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Research

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

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Summary

- Bud dormancy release in many woody perennial plants responds to the seasonal accumulation of chilling stimulus. MADS-box transcription factors encoded by *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes in peach (*Prunus persica*) are implicated in this pathway, but other regulatory factors remain to be identified. In addition, the regulation of *DAM* gene expression is not well known at the molecular level.
- A microarray hybridization approach was performed to identify genes whose expression correlates with the bud dormancy-related behaviour in 10 different peach cultivars. Histone modifications in *DAM6* gene were investigated by chromatin immunoprecipitation in two different cultivars.
- The expression of *DAM4–DAM6* and several genes related to abscisic acid and drought stress response correlated with the dormancy behaviour of peach cultivars. The trimethylation of histone H3 at K27 in the *DAM6* promoter, coding region and the second large intron was preceded by a decrease in acetylated H3 and trimethylated H3K4 in the region of translation start, coinciding with repression of *DAM6* during dormancy release.
- Analysis of chromatin modifications reinforced the role of epigenetic mechanisms in *DAM6* regulation and bud dormancy release, and highlighted common features with the vernalization process in *Arabidopsis thaliana* and cereals.

Introduction

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

In *Arabidopsis thaliana*, the MADS-box transcription factor FLOWERING LOCUS C (FLC) prevents the flowering transition by repressing the floral integrator genes *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (Searle *et al.*, 2006). The *FLC* transcript decreases quantitatively during cold exposure by an epigenetic mechanism involving the synthesis of noncoding RNAs and the binding of the Polycomb group (PcG) proteins (De Lucia *et al.*, 2008; Swiezewski *et al.*, 2009; Heo & Sung, 2011). The PcG complexes ensure stable repression of *FLC* after subsequent cell divisions by means of chromatin modifications including trimethylation of histone H3 at lysine 27 (H3K27me3) (Doyle & Amasino, 2009). In cereals, vernalization is controlled by *VERNALIZATION1* (*VRN1*), a *FRUITFULL*-like MADS-box gene that, contrary to *FLC*, responds to cold by increasing its expression. Upregulation of *VRN1* during vernalization is associated with reduced H3K27me3 and increased histone H3 lysine 4 trimethylation (H3K4me3) levels at *VRN1*, a typical indication of transcriptionally active chromatin also found in *FLC*. These histone modifications suggest participation of PcG-like complexes in repression of *VRN1* before winter (Oliver *et al.*, 2009; Hemming & Trevaskis, 2011).

In peach (*Prunus persica*), a set of six tandemly repeated *SHORT VEGETATIVE PHASE*-like MADS-box genes, named *DORMANCY ASSOCIATED MADS-box1-6* (*DAM1-6*), have been found partially deleted in the *evergrowing* (*evg*) mutant showing nondormant behaviour (Bielenberg *et al.*, 2008). The expression of *DAM* genes is highly dependent on the establishment, maintenance and release of bud dormancy. Length of photoperiod

and chilling accumulation have been postulated as the major inputs conditioning seasonal fluctuations in the expression of these genes (Li *et al.*, 2009). *DAM1* and *DAM6* are upregulated during cessation of bud growth, following a change from a long- to a short-day photoperiod (Jiménez *et al.*, 2010a), and down-regulated during dormancy release in two different peach cultivars (Leida *et al.*, 2010). In a recent work, the expression of *DAM5* and *DAM6* have been found associated to the dormancy status of peach plants treated with prolonged low temperature and with the dormancy-breaking reagent cyanamide (Yamane *et al.*, 2011).

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

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

Materials and Methods

Plant material and growth conditions

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

Incubation of shoots for the determination of bud break and dormancy parameters was performed in a phytotron set at 25°C, with a 12 h : 12 h light : dark cycle and 70% humidity.

