015-2016. Cultivar 'Red Candem' (low CR) was harvested on November 20 (RC1), November 30 (RC2), December 14 (RC3), December 21 (RC4) and January 4 (RC5), whereas cv 'Crimson Baby' (medium CR) was harvested on Novemeber 30 (CB1), December 14 (CB2), December 21 (CB3), January 4 (CB4) and January 19 (CB5). In order to evaluate the dormancy status, 10 budsticks from three different trees with no less than 6 flower buds were placed with their basal end in water in a chamber set at 24°C 12h:12h light:dark cycle. Routinely the base of budsticks was cut and the water replaced with fresh one. Dormancy release was considered when more than 50 % of buds showed at least the green tip of the sepals after 14 days. For tissue dependent expression analysis, samples required were obtained from buds (collected on January 12, 2010), leaves (November 6, 2012), embryos, flower parts (March 26, 2010) and fruit tissues (June 29, 2010) of cv ‘Big Top’. Finally, for plasmid constructions required in this study, we used leaves collected on April 11, 2012 and buds collected on autumn-winter 2009-2010 of cv ‘Big Top’.

DNA Isolation, RNA Isolation and RT-qPCR Analysis

Leaf DNA extraction was performed according to Doyle and Doyle (1987). DNA quality and quantity were checked by agarose gel electrophoresis and Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies).

For RNA isolation from peach buds, 100 mg of powdered buds were extracted using RNeasy Plant Mini Kit (Qiagen), adding 1 % (w:v) polyvinylpyrrolidone (PVP-40) to the kit extraction buffer before use. On the other hand, leaf plum RNA extraction was performed according to Gambino et al. (2008). Both extractions were treated with the RNase-Free DNase Set (Qiagen). RNA quality and quantity was assessed similarly to DNA isolation procedure.

For expression analysis, 500 mg of total RNA were reverse transcribed to cDNA using PrimeScript RT reagent kit (Takara Bio) in a final volume of 10 µl. The qPCR was performed with 2 µl of a 10x diluted first strand cDNA in StepOnePlus Real-Time PCR System (Life Technologies), utilizing SYBR premix Ex Taq (Tli RNaseH plus) (Takara Bio) in a final volume of 20 µl. Cycling conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. Specificity of the amplification was evaluated by the presence of a single peak in the melting curve after PCR and by size estimation of the amplified product in agarose gel. Housekeeping genes were selected according to Lloret et al. (2017). Relative expression was measured using a relative standard curve and three biological replicates each one with two technical replicates. All the primers used in this study were listed at Supplementary Table S8.

Analysis of protein-DNA interaction by Y1H system

Y1H screening was assayed to detect DNA binding regulatory proteins interacting with a genomic region of *PpeDAM6* that is enriched in H3K27me3 modification concurrently with dormancy release. We selected two fragments of this genomic region to perform the Y1H. The first fragment, called Reg1 (-316 to -1 relative to the translation initiation codon), contains two CArG box motifs associated with MADS-box interactions (Fig. 22A). The second one, called Reg2 (+182 to +575 relative to the translation initiation codon), includes one CArG box

motif and two GAGA motifs (Fig. 22A). For cloning both regulatory regions into the bait plasmid, genomic DNA from leaves was amplified and inserted into pAbAi vector. The reporter constructions were linearized and integrated into the genome of *Saccharomyces cerevisiae* strain Y1HGold following the Yeastmaker yeast Transformation System 2 (Clontech-Takara Bio) to create Y1H bait strains. Next, they were tested to determine the minimal inhibitory concentration of Aureobasidin A (AbA) that completely suppresses the growth of the transformed yeast, being in both cases 200 ng/mL of AbA. Two µg of total RNA obtained from a mix of dormant and non-dormant flower buds was reverse transcribed to generate the library by recombining the cDNAs with the pGADT7-rec linearized vector. The Y1H screening assay was performed following the Matchmaker® Gold Yeast One-Hybrid Library Screening System (Clontech-Takara Bio) in minimal medium without leucine and supplemented with 200 ng/mL of AbA. The protein interaction was confirmed by subsequent transformation of Y1HGold containing bait constructions and the pGADT7-positive clones, using yeast strain Y1HGold with empty bait as a negative control.

To determine the DNA-binding specificity of the PpeBPCs, we used yeast strains containing reporter vector with seven different fragments derived of the pABAi-Reg2 (Fig. 22C). In addition, the whole coding region of *PpeBPC3* (Prupe.8G082900.1) was PCR-amplified from cDNA from non dormant buds and cloned into pGADT7 vector. Both pGADT7-*PpeBPC1,2*$_{(parcial)}$ and pGADT7-*PpeBPC3* were introduced into the Y1H bait strains with the seven different pAbAi-Reg2-derived plasmids. The positive interactions were tested in the same minimal medium employed in the screening.

Analysis of protein interaction by Y2H system

In order to confirm the direct interaction between PpeBPCs and regulatory proteins reported as putative interactors in other species (Simonini et al., 2012; Hecker et al., 2015; Mu et al., 2017), a Y2H for analizing protein-protein interaction was performed. Firstly, the full length coding sequence of *PpeBPC1, PpeBPC2, PpeLHP1, PpeSWN and PpeSEUSS* were introduced into pGADT7 with

the same procedure followed for pGADT7-*PpeBPC3* construction. Subsequently, *PpeBPC1*, *PpeBPC2* and *PpeBPC3* genes obtained from pGADT7-cloned plasmids were inserted into pGBKT7 and introduced into yeast strain Y2HGold following the described protocol, Yeastmaker yeast Transformation System 2 (Clontech-Takara Bio). Previously to Y2H experiment, it is imperative to verify that our gene does not autonomously activate the reporter genes in the manufacturer's recommended mediums with minor modifications in AbA concentration (125 ng/mL). Since none of the bait construction auto-activated the reported genes, pGADT7-cloned plasmids were sequentially introduced into the pGBKT7-transformed yeast strains. Two-hybrid interactions were tested in minimal medium without tryptophan, leucine, histidine and adenine, and supplemented with AbA (125 ng/ml) and X-α-Gal (40 µg/ml).

On the other hand, a Y2H library screening was also executed. Firstly, the whole ORF of *PpeDAM6* (1-717 nucleotides relative to the coding sequence) was amplified using cDNA from dormant buds and introduced into pGBKT7 vector, obtaining pGBKT7-*PpeDAM6*. From that plasmid, a new one was generated but adding a premature stop codon, pGBKT7-*PpeDAM6*$^{1\text{-}537}$ (1-537 nucleotides relative to the coding sequence). Then, yeast strain Y2HGold was transformed with both constructions following the manual of yeast transformation in order to test for self-activation. In this case, as PpeDAM6 is a transcription factor with a transcription activation domain at the end of the coding sequence, only the construction with the truncated sequence of *PpeDAM6* (pGBKT7-*DAM6*$^{1\text{-}537}$) did not auto activate the reporter genes and was suitable to perform the screening assay. The library construction and the Y2H screening was performed following Make Your Own "Mate & Plate™" Library System and Matchmaker® Gold Yeast Two-Hybrid System (Clontech-Takara Bio) previously described in Lloret et al. (2017).

Phylogenetic analysis

For the phylogenetic analysis, apart from peach BPCs protein sequence, *Hordeum vulgare, Arabidopsis thaliana, Vitis vinifera* and *Populus trichocarpa*

BPCs were also downloaded from the TAIR10 database and NCBI database, respectively. We used ClustalW (Larkin et al., 2007) to perform multiple sequence alignment and Gblocks to remove poorly aligned positions and divergent regions of the alignment (Talavera and Castresana, 2007). For phylogenetic tree construction, MEGA 7 (Kumar et al., 2016b) was used with Maximum Likelihood method and tested using a Bootstrap with 1000 replicates. Nodes with less than 70 % bootstrap support were eliminated.

Dual luciferase assay

In order to confirm *PpeDAM6* gene expression regulation by BPCS a dual luciferase assay was performed. Firstly, the whole cDNA of the three *PpeBPCs* were subcloned into effector pGreenII-62sk vector under 35S promoter using pGADT7-*PpeBPCs* plasmids. On the other hand, from the promoter until the end of the second intron of *PpeDAM6* (-1869 to + 3575 relative to the start site) was inserted into reporter pGreenII-0800luc vector so that this regulatory region controls the firefly luciferase (LUC) expression. This vector also contains the renilla luciferase gene (REN) under a constitutive promoter which is used as an internal control to normalize the values of the experimental reporter gene for variations that could be caused by transfection efficiency and sample handling. All recombinant plasmids were individually introduced in *Agrobacterium tumefaciens* strain C58 already transformed with pSOUP, a helper plasmid that enables binary replication of pGreenII construction inside the bacterium. *Nicotiana benthamina* plants grown during six weeks were infected with a mix of transformant *Agrobacterium*. In each mix, apart from the different effector/reporter plasmids combinations, one extra *Agrobacterium* strain expressing the supressor protein HcPro was included. HcPro mitigates the defense response of the plant and improves infection. In the inoculum, overnight culture of confluent bacterium were resuspended in the infiltration media (10 mM $MgCl_2$, 10 mM MES pH 5.6) to an OD_{600} of 0.5 (except *Agrobacterium* with HCpro that was resuspended to an OD_{600} of 0.1). This *Agrobacterium* inoculum was infiltrated on the abaxial side supported by 1 ml syringe and a needle to do a small cut. After inoculation and a

transient incubation of 3 days, LUC activity was measured using the dual luciferase reporter assay system (Promega) with minor modifications. Two cm leaf discs were harvested, ground and resuspended in 300 µl of Lysis Buffer. Ten µl of this crude extract was assayed in 40 µl of Luciferase Assay Buffer, and the chemiluminescence measured in the PROMEGA GloMax Multi Microplate Reader luminometer. A volume of 40 µl of Stop and Glow™ buffer was then added and a second chemiluminescence measurement made. This second measurement corresponds to REN activity and was used to normalize the luciferase data. Four biological replicates were measured for each combination.

Genetic transformation of *Arabidopsis thaliana*

To overexpress *PpeDAM6* in *Arabidopsis*, a fragment containing *PpeDAM6* fused to N-terminal c-myc tag was excised from the pGBKT7-*PpeDAM6* plasmid and then introduced into binary pROK2 vector under the 35S promoter. In addition, the pROK2 construction with the epitope fused to the C-terminal end of *PpeDAM6* was also obtained using pGBKT7-*PpeDAM6* as a template in a PCR amplification with specific primers that directly generate *PpeDAM6* fused to the c-myc tag at the end of the sequence. Both constructions were introduced into *Agrobacterium* strain EHA105. Overexpression of *PpeDAM6* gene in *Arabidopsis* was carried out in wild-type Columbia. *Agrobacterium tumefaciens*-mediated plant transformation was performed by floral dipping method (Mara et al., 2010). The transformed seeds were selected on Murashige and Skoog (MS) agar with 50 µg/ml of kanamycin. Floral phenotype was evaluated directly in the T0 generations since many of them are sterile. However, for measuring flowering time 10 plants of the T2 generation from one line with abnormal phenotype but with viable seeds were used. Seedlings were grown in a chamber at 24°C 16h:8h light:dark cycle.

Genetic transformation of plum

Transgenic plant regeneration of plum (*Prunus domestica* cv. Claudia Verde) was performed according to Petri et al. (2008b). Briefly, the endocarp was

removed with a nutcracker, and the seeds were surface-sterilized for 30 min using 1 % sodium hypochlorite solution containing 0.02 % of Tween-20. Disinfected seeds were soaked in sterile distilled water overnight at room temperature in order to ease the removing of seed coats with a scalpel. The radicle and the epicotyl were discarded, and the hypocotyl was sliced into several cross sections (less than 1 mm), which were used for co-transformation. *Agrobacterium tumefaciens* strain LBA4404, carrying the binary vector pROK2-*c-myc-DAM6* was overnight culture until reach an OD_{600} of 0.2-1.0. Then was centrifuged at 5.000xg for 10 min and resuspended in 50 ml of medium consisting of MS salts, 2 % (w/v) sucrose and 100 µM acetosyringone. This culture was shaken (175 rpm) at 25°C for 5 h before co-culture with the slices of hypocotyl.

After 3 days on shoot regenerating medium (SRM: ¾ MS based medium with 7.5 µM thidiazuron (TDZ), 0.25 µM indole butyric acid (IBA), 9.05 µM 2,4-D and 100 µM acetosyringone), the hypocotyl co-culture slices were transferred to SRM selective medium without 2,4-D and acetosyringone, and containing timentin (600 mg/l) and kanamycin (80 mg/l) during 8 weeks. Then regenerated shoots were transferred to the shoot growing medium (SGM), in which TDZ was replaced by 1.0 µM 6-benzylaminopurine (BAP) where they were sub-cultured at 4-week intervals at 24°C under a 16-h photoperiod. When shoots reached 2-3 cm long they were separated from the cluster and transferred to rooting media (RM) (Gonzalez-Padilla et al., 2003) supplemented with kanamycin (40 mg/l) and timentin (300 mg/l). After 5-7 weeks, shoots with roots were ready for acclimatization and *in vitro* plants were removed from culture pots and transplanted into pots containing sterilized topsoil sand (4:1) mixture. Plants were covered with transparent plastic pots and progressively removed as plants hardened-off.

Western blot analysis

Protein extracts were obtained from 50 µg of ground leaf boiled in Laemmli buffer during 10 min at 95°C. The samples were resolved on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on 15 % resolving gel and

3.5 % stacking gel (Laemmli, 1970), before transfer onto a PVDF membrane (GE Healthcare- Life sciences). The proteins were detected with BM Chemiluminecnce Western Blotting kit (Mouse/Rabbit) (Roche) following the manufacturer's protocol with minor modifications. Membranes were blocked in 1 % of Blocking Solution overnight at 4°C and then incubated with Anti-myc Tag clone 4A6 (EMD Millipore) for 1.5 h. The membranes were subsequently washed and then incubated for 1 h with anti-mouse IgG POD-secondary antibody (Roche). They were then washed and briefly incubated with the detecting solutions (Roche). For the detection, X-ray films were used following standard protocols.

RNA-Seq analysis

Total RNA isolation was extracted from transgenic plum leaves with the previously described plum RNA extraction protocol. RNA-quality was evaluated by agarose gel electrophoresis and Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) while Invitrogen Qubit 2.0 Fluorometer, which measures the concentration of extracted RNA using the Qubit RNA HS Assay Kit (ThermoFisher Scientific) checked RNA-quantity. Library preparation and transcriptome sequencing by paired-end 150 pb sequence using Illumina HiSeqTM 2500 were conducted by Novogene Corporation. Three biological replicates from control 'Claudia Verde', 35S::*PpeDAM6* #1 and 35S::*PpeDAM6* #2 were sequenced.

Raw reads with sequenced adapters, with more than 10 % of uncertain bases and more than 50% of low-quality bases were removed from the analysis. Clean reads of all samples were combined and de novo transcriptome assembled by Trinity (Grabherr et al., 2011) and filtered by CORSET (Davidson and Oshlack, 2014) was used as a reference transcriptome. To achieve comprehensive gene functional annotation, seven databases were applied (Supplementary Table S9). Cleaned RNA-seq reads were aligned to the assembled transcriptome using Bowtie (Langmead and Salzberg, 2012) through the Trinity software. Once they were mapped, reads per gene were counted by RSEM (Li and Debey, 2011) and differential expression analysis was performed on raw counts using DESeq (Anders

et al., 2010). KEGG enrichment was assessed by KOBAS (Xie et al., 2011). Version and parameters used in each software are listed in Supplementary Table S10. All the studied differentially expressed unigenes were assayed by RT-qPCR.

Measurement of phytohormones

Frozen plant material from transgenic plants was ground to fine powder and weighed directly in 2 mL-microtubes recording the actual sample weight. Before extraction, plant samples were spiked with 25 µl of an internal standard mixture (containing ABA-d_6, DHJA, IAA-d_5, GA1-d_2 and GA4-d_2 at concentration of 1 mg/L) to correct for analyte loses. Extraction was carried out in 1 mL ultrapure water for 10 min in a ball mill at room temperature using 2 mm glass beads. After extraction, homogenates were centrifuged at 10,000 rpm for 10 min at 4°C to remove debris and supernatants recovered. The resulting solutions were partitioned twice against an equal volume of di-ethyl ether after adjusting pH to 3.0 with 30% acetic acid. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan, Sant Germaine Cedex,) and the dry residues reconstituted in 0.5 mL of a 10% aqueous methanol solution. Prior to injection in the analytical system, extracts were filtered through 0.20 µm PTFE syringe membrane filters and filtrates recovered in chromatography amber glass vials. Samples were analyzed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp.,) coupled to a TQS triple quadrupole mass spectrometer (Micromass Ltd.) through an electrospray ionization source. Separations were carried out on a C18 column (Luna Omega Polar C18, 50×2.1 mm, 1.6 µm particle size, Phenomenex) using a linear gradient of ultrapure acetonitrile and water, both supplemented with formic acid to a 0.1% (v/v) concentration, at a constant flow rate of 0.3 mL min^{-1}. During analyses, column temperature was maintained at 40°C and samples at 10°C to slow down degradation. Plant hormones were detected in negative electrospray mode following their specific precursor-to-product ion transitions (JA, 209>59; OPDA, 291>165; JA-Ile, 322>130; GA1, 347>229; GA4, 331>213; GA7, 329>223; iPA, 204>136; iPR 336>204) and quantitated using an external calibration curve with standards of known amount.

GA treatment of transgenic plants

For GA treatment, five transgenic plum seedlings of each sample were sprayed with a solution of gibberillic acid (100 mg/ L GA_3, 0.05% Tween 20 pH 6-7) repeatedly once per week during one month. Each week, the height of the plant was measured.

Statistical analysis

Statgraphics XVI.I package 324 was used to assess the statistics significance in each analysis (Statpoint Technologies). The means of two samples were compared using non-parametric Man-Whitney U test and comparisons of multiple samples were evaluated by non-parametric Klustal-Wallis test with a confidence level of 95 %. Significantly different samples were labelled with asterisks or different letters.

GENERAL DISCUSSION

In trees from temperate climates, meristem survival to unfavorable conditions during winter and a proper growth resumption and flowering in spring is dependent on bud dormancy. A better understanding of the molecular bases of bud dormancy will strongly facilitate plant breeding tasks aimed at assessing the potential for environmental adaptability of particular genotypes, and to evaluate the impact of climate change on crop yields.

In this thesis we focus on the systematic study of three differentially-expressed transcripts identified in reproductive buds of peach that are expected to act in one or more of three different processes: dormancy regulation, stress tolerance and flowering. Inside a bud, dormancy and tolerance to abiotic stress are overlapping processes, while flowering event mainly happens before dormancy (flowering induction and differentiation) and after dormancy (growth resumption and gametogenesis). Since these processes converge temporally and spatially in a reproductive bud, the integration and the interaction of each other is fundamental.

PpeDAM6, one of the main regulators of dormancy, and *PpeS6PDH,* an enzyme putatively involved in the stress tolerance response during dormancy, have been found affected by similar chromatin modification mechanisms. A previous work describes H3K27me3 increase and H3K4me3 reduction in a regulatory region of *PpeDAM6*, coinciding with repression of *PpeDAM6* during dormancy release (Leida et al., 2012). Similarly, we have shown that peach *PpeS6PDH* gene is down-regulated in flower buds after dormancy release, concomitantly with changes in the methylation level at specific lysine residues of

histone H3 (H3K27 and H3K4) in the chromatin around the translation start site of the gene (Fig. 36). Likewise, CBFs are transcription factors responsible of different plant responses related to low temperature and freezing and, in addition, they have been identified as direct regulators of *DAM* genes in different species (Saito et al., 2015; Niu et al., 2016; Zhao et al., 2018b). In fact, the overexpression of a peach CBF in apple affects both freezing tolerance and bud break (Wisniewski et al., 2015). These facts reveal that dormancy and stress tolerance in woody plants are closely related and suggest the existence of concerted regulatory mechanism in these physiologic processes in buds.

On the other hand, growth cessation is required for bud dormancy and optimal stress tolerance, but indirectly also affects flowering since it implies the growth and elongation of floral organs and the cell division events leading to gametophytes.

PpeDAM6 has been proposed as a central regulator integrating the control of different processes in a dormant bud through the control of different hormone pathways. Low GA contents favour dormancy maintenance, whereas CK and JA levels impinge on stress tolerance responses, and a decrease of GA and CK prevent respectively flowering initiation and development (Fig. 36).

We have postulated that *PpSAP1* also impacts on more than one process in the developing bud. On one hand, it has been proposed to be involved in the stress resistance response by conferring tolerance to drought stress, and on the other hand it could control growth cell through the down-regulation of genes involved in cell expansion (Fig. 36).

The study of both *PpeDAM6* and *PpSAP1* clearly exposes that the whole succession of events from flower bud induction to blooming can be interpreted as a trade-off between defense factors leading to stress tolerance and dormancy and growing factors leading to dormancy release and flowering.

In conclusion, this novel approach provides new perspectives in the study of dormancy in reproductive buds of temperate trees. The three studied genes have crucial roles during peach dormancy, being potential candidate genes to obtain improved plants more adapted to changing environmental conditions. In addition,

the data obtained in these studies have provide a dynamic snapshot of biological processes that take place inside the bud, including regulation of dormancy, tolerance to abiotic stresses and flowering and highlight the interaction between the different signaling pathways and regulatory mechanisms.

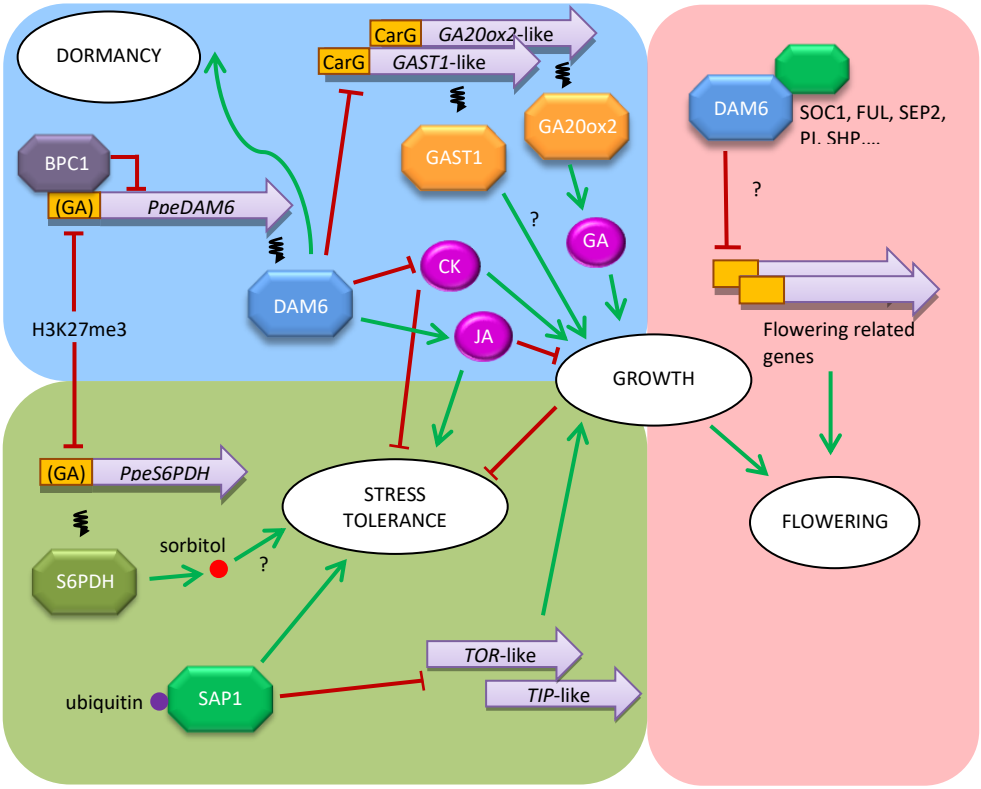

Figure 36. An overview of the main processes converging in a flower bud of peach: dormancy, stress tolerance and flowering. Green arrows indicate genetic or biochemical activation while red ones repression. Question marks tag the relations that have not been confirmed yet.

CONCLUSIONS

-*PpSAP1* is expressed in dormant buds and down-regulated concomitantly with dormancy release. PpSAP1 protein belongs to the *STRESS ASSOCIATED PROTEIN* family containing Zn-finger domains A20 and AN1. These domains have been found to regulate the abiotic stress response in different species, most likely by an ubiquitin-related mechanism.

-The ectopic expression of *PpSAP1* in plum alters water loss and leaf morphology. This effect on cell growth could be mediated by down-regulation of *TARGET OF RAPAMYCIN* (*TOR*)-like, a key regulator of cell growth and metabolism in eukaryotic cells, and *TONOPLAST INTRINSIC PROTEIN* (*TIP*)-like, a tonoplast aquaporin affecting water permeability and cell turgor.

-*PpeS6PDH* is expressed in dormant buds and down-regulated in dormancy released buds concomitantly with an increase in H3K27me3 modification.

-*PpeS6PDH* codifies a sorbitol-6-phosphate dehydrogenase involved in sorbitol synthesis. This correlates with sorbitol accumulation in dormant buds, and has prompted us to postulate a role of *PpeS6PDH* and sorbitol in protection against cold and hydric stresses.

-*PpeDAM6*, belonging to the family of 6 tandemly arrayed *DAM* genes, has been found down-regulated in flower buds of peach following dormancy release, and differentially expressed in cultivars with different chilling requirements.

-*PpeBPC1* down-regulates *PpeDAM6* expression by binding to GAGA motifs present in an intronic regulatory region. This repression could be mediated by direct control of chromatin structure.

-The over-expression of *PpeDAM6* in *Arabidopsis* modifies flowering development producing abnormal flowers with vegetative traits, according to 35S::*AtSVP* phenotype. This atypical phenotype could be mediated by PpeDAM6 interaction with floral organ identity proteins or flowering regulators.

-*PpeDAM6* over-expression in transgenic plum impairs growth and meristem development. This alteration could be due to changes in the hormone homeostasis. These results suggest that *PpeDAM6* works as a central regulator of dormancy controlling various hormone pathways during dormancy in peach. In addition, we propose the GA synthesis and response genes *PpeGA20OX2*-like and *PpeGAST*-like as putative direct transcriptional targets of *PpeDAM6*.

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SUPPLEMENTARY MATERIAL

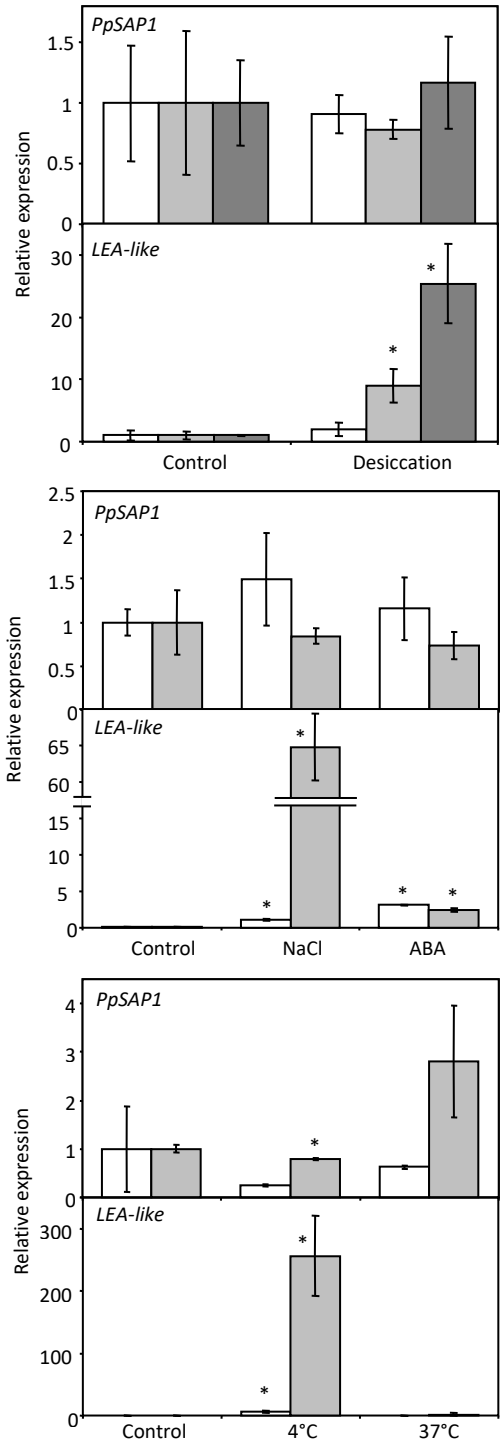
PpSAP1
LEA-like
Relative expression
Control
Desiccation
PpSAP1
LEA-like
Relative expression
Control
NaCl
ABA
PpSAP1
LEA-like
Relative expression
Control
4°C
37°C

Figure S1. *PpSAP1* gene expression in detached leaves and leaf discs under abiotic stresses. In a, detached leaves were desiccated for one (white bars), three (light grey bars) and seven days (dark grey bars). In b and c, leaf discs were treated for four (white bars) and 24 hours (grey bars). An expression value of one is assigned to the control. *LEA*-like gene (ppa008651m) expression is included as a positive control of stress response. An asterisk indicates significant difference with the control with a confidence level of 95 %.

ppa005503m

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ppa009116m

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ppa005507m

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ppa007117m

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Figure S2. Sequence of yeast two-hybrid clones.

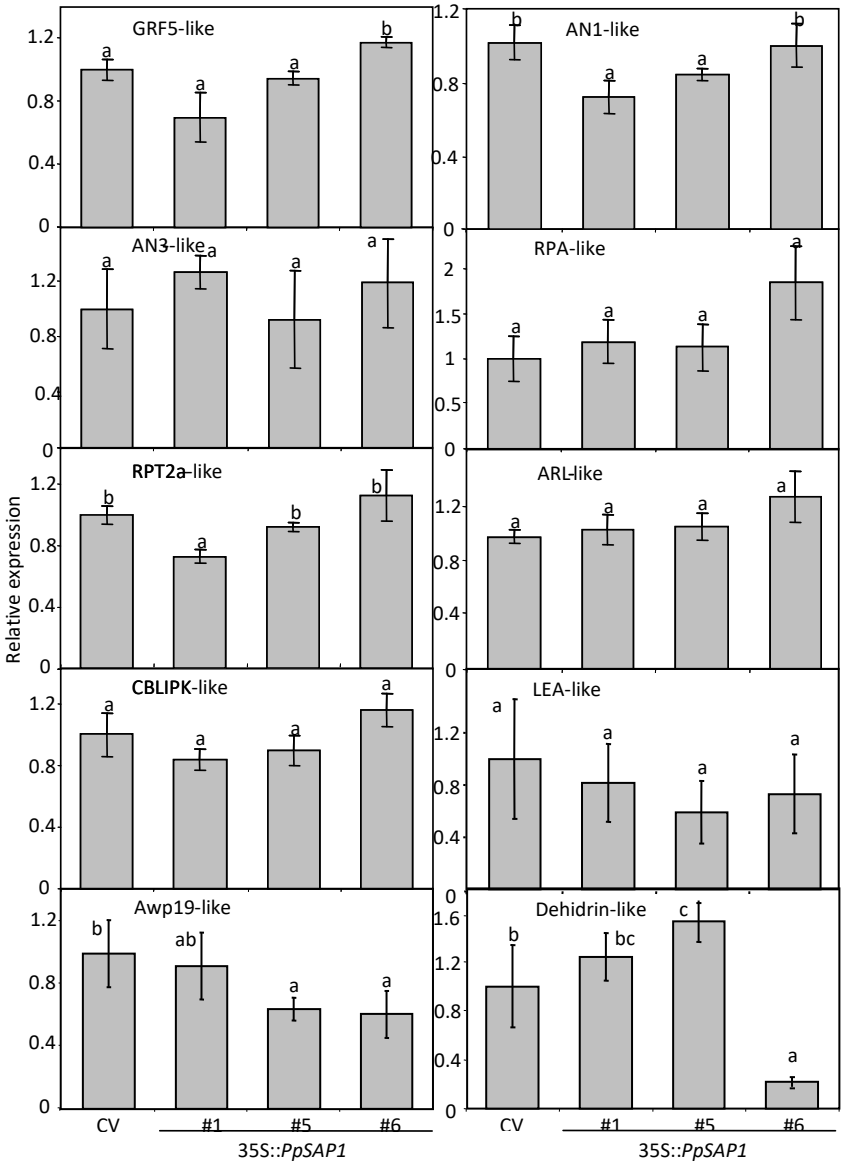
'Claudia Verde'

35S::*PpSAP1*#1

35S:: *PpSAP1* #5

35S:: *PpSAP1* #6

Figure S3. Drought stressed plants for seven days. Two month old plants of control 'Claudia Verde' and transgenic lines 35S::*PpSAP1 #1*, *#5* and *#6* are shown. scale bar, 5 cm.



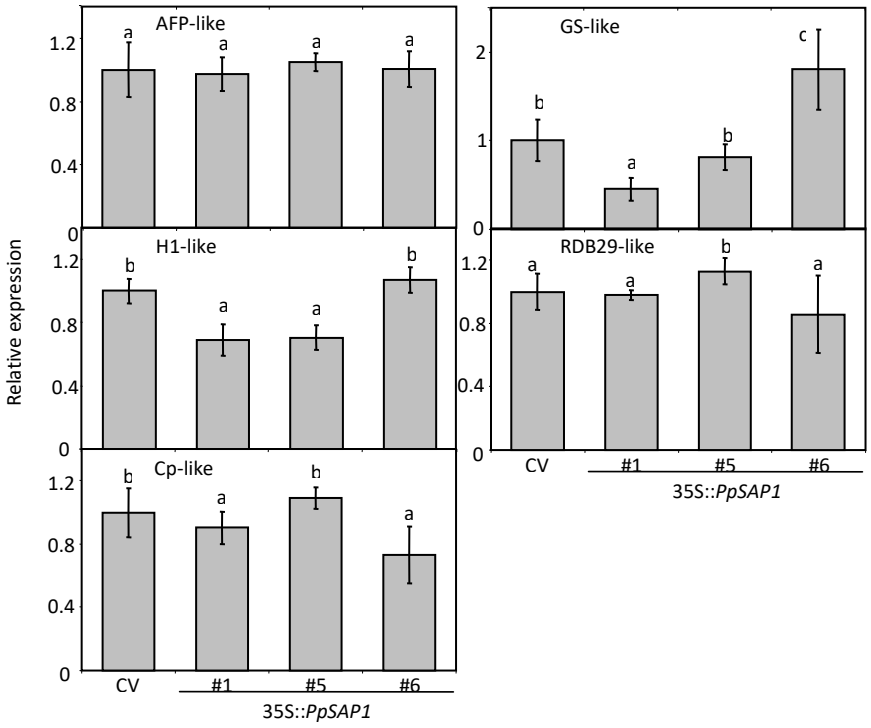


Figure S4. Expression of several genes assayed in transgenic plum overexpressing *PpSAP1*. An expression value of one is assigned to the control 'Claudia Verde' (CV). Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Letters (a-b) indicate significant difference between samples.

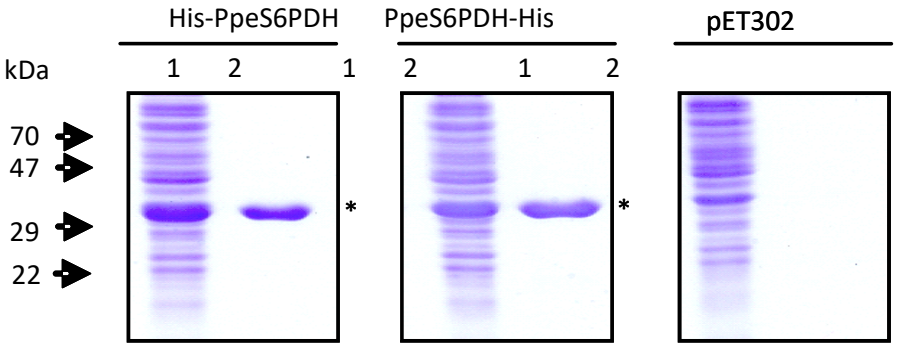
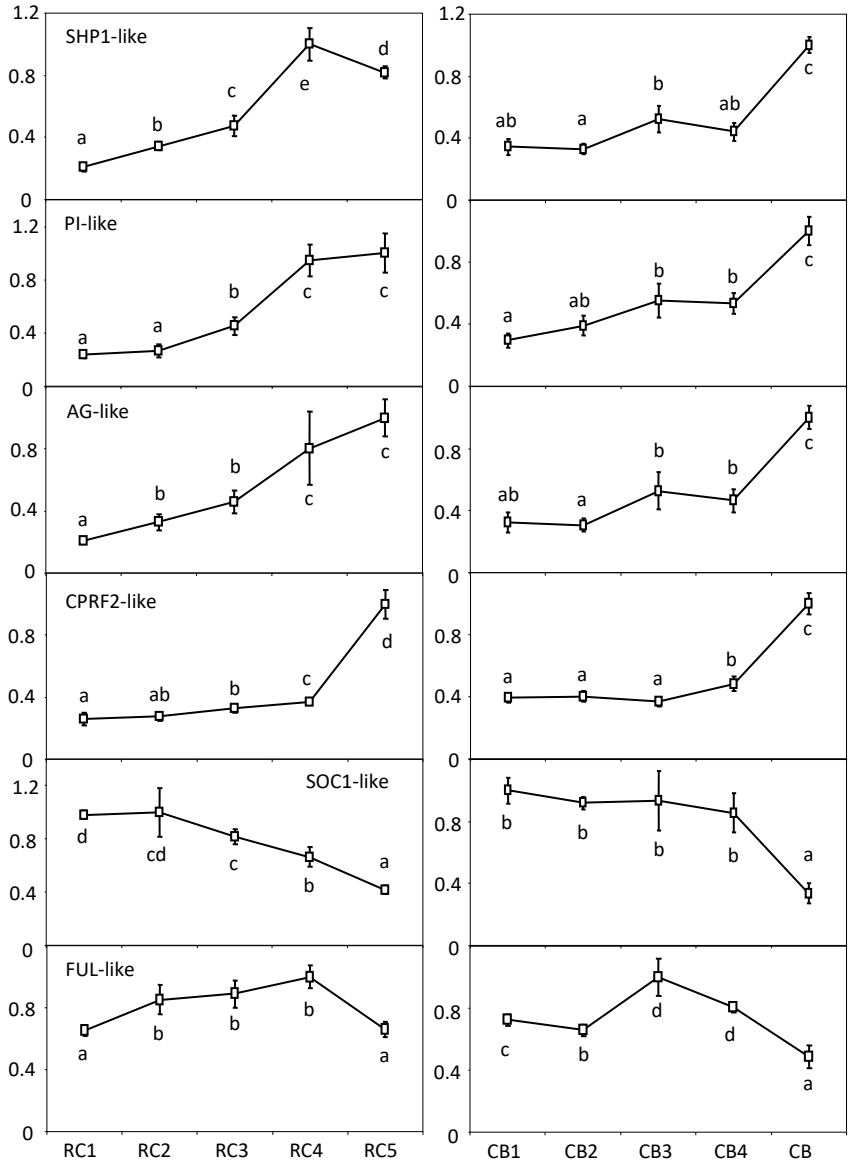


Figure S5 Expression and purification of recombinant protein. Total protein extracts (1) and affinity purified solutions (2) of BL21 (DE3) cells expressing PpeS6PDH His-tagged at the N-terminus (His- PpeS6PDH) and C-terminus (PpeS6PDH-His), and with the control plasmid pET302. Proteins were separated by SDS-PAGE and stained using coomassie brilliant blue R-250. The position of molecular weight markers is shown on the left. A band with the expected molecular weight is labelled with an asterisk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AG-like
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SOC1-like
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RC5
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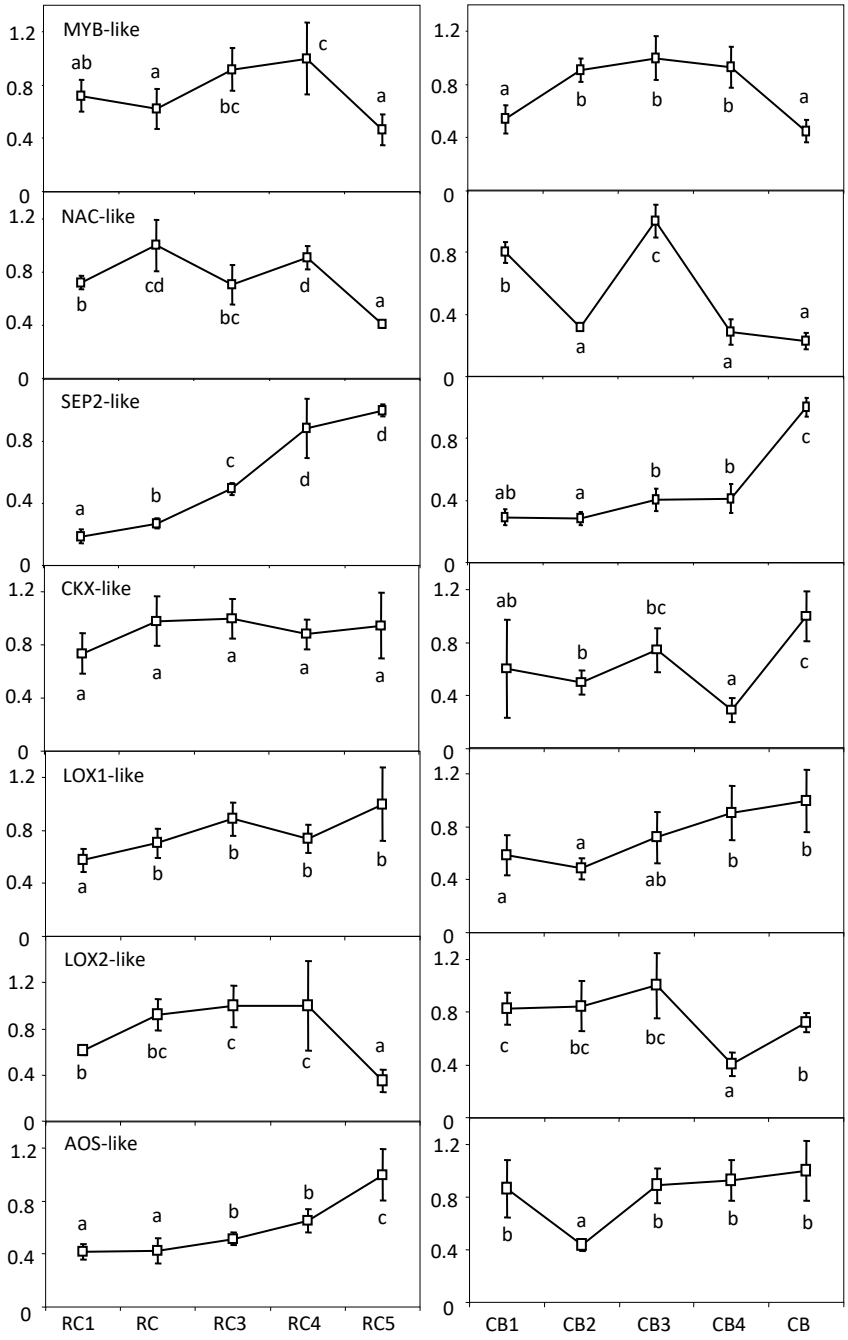
CB4
CB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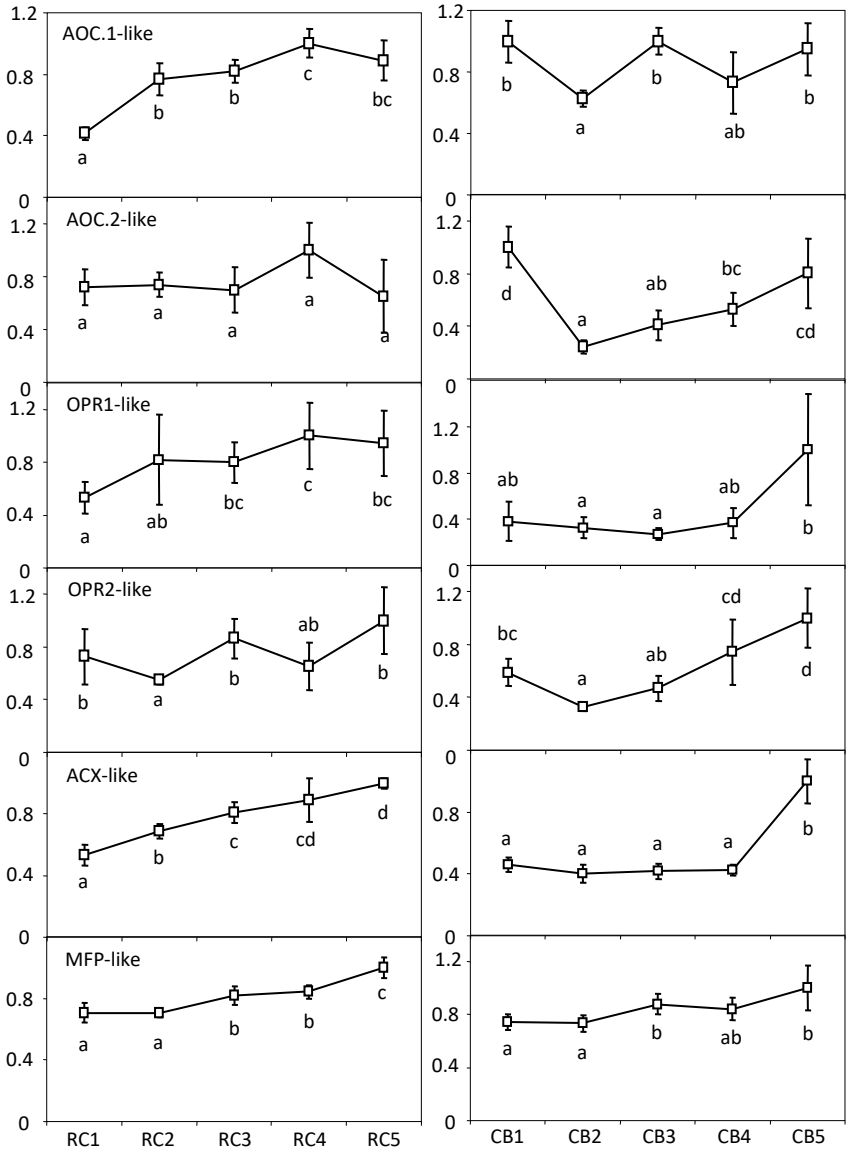
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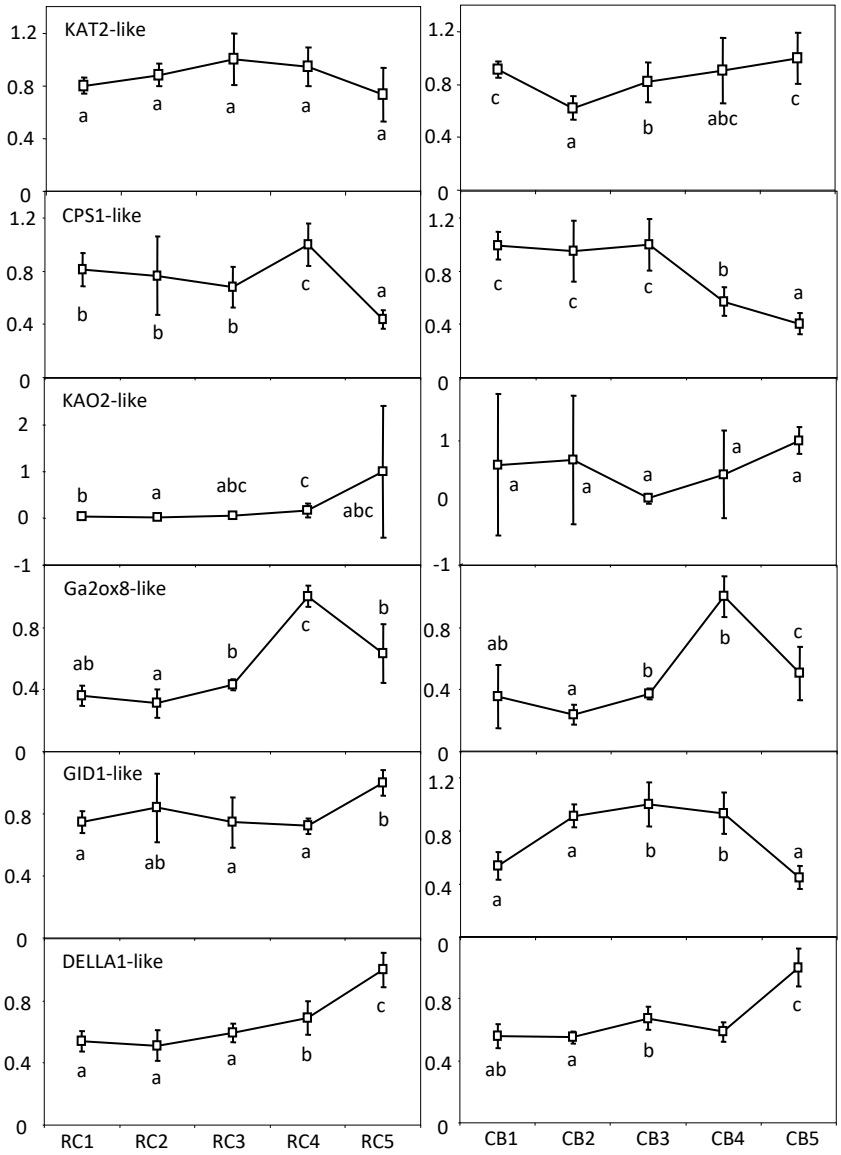

Figure S6. Relative expression of Y2H positives and DEUs in 35S::PpeDAM6 lines related to hormone pathways along flower bud development in peach. The relative expression of *PpeDAM6* (dark square), *GAST1*-like (white rhomb) and *GA20ox2*-like genes (white triangle) was measured along bud development in cultivars 'Red Candem' (RC) and 'Crimson Baby' (CB). Bud sample code used in Fig. 21 also applies to this figure. SAND-like gene was used as

reference gene. An expression value of one is assigned to the highest value per gene. Data are means from three biological samples with two technical replicates each, with error bars representing standard deviation. Different letters (a–e) indicate significant difference between samples for each gene, at a confidence level of 95%.

Table S1. Primers used in *PpSAP1* study.

	Forward	**Reverse**
qRT-PCR in peach		
PpSAP1	ACACAGGCTTCCTCTACTCCATCTTT	GAACCCTCATTCCGAGACATTTATCAG
LEA-like	TCATCTTCCGCTGCCTTTGTAGCCT	GACACTGCCAAGAACACCAAGGACA
Tubulin-like	CAGATGCCCAGTGATGCCTCAG	TGCTTGCCTGATCCAGTCTCAC
AGL26-like	ACCACCTGAAGTCCTCCAAGATTG	GCTTCATACAAAGCAATGCCAACAC
SAND-like	TCGTGGGTACCAGGAAAACGACAT	CCTGCTAGCTTGTGTTCATCTCCA
Actin-like	CTTCTTACTGAGGCACCCCTGAAT	AGCATAGAGGGAGAGAACTGCTTG
qRT-PCR in plum		
PpSAP1	TGATCTCAGAGGAGGACCTGCATAT	CAAGTCCTTGTGGCATTTGGAACAC
Plum *SAP1*-like	ACACAGGCTTCCTCTACTCCATCTTT	GAACCCTCATTCCGAGACATTTATCAG
PpSAP1 + plum *SAP1*-like	AACGTGTTGGTCTAACCGGCTTCAAT	TTGTCCAGCTTTTCTGCCTTCACAAC
CBLIPK-like	ACCCCAATGTGGTTCAGCTCTTTGA	AGTAATCAACAGCCCCAACCAACTG
TIP-like	AGTTGTTGCTTGCTTGCTACTCAAG	GCACCAACAATCAAACCAATTGCGA
GRF1-like	GGGGTTCTATCCATTTGGGATTCTC	TTTTTGGTCAGGAACGGCATCTCTC
GRF5-like	GGAGGTGCAGAAGAACAGATGGTAA	TTGAACGGTTTCTGCCTCTGTGCAT
RPA-like	GGGTTGCTAACAAGCAGTTTAAGAC	GGGACCTAACATATCAACTGGAACA
AN-like	AAAGCCTGGTGAGCTGTTGAAGGAT	AGTATCTAGGAACCAACCACCACCT
AN3-like	ATGTACCTGGCTGCCATTGCTGATT	GCTTGTTGCTGCATGTAATGTGCTC
TOR-like	GCAGTACCAAAGAAGATTGGGCAGA	GCAAATAACTCGCGCCCAACAAAT
ARL-like	TCGTCGAACACTATCACACGGTAG	CAACAGAGTCGCCGTGAGACATATA
RPT2a-like	GTGGCAAACTCAACATCTGCAACT	GTGAGAGGTCATCAGCAACCCTAA
AWPM19-like	CCCAGCCAATATGGCGAATATCAGAA	CATAGTGAGCAGCAGTAAGTTTGTGCT
Dehydrin-like	GTACTCTCATGACACCCACAAAACTAC	CCCGGCCCCACCGTAAGCTCCAGTT
AFP-like	TTCCGTTGGTGGTGGAGTGGATGCA	TTACTAGCAGGGCTTCTTGCTTCAC
H1-3-like	AAACCGCTGCTCATCCTCCATACT	TGCTTCTCCTCCATGTACTTGGCT
RD29B-like	ATCTGCTAAGAACGTCGTCGCTTC	GGCTTTGCTGTAACCTCCTGATGA

ATCP1-like	AAGGTTATGGACAAGGACGGGGAT	CATGGCCTTGATGTCTTCATCAGTG
GOLS2-like	TGACCACCTGTTTGACTACCCAGA	GCTGGCAGTACCCAATCTTGTACT
Two-hybrid cloning		
	CCAGAATTCATGGAGCACAACGAGACAG	GCCGGATCCTCAGATTTTGTCCAGCTTTTC
pROK2 cloning		
	CAGTCTAGATGGAGGAGCAGAAGCTGATCTC	GCCGGATCCTCAGATTTTGTCCAGCTTTTC

Table S2. List of genes tested by qRT-PCR in transgenic plum and peach.

Name	Rice or *Arabidopsis* gene/protein	Peach putative ortholog	Protein similarity	Reference
CBLIPK-like	LOC_Os01g10890	ppa005365m	CBL-interacting protein kinase	(1)
TIP-like	LOC_Os01g74450	ppa010364m	Tonoplast intrinsic protein1-3	(1)
GRF1-like	LOC_Os03g51970	ppa019623m	Growth-regulating factor 1	(1)
GRF5-like	LOC_Os02g53690	ppa017593m	Growth-regulating factor 5	(1)
RPA-like	LOC_Os03g11540	ppa003038m	Replication protein A subunit B	(1)
AN-like	AT1G01510	ppa003091m	Angustifolia	(2)
AN3-like	AT5G28640	ppa011329m	Angustifolia3	(3)
TOR-like	AT1G50030	ppa000022m	Serine/threonine-protein kinase TOR	(4)
ARL-like	AT2G44080	ppa013582m	Argos-like	(5)
RPT2a-like	AT4G29040	Prupe.8G208900	26S proteasome regulatory subunit	(6)
LEA-like		ppa008651m	Late embryogenesis abundant	(7)
AWPM19-like		ppa012188m	AWPM19	(7)
Dehydrin-like		Prupe.7G161100	Dehydrin	(7)
AFP-like		ppa006974m	ABI5 binding protein	(7)
H1-3-like	AT2G18050	ppa011941m	Histone H1-3	(8)
RD29B-like	AT5G52300	ppa001989m	Responsive to desiccation 29B	(8)
ATCP1-like	AT5G49480	ppa012594m	NaCl-inducible calcium-binding protein	(9)
GOLS2-like	AT1G56600	ppa008294m	Galactinol synthase 2	(9)

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Table S3. Stability index of reference genes for real-time experiments using three methods.

	NormFinder	BestKeeper	ΔCt
Tissues			
Tubulin-like	0.29	1.48	0.91
AGL26-like	0.41	1.91	1.17
SAND-like	0.30	1.61	0.92
Actin-like	0.37	1.39	1.02
Reproductive buds			
Tubulin-like	0.19	1.51	0.52
AGL26-like	0.33	1.73	0.64
SAND-like	0.18	1.21	0.46
Actin-like	0.28	1.15	0.58
Stress assays			
Tubulin-like	0.45	1.69	1.07
AGL26-like	0.25	1.25	0.75
SAND-like	0.14	1.10	0.60
Actin-like	0.29	1.23	0.76

Table S4. Primers used in *PpeS6PDH* study.

	Forward	Reverse
	qRT-PCR	
PpeS6PDH	CTACATGGCACGACATGGAAAAGAC	GGCGTAAGATAAGCAATCTCTGGTC
ppa008399m	CGTTTGGCGTTCTGTCTTTCTGCTT	AAAGTGACAACGGTGCTGCTGTGTA
Tubulin-like	CAGATGCCCAGTGATGCCTCAG	TGCTTGCCTGATCCAGTCTCAC
AGL26-like	ACCACCTGAAGTCCTCCAAGATTG	GCTTCATACAAAGCAATGCCAACAC
SAND-like	TCGTGGGTACCAGGAAAACGACAT	CCTGCTAGCTTGTGTTCATCTCCA
Actin-like	CTTCTTACTGAGGCACCCCTGAAT	AGCATAGAGGGAGAGAACTGCTTG
	Chromatin immunoprecipitation (ChIP)	
	GTCCACCATAACTCTCAACAATGGCT	TGGCACTTCTGAGCTCTTCTTTCT
	pET302 cloning (His- PpeS6PDH)	
	AACCTCGAGTCCACCATAACTCTCAACAATGG	TTGGGATCCTTATGCATAAACATCTACTCCCCAAG
	pET303 cloning (PpeS6PDH-His)	
	CGGTCTAGAAATATGTCCACCATAACTCTCAAC	AATCTCGAGTGCATAAACATCTACTCCCCAAG

Table S5. Positive clones of yeast experiments

Experiment	Name	Peach model	n° of positives
Y1H	PpeBPC1$_{(parcial)}$	Prupe.1G338500.2	1
Y1H	PpeBPC2$_{(parcial)}$	Prupe.1G369400.1	1
Y2H	TT2-like	Prupe.1G405400	1
Y2H	SHP1-like	Prupe.3G170600	3
Y2H	PI-like	Prupe.1G489400	5
Y2H	AG-like	Prupe.4G070500	2
Y2H	CPRF2-like	Prupe.6G217300	1
Y2H	SOC1-like	Prupe.2G287500	1
Y2H	FUL-like	Prupe.5G208500	1
Y2H	Myb-like	Prupe.1G415100	1
Y2H	NAC-like	Prupe.1G493100	1
Y2H	SEP2-like	Prupe.3G249400	1
Y2H	ATPsynthase-like	Prupe.6G296700	6

Table S6. Flowering time of the only transgenic *Arabidopsis* line with seeds. Flowering time was recorded as the rosette leaf number when the primary inflorescence stems apprears. An asterisk indicates significant difference with the control at a confidence level of 95%

Genotype	T1 Line	T2 Rossette leaves No. (n=10)
Columbia		8.1 ± 1.4
35S::*DAM6-c-myc#15*	1	6.9 ± 1.4*
	2	6.8 ± 1.0*
	3	7.5 ± 0.5
	4	6.5 ± 1.0
	5	7.0 ± 1.3*
	6	6.8 ± 1.1*
	7	7.5 ± 1.5
	8	6.9 ± 1.1
	9	7.3 ± 1.2

Table S7. Identified DEUs associated with various aspects of hormone homeostasis and response

Unigene	Gene Length (bp)	DAM2vsCV Regulation	DAM2vsCV log2ratio	DAM2vsCV q value	DAM1vsCV Regulation	DAM1vsCV log2ratio	DAM1vsCV q value	Hormone	Functional description	RT Abbreviature	Peach model
Cluster-22554.126056	987	DOWN	-1,9792	0,007862	DOWN	-1,6712	0,037699	Abscisic acid	Abscisic acid receptor PYL2	PYL2-like	Prupe.1G413500
Cluster-22554.89003	1056	DOWN	-Inf	1,41E-05	DOWN	-Inf	3,54E-05	Abscisic acid	Carotenoid 9,10(9',10')-cleavage dioxygenase	NCED-like	Prupe.2G014700
Cluster-22554.89006	1266	UP	Inf	3,13E-05	UP	Inf	6,09E-07	Abscisic acid	Carotenoid 9,10(9',10')-cleavage dioxygenase	NCED-like	Prupe.2G014700
Cluster-22554.80257	2434	UP	1,0533	0,014015	UP	1,0614	0,03862	Abscisic acid	Lycopene β-cyclase		Prupe.7G046100
Cluster-22554.70311	2631	DOWN	-Inf	4,97E-93	DOWN	-6,6717	1,62E-56	Abscisic acid	Phytoene synthase		Prupe.3G013200
Cluster-22554.132185	1206	DOWN	-2,1401	1,94E-06	DOWN	-1,4702	0,002018	Abscisic acid	violaxanthin de-epoxidase	VDE-like	Prupe.6G356100
Cluster-22554.87201	3131	UP	2,5515	5,33E-11	UP	2,4626	3,72E-09	Abscisic acid	Zeaxanthin epoxidase	ZEP.1-like	Prupe.7G133100
Cluster-22554.75800	1969	DOWN	-2,3665	4,05E-06	DOWN	-1,3709	0,017338	Abscisic acid	Zeaxanthin epoxidase	ZEP.2-like	Prupe.8G046000
Cluster-22554.119625	1868	UP	1,0088	0,007864	UP	1,0642	0,010517	Abscisic acid	Zeaxanthin epoxidase	ZEP.3-like	Prupe.8G167400
Cluster-22554.101651	422	DOWN	-3,6154	0,000784	DOWN	-4,2381	0,00023	Auxin	Auxin binding protein		
Cluster-22554.105259	386	DOWN	-4,3081	0,012204	DOWN	-4,3067	0,016379	Auxin	Auxin binding protein		
Cluster-22554.105260	1016	DOWN	-9,4396	1,71E-38	DOWN	-8,0055	5,82E-35	Auxin	Auxin binding protein		
Cluster-22554.105262	1003	DOWN	-2,06	0,000196	DOWN	-2,0259	0,000173	Auxin	Auxin binding protein		
Cluster-22554.105264	455	DOWN	-3,6272	0,00262	DOWN	-3,4419	0,005774	Auxin	Auxin binding protein		

Cluster-22554.105265	371	DOWN	-3,9393	3,59E-08	DOWN	-3,3228	5,01E-06	Auxin	Auxin binding protein
Cluster-22554.74947	589	DOWN	-5,1261	1,78E-06	DOWN	-2,6213	0,006793	Auxin	Auxin binding protein
Cluster-22554.96009	1340	DOWN	-1,3568	0,002047	DOWN	-1,3655	0,004075	Auxin	Auxin binding protein
Cluster-22554.130487	1755	DOWN	-2,106	6,65E-05	DOWN	-1,5759	0,010334	Auxin	Auxin efflux carrier component
Cluster-22554.130488	2186	DOWN	-2,8861	3,24E-17	DOWN	-2,6877	2,72E-13	Auxin	Auxin efflux carrier component
Cluster-22554.97890	1623	DOWN	-Inf	1,61E-12	DOWN	-Inf	3,01E-12	Auxin	Auxin efflux carrier component
Cluster-22554.114870	2741	DOWN	-1,3254	0,000501	DOWN	-1,6292	4,80E-05	Auxin	Auxin response factor
Cluster-22554.114872	2529	DOWN	-1,2551	0,000423	DOWN	-1,3956	0,000275	Auxin	Auxin response factor
Cluster-22554.114873	2848	DOWN	-1,3314	0,037127	DOWN	-2,6969	1,89E-05	Auxin	Auxin response factor
Cluster-22554.24503	608	UP	Inf	7,85E-05	UP	Inf	0,000342	Auxin	Auxin response factor
Cluster-22554.39203	5784	DOWN	-1,4489	3,91E-05	DOWN	-1,5349	4,37E-05	Auxin	Auxin response factor
Cluster-22554.39204	5865	UP	7,6839	1,42E-54	UP	7,6433	7,70E-49	Auxin	Auxin response factor
Cluster-22554.47139	1431	DOWN	-1,3684	0,002686	DOWN	-1,5432	0,00223	Auxin	Auxin response factor
Cluster-22554.84396	3450	DOWN	-1,2556	0,00111	DOWN	-1,1758	0,006525	Auxin	Auxin response factor
Cluster-22554.85681	777	UP	Inf	4,21E-06	UP	Inf	1,03E-05	Auxin	Auxin response factor
Cluster-22554.90976	1793	DOWN	-1,9782	1,40E-07	DOWN	-1,2632	0,004564	Auxin	Auxin response factor
Cluster-22554.96517	2676	DOWN	-1,1514	0,035668	DOWN	-2,1562	6,03E-05	Auxin	Auxin response factor
Cluster-22554.64900	715	DOWN	-Inf	5,76E-27	DOWN	-Inf	8,33E-25	Auxin	Auxin response factor
Cluster-24036.1	866	DOWN	-3,0372	2,03E-05	DOWN	-2,0657	0,003571	Auxin	Auxin responsive protein
Cluster-22554.138336	1740	DOWN	-2,8979	1,18E-07	DOWN	-2,6768	3,29E-06	Auxin	Auxin transport protein
Cluster-22554.26687	1870	DOWN	-5,7443	1,68E-15	DOWN	-Inf	8,44E-19	Auxin	Auxin transport protein

Cluster-22554.133366	3946	DOWN	-5,3382	1,27E-11	DOWN	-Inf	4,35E-18	Auxin	Auxin transporter
Cluster-22554.133369	2925	DOWN	-4,7629	0,000397	DOWN	-4,9412	0,000319	Auxin	Auxin transporter
Cluster-22554.133370	1771	DOWN	-1,3815	0,001494	DOWN	-1,2072	0,015386	Auxin	Auxin transporter
Cluster-22554.27794	2622	DOWN	-0,95603	0,022587	DOWN	-1,0886	0,020983	Auxin	Auxin transporter
Cluster-22554.51058	1385	DOWN	-1,27	0,00148	DOWN	-1,1626	0,010855	Auxin	Auxin transporter
Cluster-22554.133364	704	DOWN	-Inf	3,77E-06	DOWN	-Inf	1,04E-05	Auxin	Auxin transporter-like protein 2
Cluster-22554.26435	719	UP	1,822	2,71E-05	UP	1,9211	0,000118	Auxin	Auxin-Induced in root cultures protein
Cluster-22554.52231	621	UP	1,8158	0,003926	UP	1,9065	0,002844	Auxin	Auxin-Induced in root cultures protein
Cluster-22554.52234	678	DOWN	-3,7318	9,24E-09	DOWN	-3,4237	4,32E-07	Auxin	Auxin-Induced in root cultures protein
Cluster-22554.101657	593	DOWN	-1,8433	4,23E-06	DOWN	-1,4735	0,00097	Auxin	Auxin-Induced protein
Cluster-22554.27105	2608	DOWN	-Inf	0,016575	DOWN	-Inf	0,02117	Auxin	Auxin-Induced protein
Cluster-22554.60052	1195	DOWN	-4,7756	1,60E-05	DOWN	-2,1763	0,046231	Auxin	Auxin-Induced protein
Cluster-22554.60053	1110	DOWN	-3,2244	1,28E-14	DOWN	-3,1708	1,15E-12	Auxin	Auxin-Induced protein
Cluster-22554.70903	285	DOWN	-1,6587	0,000477	DOWN	-1,6995	0,000919	Auxin	Auxin-Induced protein
Cluster-22554.7201	871	DOWN	-2,1947	0,002765	DOWN	-1,9018	0,006764	Auxin	Auxin-Induced protein
Cluster-22554.72679	396	DOWN	-1,5648	0,003243	DOWN	-1,6275	0,004029	Auxin	Auxin-Induced protein
Cluster-22554.72683	449	DOWN	-2,1339	1,34E-09	DOWN	-1,8279	1,68E-06	Auxin	Auxin-Induced protein
Cluster-22554.72684	888	DOWN	-1,9651	1,45E-07	DOWN	-1,7177	2,63E-06	Auxin	Auxin-Induced protein
Cluster-22554.81470	1718	DOWN	-1,5622	1,36E-06	DOWN	-1,4034	0,000109	Auxin	Auxin-Induced protein
Cluster-22554.90422	1604	DOWN	-1,8152	1,50E-05	DOWN	-1,6246	4,66E-05	Auxin	Auxin-Induced protein
Cluster-22554.83208	909	DOWN	-Inf	0,000215	DOWN	-5,1534	0,00274	Auxin	Auxin-repressed 12.5 kDa protein

Cluster-22554.95337	1472	DOWN	-3,0077	0,001357	DOWN	-3,0028	0,001551	Auxin	Auxin-repressed 12.5 kDa protein
Cluster-15465.0	778	DOWN	-5,1251	0,00117	DOWN	-3,0441	0,04424	Auxin	Auxin-responsive protein
Cluster-22554.101644	585	DOWN	-3,3659	2,20E-07	DOWN	-2,4434	0,000301	Auxin	Auxin-responsive protein
Cluster-22554.104270	513	DOWN	-3,6349	2,56E-10	DOWN	-3,1783	1,24E-07	Auxin	Auxin-responsive protein
Cluster-22554.109367	765	DOWN	-Inf	4,87E-07	DOWN	-2,9084	0,008328	Auxin	Auxin-responsive protein
Cluster-22554.115322	625	DOWN	-3,3722	1,98E-08	DOWN	-2,4337	1,43E-05	Auxin	Auxin-responsive protein
Cluster-22554.130967	694	DOWN	-4,3243	3,00E-15	DOWN	-3,3714	2,56E-11	Auxin	Auxin-responsive protein
Cluster-22554.130968	761	DOWN	-1,7558	0,002875	DOWN	-1,3436	0,048817	Auxin	Auxin-responsive protein
Cluster-22554.131160	1107	DOWN	-1,1742	0,004301	DOWN	-0,99176	0,043212	Auxin	Auxin-responsive protein
Cluster-22554.148186	1187	UP	3,4258	0,032379	UP	3,2739	0,017218	Auxin	Auxin-responsive protein
Cluster-22554.33785	1387	DOWN	-1,036	0,003964	DOWN	-0,9069	0,036509	Auxin	Auxin-responsive protein
Cluster-22554.33787	1375	UP	0,85365	0,044732	UP	1,1977	0,008662	Auxin	Auxin-responsive protein
Cluster-22554.55335	1016	DOWN	-1,6159	4,77E-05	DOWN	-1,3497	0,004312	Auxin	Auxin-responsive protein
Cluster-22554.55338	1133	DOWN	-3,077	4,10E-10	DOWN	-1,5978	0,002869	Auxin	Auxin-responsive protein
Cluster-22554.6079	811	DOWN	-4,08	0,001242	DOWN	-2,9924	0,019809	Auxin	Auxin-responsive protein

Cluster-22554.72678	546	DOWN	-2,7344	0,000403	DOWN	-2,0201	0,014398	Auxin	Auxin-responsive protein
Cluster-22554.72685	2224	DOWN	-Inf	0,011748	DOWN	-Inf	0,015521	Auxin	Auxin-responsive protein
Cluster-22554.78125	1247	DOWN	-1,4443	5,89E-06	DOWN	-1,1836	0,001393	Auxin	Auxin-responsive protein
Cluster-22554.88296	1789	UP	3,0203	1,12E-07	UP	2,6859	1,23E-06	Auxin	Auxin-responsive protein
Cluster-28811.0	1133	DOWN	-2,4203	0,004104	DOWN	-4,329	8,11E-06	Auxin	Auxin-responsive protein
Cluster-22554.122769	1704	UP	2,3315	3,44E-06	UP	2,1127	0,000182	Auxin	IAA-amino acid hydrolase
Cluster-22554.24681	1083	UP	1,25	0,004755	UP	1,2286	0,012911	Auxin	IAA-amino acid hydrolase
Cluster-22554.98875	2546	DOWN	-7,0193	5,92E-30	DOWN	-Inf	3,89E-32	Auxin	IAA-amino acid hydrolase
Cluster-19520.0	641	DOWN	-4,0749	0,001748	DOWN	-3,8695	0,003944	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.101648	978	DOWN	-1,1999	0,000559	DOWN	-0,97028	0,018338	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.101652	840	DOWN	-2,4154	0,004777	DOWN	-2,3042	0,007236	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.101653	794	DOWN	-4,6132	4,56E-16	DOWN	-4,0725	6,95E-14	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.101656	707	DOWN	-4,0641	2,24E-05	DOWN	-3,5566	0,000191	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.101659	965	DOWN	-3,4798	1,28E-24	DOWN	-3,1671	1,14E-18	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.141308	2133	UP	Inf	0,000114	UP	Inf	9,48E-09	Auxin	Indole-3-acetic acid-Induced protein

Cluster-22554.141309	1979	UP	2,7734	0,0021	UP	2,8721	0,001796	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.21621	1360	DOWN	-1,2013	0,001656	DOWN	-1,2042	0,004751	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.4463	2037	UP	3,6631	1,42E-13	UP	3,5608	1,82E-11	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.49356	809	DOWN	-3,4658	3,49E-06	DOWN	-2,3202	0,003732	Auxin	Indole-3-acetic acid-Induced protein
Cluster-26328.0	2193	UP	4,4668	0,000484	UP	5,0915	3,18E-05	Auxin	Indole-3-acetic acid-Induced protein
Cluster-22554.70989	1891	DOWN	-4,7046	4,39E-12	DOWN	-4,7489	3,55E-12	Auxin	receptor-like protein kinase
Cluster-22554.36737	2214	DOWN	-0,88332	0,016119	DOWN	-0,88873	0,031556	Auxin	Transport inhibitor response
Cluster-22554.45515	1365	UP	2,0231	0,008302	UP	2,9096	0,007224	Auxin	Tryptophan aminotransferase-related protein
Cluster-22554.687	1860	UP	Inf	3,66E-06	UP	Inf	7,03E-07	Auxin	Tryptophan aminotransferase-related protein
Cluster-22554.689	2113	UP	Inf	3,00E-10	UP	Inf	0,040252	Auxin	Tryptophan aminotransferase-related protein
Cluster-22554.690	2086	UP	5,1733	0,000567	UP	5,5334	0,000991	Auxin	Tryptophan aminotransferase-related protein
Cluster-27305.7	1152	DOWN	-2,7305	0,00309	DOWN	-2,9074	0,003302	Auxin	Tryptophan aminotransferase-related protein

Cluster-22554.61360	1501	UP	1,2801	0,003526	UP	0,92352	0,035004	Cytoquinin	Adenylate isopentenyltransferase		
Cluster-22554.19990	1780	UP	2,0998	0,000401	UP	2,2574	0,0046	Cytoquinin	Cytokinin dehydrogenase	CKX-like	Prupe.1G373300
Cluster-22554.35929	2433	UP	1,8674	1,76E-06	UP	1,8633	0,018383	Cytoquinin	Cytokinin dehydrogenase	CKX-like	Prupe.1G373300
Cluster-22554.57997	1450	UP	1,413	0,000164	UP	1,0342	0,028995	Cytoquinin	Cytokinin dehydrogenase		Prupe.1G404300
Cluster-22554.57998	2206	UP	1,6931	1,10E-06	UP	1,3319	0,001067	Cytoquinin	Cytokinin dehydrogenase		Prupe.1G404300
Cluster-22554.74702	1627	UP	1,857	1,97E-07	UP	1,4353	0,000652	Cytoquinin	Cytokinin dehydrogenase		Prupe.1G404300
Cluster-22554.114841	1064	DOWN	-2,7579	1,66E-07	DOWN	-2,2279	9,10E-06	Cytoquinin	Cytokinin riboside 5'-monophosphate phosphoribohydrolase		
Cluster-22554.127113	1384	DOWN	-1,0631	0,04643	DOWN	-1,1147	0,04805	Cytoquinin	Cytokinin riboside 5'-monophosphate phosphoribohydrolase		
Cluster-22554.13145	1445	UP	Inf	2,37E-06	UP	Inf	4,83E-05	Cytoquinin	Cytokinin riboside 5'-monophosphate phosphoribohydrolase		
Cluster-22554.17424	2602	UP	8,1547	1,95E-31	UP	8,6724	3,21E-08	Cytoquinin	Cytokinin riboside 5'-monophosphate phosphoribohydrolase		
Cluster-24769.1	1028	DOWN	-3,3379	1,08E-06	DOWN	-2,2912	0,000382	Cytoquinin	Cytokinin riboside 5'-monophosphate phosphoribohydrolase		
Cluster-22554.103832	3278	DOWN	-1,4135	0,000169	DOWN	-1,2321	0,002936	Ethylene	Aminotransferase		
Cluster-22554.118619	3497	UP	0,9585	0,048559	UP	1,2899	0,004592	Ethylene	Aminotransferase		
Cluster-22554.57801	3413	DOWN	-1,5593	2,35E-05	DOWN	-1,2752	0,002177	Ethylene	Aminotransferase		

Cluster-22554.57803	3204	UP	5,4923	4,23E-33	UP	5,3147	1,14E-27	Ethylene	Aminotransferase
Cluster-20190.0	1189	DOWN	-3,7291	0,000799	DOWN	-4,9772	4,80E-05	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.118371	535	UP	1,1154	0,026693	UP	1,2595	0,013515	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.122286	4325	UP	4,1189	4,42E-11	UP	4,3038	6,20E-12	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.136075	1117	UP	5,9864	0,017591	UP	6,3809	2,26E-06	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.141265	2729	UP	Inf	3,89E-06	UP	Inf	0,019816	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.19433	414	UP	Inf	0,001492	UP	Inf	6,38E-05	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.26041	1977	UP	6,4331	5,38E-06	UP	6,2966	5,91E-07	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.26682	1288	UP	Inf	0,000528	UP	Inf	0,000421	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.39671	1940	UP	1,7298	0,000248	UP	1,6271	0,000323	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.39673	2038	UP	1,2407	0,016303	UP	1,6216	4,25E-05	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.69866	858	DOWN	-2,7027	3,62E-11	DOWN	-1,4345	0,002043	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.77378	1679	DOWN	-2,3542	2,36E-14	DOWN	-1,2907	0,000428	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.8579	1696	UP	5,1354	3,34E-13	UP	5,506	1,13E-26	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.8580	1567	UP	9,9922	3,77E-22	UP	10,406	9,57E-20	Ethylene	1-aminocyclopropane-1-carboxylate oxidase
Cluster-22554.89351	644	UP	1,757	2,13E-06	UP	1,6975	2,68E-05	Ethylene	1-aminocyclopropane-1-carboxylate oxidase

Cluster-22554.89360	499	DOWN	-1,3377	0,000672	DOWN	-1,186	0,007973	Ethylene	1-aminocyclopropane-1-carboxylate oxidase		
Cluster-22554.35948	3429	UP	2,7204	0,001741	UP	2,5117	0,002301	Ethylene	Ethylene-overproduction protein		
Cluster-22554.87180	5608	UP	Inf	1,16E-69	UP	Inf	3,13E-24	Gibberellins	DELLA protein	DELLA-like	Prupe.3G162500
Cluster-13895.0	2529	DOWN	-Inf	0,001843	DOWN	-Inf	0,003314	Gibberellins	Ent-copalyl diphosphate synthase	CPS-like	Prupe.8G239900
Cluster-17541.0	1234	DOWN	-5,4364	0,000386	DOWN	-3,0079	0,029471	Gibberellins	Ent-kaurenoic acid oxidase	KAO-like	Prupe.5G041400
Cluster-22554.14055	1940	DOWN	-1,7836	0,007805	DOWN	-2,6358	0,000157	Gibberellins	Ent-kaurenoic acid oxidase		Prupe.2G109700
Cluster-28710.6	933	DOWN	-2,239	0,001656	DOWN	-3,0251	0,000208	Gibberellins	Gibberellin 20 oxidase 2	Ga20ox2-like	Prupe.2G150700
Cluster-28710.7	3015	DOWN	-3,1944	0,000454	DOWN	-2,4673	0,010522	Gibberellins	Gibberellin 20 oxidase 2	GA20ox2-like	Prupe.2G150700
Cluster-28710.8	2602	DOWN	-2,3253	8,60E-08	DOWN	-1,5659	0,000992	Gibberellins	Gibberellin 20 oxidase 2	GA20ox2-like	Prupe.2G150700
Cluster-28710.9	793	DOWN	-2,4228	0,007732	DOWN	-1,9732	0,049941	Gibberellins	Gibberellin 20 oxidase 2	GA20ox2-like	Prupe.2G150700
Cluster-22554.10017	1642	UP	Inf	7,15E-05	UP	Inf	5,25E-07	Gibberellins	Gibberellin 2-beta-dioxygenase 8	GA2ox8-like	Prupe.1G344000
Cluster-22554.144616	1814	UP	4,4414	0,001471	UP	3,9927	0,000244	Gibberellins	Gibberellin 2-beta-dioxygenase 8	GA2ox8-like	Prupe.1G344000
Cluster-22554.134519	334	UP	2,3763	0,000814	UP	2,2753	0,015597	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.134520	2438	UP	2,6165	0,000619	UP	2,4502	1,90E-05	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.134522	2998	UP	5,0125	2,53E-11	UP	4,8792	4,55E-10	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800

Cluster-22554.134523	2349	UP	2,3697	7,34E-07	UP	2,0719	4,24E-05	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.134525	2427	UP	Inf	6,90E-41	UP	Inf	1,46E-20	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.134526	2974	UP	Inf	4,00E-13	UP	Inf	7,45E-09	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.134527	3099	UP	Inf	0,015253	UP	Inf	0,020502	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.134529	3063	UP	Inf	5,11E-13	UP	Inf	7,24E-07	Gibberellins	Gibberellin receptor GID1B	GID1b-like	Prupe.8G249800
Cluster-22554.56226	341	DOWN	-Inf	0,001512	DOWN	-Inf	0,00276	Gibberellins	Gibberellin-regulated protein	GAST1-like	Prupe.4G257500
Cluster-22554.56227	1220	DOWN	-Inf	3,60E-12	DOWN	-5,0931	5,25E-08	Gibberellins	Gibberellin-regulated protein	GAST1-like	Prupe.4G257500
Cluster-22554.85674	1430	UP	1,4199	2,58E-05	UP	1,4955	3,62E-05	Jasmonic acid	12-oxophytodienoate reductase	OPR.1-like	Prupe.1G549000
Cluster-22554.85678	1312	UP	1,3235	0,000294	UP	1,7045	0,000198	Jasmonic acid	12-oxophytodienoate reductase	OPR.1-like	Prupe.1G549000
Cluster-22554.110632	1725	UP	1,1782	0,000613	UP	1,0492	0,007651	Jasmonic acid	12-oxophytodienoate reductase	OPR.2-like	Prupe.7G200800
Cluster-22554.110635	2276	UP	1,0788	0,010529	UP	1,0186	0,032126	Jasmonic acid	12-oxophytodienoate reductase	OPR.2-like	Prupe.7G200800
Cluster-22554.78185	858	UP	3,8847	0,042916	UP	4,0279	0,012439	Jasmonic acid	12-oxophytodienoate reductase		Prupe.1G548700
Cluster-22554.73792	1353	UP	Inf	6,18E-05	UP	Inf	0,030345	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.1-like	Prupe.1G003300
Cluster-22554.73793	546	UP	1,3019	7,48E-05	UP	0,8841	0,038308	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.1-like	Prupe.1G003300
Cluster-22554.73798	1621	UP	1,3711	0,002503	UP	1,3122	0,010011	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.1-like	Prupe.1G003300

Cluster-22554.73800	2024	UP	3,9826	1,08E-06	UP	4,1555	0,004605	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.1-like	Prupe.1G003300
Cluster-22554.113980	1551	UP	3,0911	0,004496	UP	3,0495	0,006714	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.66661	1822	UP	1,2296	0,000265	UP	1,0857	0,00458	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.74365	1899	UP	1,4063	1,79E-05	UP	1,2956	0,000364	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.74366	1892	UP	1,5269	0,027849	UP	1,5849	0,013036	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.74369	1904	UP	1,6472	0,000994	UP	1,3136	0,033797	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.74373	1395	UP	Inf	9,33E-32	UP	Inf	2,48E-27	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.93522	1946	UP	4,9442	5,09E-16	UP	5,345	2,90E-19	Jasmonic acid	3-ketoacyl-CoA thiolase 2	KAT.2-like	Prupe.8G206400
Cluster-22554.98924	1428	UP	1,7718	0,04422	UP	1,8469	0,010013	Jasmonic acid	Allene oxide cyclase	AOC.1-like	Prupe.1G306100
Cluster-22554.98913	1337	UP	1,4449	7,67E-06	UP	1,2276	0,001076	Jasmonic acid	Allene oxide cyclase	AOC.1-like	Prupe.1G306100
Cluster-22554.128014	887	UP	2,7947	2,24E-18	UP	2,8371	8,09E-06	Jasmonic acid	Allene oxide cyclase	AOC.2-like	Prupe.3G239900
Cluster-22554.136151	2037	UP	0,93547	0,012856	UP	1,1747	0,002292	Jasmonic acid	Allene oxide synthase	AOS-like	Prupe.1G386300
Cluster-22554.55584	350	UP	1,4597	0,000257	UP	1,2733	0,005831	Jasmonic acid	Allene oxide synthase	AOS-like	Prupe.1G386300
Cluster-20219.0	1335	DOWN	-3,5877	0,009851	DOWN	-3,8411	0,007534	Jasmonic acid	jasmonate O-methyltransferase		Prupe.1G375700
Cluster-22554.32274	306	UP	3,4307	3,27E-10	UP	3,084	1,02E-06	Jasmonic acid	jasmonate O-methyltransferase		Prupe.8G093500

Cluster-22554.37079	290	UP	3,4732	9,54E-10	UP	3,0856	1,54E-06	Jasmonic acid	jasmonate O-methyltransferase		Prupe.8G093600
Cluster-22554.76170	395	UP	2,9864	4,21E-06	UP	2,4082	0,003369	Jasmonic acid	jasmonate O-methyltransferase		Prupe.8G093600
Cluster-22554.79215	1510	UP	3,4146	1,40E-10	UP	3,1087	2,56E-10	Jasmonic acid	jasmonate O-methyltransferase		Prupe.8G093600
Cluster-22554.104348	2829	UP	1,2239	0,004396	UP	1,3838	0,001578	Jasmonic acid	Jasmonic acid-amido synthetase		Prupe.2G184100
Cluster-22554.86979	1779	DOWN	-1,5222	0,000185	DOWN	-1,4587	0,000262	Jasmonic acid	Jasmonic acid-amido synthetase		Prupe.3G233900
Cluster-22554.86982	3291	DOWN	-1,1248	0,009702	DOWN	-1,2208	0,002045	Jasmonic acid	Jasmonic acid-amido synthetase		Prupe.3G233900
Cluster-22554.86196	333	UP	1,4024	0,000145	UP	1,2179	0,004088	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1	LOX1-like	Prupe.2G005300
Cluster-22554.86201	2885	UP	1,3794	1,51E-05	UP	1,2917	0,000276	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1	LOX1-like	Prupe.2G005300
Cluster-22554.86206	2874	UP	2,3681	9,76E-15	UP	2,3515	9,91E-06	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1	LOX1-like	Prupe.2G005300
Cluster-22554.86213	3147	UP	1,6051	2,77E-07	UP	1,504	1,30E-05	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1	LOX1-like	Prupe.2G005300
Cluster-22554.88979	406	UP	1,5864	0,000104	UP	1,4158	0,000583	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1	LOX1-like	Prupe.2G005300
Cluster-22554.19621	3327	UP	Inf	4,43E-05	UP	Inf	0,001431	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1		Prupe.4G047800
Cluster-22554.19624	3609	UP	4,7036	1,42E-15	UP	4,7489	1,12E-16	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1		Prupe.4G047800
Cluster-22554.19625	3200	UP	Inf	1,23E-07	UP	Inf	0,000685	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1		Prupe.4G047800
Cluster-22554.19627	1374	UP	4,5419	2,02E-16	UP	4,2521	1,37E-08	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1		Prupe.4G047800

Cluster-22554.87976	3028	UP	0,87266	0,021454	UP	1,1845	0,001606	Jasmonic acid	Linoleate 13S-lipoxygenase 2-1		Prupe.3G039200
Cluster-22554.27253	1050	UP	4,9816	0,034439	UP	4,6837	0,021932	Jasmonic acid	Linoleate 9S-lipoxygenase 1	LOX2-like	Prupe.6G324100
Cluster-22554.106998	781	DOWN	-Inf	0,023755	DOWN	-Inf	0,035231	Jasmonic acid	Linoleate 9S-lipoxygenase 1		Prupe.6G324400
Cluster-22554.99449	1000	DOWN	-3,7642	0,0173	DOWN	-4,7668	0,009097	Jasmonic acid	Linoleate 9S-lipoxygenase 1		Prupe.6G324400
Cluster-22554.25672	1345	UP	3,1329	4,09E-05	UP	3,5491	1,39E-05	Jasmonic acid	Protein TIFY		
Cluster-22554.25674	1039	UP	2,6516	2,59E-07	UP	2,9267	0,000157	Jasmonic acid	Protein TIFY		
Cluster-22554.25675	1362	UP	3,1877	8,72E-08	UP	3,3667	0,049819	Jasmonic acid	Protein TIFY		
Cluster-22554.25677	1146	UP	Inf	0,000808	UP	Inf	0,001861	Jasmonic acid	Protein TIFY		
Cluster-22554.82324	570	UP	2,8321	0,000191	UP	2,42	0,008953	Jasmonic acid	Protein TIFY		
Cluster-22554.82531	2183	UP	2,3079	1,77E-13	UP	2,4066	1,04E-12	Jasmonic acid	Protein TIFY		
Cluster-22554.82532	2076	UP	3,3797	0,000162	UP	3,7126	1,11E-12	Jasmonic acid	Protein TIFY		
Cluster-22554.82533	1526	DOWN	-1,4419	1,02E-05	DOWN	-1,3435	0,000218	Jasmonic acid	Protein TIFY		
Cluster-22554.82534	2349	DOWN	-7,2399	1,18E-67	DOWN	-Inf	1,22E-79	Jasmonic acid	Protein TIFY		
Cluster-22554.82535	2063	UP	4,438	4,71E-12	UP	5,115	7,23E-18	Jasmonic acid	Protein TIFY		

Table S8. Primers used in *PpeDAM6* study

	Forward	Reverse
qRT-PCR		
DAM6	TACTGGACCTGCGTTTGTGGAGCC	TGTTGCAGCTGGTGGAGGTGGCAATT
DAM6 transgen	TGATCTCAGAGGAGGACCTGCATAT	GAGCTTGTCAGTAGCAGAGAAGATG
PdoDAM6-like	CCAAAACTTCAGACCGGGCTGAAA	GAGTAAACTTTCTTCCTTGTCCACTTC
DAM6 + *PdoDAM6*-like	TACTGGACCTGCGTTTGTGGAGCC	TGTTGCAGCTGGTGGAGGTGGCAATT
Tubulin-like	CAGATGCCCAGTGATGCCTCAG	TGCTTGCCTGATCCAGTCTCAC
SAND-like	TCGTGGGTACCAGGAAAACGACAT	CCTGCTAGCTTGTGTTCATCTCCA
Actin-like	CTTCTTACTGAGGCACCCCTGAAT	AGCATAGAGGGAGAGAACTGCTTG
AGL26-like	ACCACCTGAAGTCCTCCAAGATTG	GCTTCATACAAAGCAATGCCAACAC
PpeBpc1	GTGATCCCGCAGTCATGGTTAG	GCATAGTTAGGATTGGCAGGCAT
PpeBPC2	GATATGGGTGGCGGAGGTGAT	CACTTGTAGCACTGCCTCAGGA
PpeBPC3	GAAGGAACCAAATGCCCTAGTCATG	GTTCACAGCATTGTCCCGCATAT
LOX1-like	CCATCCTTCTCTCTCTTACCAAAATCCT	CAGATGATCCGAGCTCACCAGAA
LOX2-like	ACAGATCCTAACACTGAGAGCCAGC	CCATCAGGCAACTCTATACCTCCTTC
AOS-like	AAATGCCTTTGATGAAATCCGTCGTG	GACAAACCGATCCGCTACAAACTC
AOC1-like	GTGTATGAGATCAACGAGAGAGACAGAG	GTTTTGGATCAGAACACATAAGCCTGC
AOC2-like	GCATATCTCAGTTTAAGCCAGAAACCTGT	TGTCCTCGTAGGTCAAATAAGGTCCT
OPR1-like	AGCAATCAAAAACCAAAACACACAAAAGGT	AGGTTGAGCAGCCATGTCAGAG
OPR2-like	CCTAGACGCTTAGAAACCCCTGA	ATCAATGGCTGGTGAGATTCTGACA
ACX-like	GAAGTTTCACAGCCATGACAGTACCT	TCCTCAGGCGTCAATAGATCATCAAAC
MFP-like	AGTCAATCATGTCTGAAGAAGGGAAGAAG	GCTTATCTGTCCTGTGAAGAGAGCG
KAT1-like	TAGTTGAATCCCCAGATCCTTGTTCATC	TGGTATACAAACATTGGAGATCAAAACTGC
KAT2-like	TGCTGCCCTGTCTGCTTCAATAT	CACAATCACTACATCGTCTCCAAAAGC
CKX-like	TACTGTATTCCAACTTTTCGGCTTTTACCA	GACATAGTCGAACTTGTGGGTGGAG
CPS1-like		
KAO2-like		
GA20ox2-like	GTTTAGTCGATGAGGCATGCAGGA	GAGAGTTGCATGCCAAAGAACAAGT
GA2ox8-like	CGAACAATCTGGGACACCGAAAG	TACACATCATTGCTCCACGCCT
GAST1-like	AGAAGCCTTGTCTGTTTTTCTGCC	GGGTCTTCCAGTTGTTGTAGCAAG
GID1b-like	CAGCCAACAGTGCCATCTATGATAC	CATGAACCTTTGAGTCCTTCCCAC
DELLA1.like	GAGAGCAGGAGAAAGCGATTGAA	TGTATGGACGAGTCTAACGCCT
TT2-like		
SHP1-like	GCTGAAAATGAGAGGGCACAACAG	CTGTCTGGTCATGGCGAGAGTAAT
Pi-like	AGAGCACTGGAAGAGGAGCATAAG	CTGAACACGAAAGGCAAACGGT
AG-like	ATAGCTGAGAATGAGAGGAGCCAG	GCGTGAATTGTACTGATGATTGGGT
CPRF2-like	CAATCCCCGAAACCCACAATCTC	TTCCTCTCGTCCACCACAGAAA
SOC1-like	CGAAATGGGTTGTTGAAGAAAGCCT	CAGAGCTGGAGAACTCATATAGCCT

FUL-like	GCCCAAGAGATTTCTGTGCTTTGTG	TTTCCATGCAGGAGTCAGTGGAGTA
MYB-like	GCCACTTCAGCGTGTTCTCAG	GTGGTGGTTGCCTTTCTTATCGTG
NAC-like	CCAAGTGGAAGGAGTGGGAGAA	CGGCGACATCTGATTACTGCTCT
SEP2-like	TTCTCTGTGATGCTGAGGTTGCT	CTTGGCAGGTTTGTTGACTTCCAC
Two-hybrid cloning		
PpeBPC1	ACAGGATCCTCATGGATGATGATGCATTGAACA	AATCTCGAGCTACCTGATTGTGACGAACTTGT
PpeBPC2	AAAGGATCCAGATGGATGATAGTGGGCATC	CTTCTCGAGCTACTTGATTGTGATGTAGCGATTTG
PpeBPC3	TCAGAATTCATGCACTCAGCAGATAGCA	CATGGATCCTACTTGATCGTTATGTAGCGATTTG
SWN-like	CGCCATATGAGCAAAACAGGGATGGTGTC	AATGGATCCTCAGTGAGATTGGTGTTTCTTCGCT
LHP1-like	TCCCATATGAAAGTGAAGGGAGGAGGA	ATTGGATCCTTACAATGTAGAATTGTACCGGAGATG
SEUSS-like	TATGGATCCTTATGGTACCTTCGGGGC	TATGTCGACTCAAGGGGAATGTTTCCAATCA
DAM6	ACCGAATTCATGATGAGGGAGAAGATCAAG	AAAGGATCCCTAGGGAAGCCCCAGTTT
DAM6(1-537)	ACCGAATTCATGATGAGGGAGAAGATCAAG	GACGGATCCTATCCTCCGGATAACATCG
One hybrid cloning		
Reg1	CAAGAGCTCTTTTTCTGGACAGACCAAAAC	ACACTCGAGCTTGAGCTTGAATAATCAAAGAG
Reg2	ATAGAGCTCGTACCAGCACCCACCA	GCACTCGAGATATGTGATAGGTGGGAGAGGA
Reg2.1	ATAGAGCTCGTACCAGCACCCACCA	GTACTCGAGCACACACACACACACACAAG
Reg2.2	ATAGAGCTCGTACCAGCACCCACCA	AGGCTCGAGAATCTCAGATTCTCTCTCTCTCTC
Reg2.3	ATAGAGCTCGTACCAGCACCCACCA	TCGCTCGAGTCTCTCTCTCTCTCTCTAGAAG
Reg2.4	GTTGAGCTCTGTGTGTGTGCGAGAGAG	GCACTCGAGATATGTGATAGGTGGGAGAGGA
Reg2.5	GAGGAGCTCGAAGCTTCTAGAGAGAGAGAGAGA	GCACTCGAGATATGTGATAGGTGGGAGAGGA
Reg2.6	GCCGAGCTCAAAATCTTTTAGATAGTTATACTCTATTTCA	GCACTCGAGATATGTGATAGGTGGGAGAGGA
Reg2.7	CGCGAGCTCATCTGAGATTCAGTCATTTTGGTAGAAA	ACCCTCGAGAGAAGCTTCTATTTGCCTTTGACA
Reg2.1		
Luciferase assay cloning		
Pro.3-LUC	ATAGTCGACCCGGAGCTTCTACAATGAG	GGTGGTAACCCTCGAACTAGTTGCct
	GCAACTAGTTCGAGGGTTACCACCTT	TATCCATGGCGCTTTGTACCTTTCAATAACATC
pROK2 cloning		
	GCGTCTAGATGATGAGGGAGAAGATCAAGATCAAG	TATGGATCCTACAGGTCCTCCTCTGAGATCAGCTTCTG CTCGGGAAGCCCCAGTTTGAG

Table S9. Databases used to annotated the transcriptome assembled

Databases	Number of Unigenes	Percentage (%)
Annotated in NR	138945	73.94
Annotated in NT	155833	82.93
Annotated in KO	55565	29.57
Annotated in SwissProt	103133	54.88
Annotated in PFAM	90455	48.13
Annotated in GO	90657	48.24
Annotated in KOG	44446	23.65
Annotated in all Databases	25454	13.54
Annotated in at least one Database	162422	86.44
Total Unigenes	187901	100

Table S10. Version and parameters used of each software used in RNA-seq analysis

Analysis	Software	Version	Parameter	Remark
Assembly	Trinity	r20140413p1	min_kmer_cov:2, SS_lib_type:RF, others are by default)	-
Hierarchical Clustering	Corset	v1.05	-m 10	remove redundancy
Gene Functional Annotation	Diamond	v0.8.22	NR, Swiss-Prot: e-value = 1e-5;KOG/COG: e-value = 1e-3	NR, KOG/COG, Swiss-Prot
	KAAS	r140224	e-value = 1e-10	KEGG Annotation
	NCBI blast	v2.2.28+	e-value = 1e-5	NT Annotation
	hmmscan	HMMER 3	e-value = 0.01	Pfam Annotation
	blast2go	b2g4pipe_v2.5	e-value = 1.0E-6	GO Annotation
Mapping and Quantification	RSEM	v1.2.26	bowtie2 mismatch 0	mapping to Corset filtered transcriptome
Differential Expression Analysis	DESeq	1.10.1	padj<0.05	For sample with bio-replicate using DESeq, samples without bio-replicate using DEGSeq. EdgeR for specific conditions.
KEGG enrichment	KOBAS	v2.0.12	Corrected P-Value<0.05	