Measurement of flowering time, bud break and chilling requirement

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

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

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

Isolation of RNA and mRNA purification

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

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

Microarray hybridization

The poly(A)+ RNA (100 ng) was reverse transcribed, amplified and labelled with the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). Cy5 and Cy3 fluorescent dyes were coupled to the amino allyl modified RNA of each sample and a mix of the samples (for reference), respectively. Purified Cy5-labelled sample and Cy3-labelled reference (200 pmol each) were combined, diluted with water to a final volume of 500 μl, and concentrated to 40 μl in a microcon YM-30 filter (Millipore, Billerica, MA, USA). Half of the mixture (20 μl) was vacuum-concentrated until *c.* 4 μl, then heat-denatured for 2 min at 80°C, mixed with 20 μl of preheated hybridization buffer (5× saline-sodium citrate (SSC) (75 mM trisodium citrate pH 7.0, 0.75 M NaCl), 50% (v : v) formamide, 0.1% (w : v) sodium dodecyl sulphate (SDS), 0.1 mg ml^{-1} salmon sperm DNA), and finally applied to the microarray slide, which was previously incubated for at least 1 h at 42°C with prehybridization buffer (5× SSC, 0.1% (w : v) SDS, 1% (w : v) BSA). The microarray contained 2496 expressed sequence tags (ESTs) obtained by a subtraction procedure from

dormant and nondormant flower buds of peach, as described by Leida *et al.* (2010). Hybridization was performed overnight at 42°C. After hybridization, slides were washed twice at 42°C for 5 min in 2× SSC–0.1% (w : v) SDS, followed by two washes at room temperature for 5 min in 0.1× SSC–0.1% (w : v) SDS, then five washes at room temperature for 3 min in 0.1× SSC, and finally rinsed briefly with 0.01× SSC before drying by centrifugation at 20 ***g*** for 5 min.

Microarray data analysis

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

For statistical analysis of microarray data, the Gene Expression Profile Analysis Suite (GEPAS) package was employed (Montaner *et al.*, 2006; http://www.gepas.org/). Normalizations within and between arrays were Loess and Scale, respectively. Signal intensities proceeding from duplicated spots were averaged. Identification of ESTs correlating with the different dormancy variables was performed by Pearson's test. The *P*-value to control the false discovery rate was adjusted with the Benjamini and Hochberg method. Expressed sequence tags showing a *P*-value < 0.05 were selected for DNA sequencing with primers NP1 or NP2R (see the Supporting Information, Table S1) and expression analysis. From 160 ESTs correlating with the chilling requirement variable, 46 showed a higher signal in dormant buds (positive correlation) and 114 in dormancy released buds (negative correlation). With respect to the percentage of bud break, 201 ESTs were found, of which 30 were more abundant in dormant buds and 171 in dormancy released buds. Similarly, from 154 ESTs correlating with the flowering time variable, 58 had a higher expression in dormant buds and 96 in dormancy released buds. After removing the clones producing a bad sequence and the ones having an internal *Rsa*I site, which was indicative of a chimeric rearrangement during the subtraction procedure, 242 ESTs were identified. The accession numbers of these ESTs are listed in Table S2. To identify the genes or transcript models containing the positive ESTs, a BLASTN analysis (Altschul *et al.*, 1990) was performed on peach genome sequence database released by the International Peach Genome Initiative (IPGI). A BLASTP analysis of the deduced protein of the different genes or ESTs was made on the non-redundant protein sequence database, to find the closest annotated hits.

To identify the varieties with similar gene expression fingerprints we applied principal component analysis to the initial matrix of 2525 available data, considering the 10 varieties as individuals and the genes as variables. The analysis was performed using STATGRAPHICS 5.1 package for windows (Statpoint Technologies, Warrenton, VA, USA).

Real-time RT-PCR

One microgram of total RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) in a total volume of 20 μl. Two microlitres of a 40× diluted first-strand cDNA was used for each amplification reaction in a final volume of 20 μl. Quantitative real-time PCR was performed on a StepOnePlus Real-Time PCR System using the Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and primers shown in Table S1. The cycling protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C for denaturation, and 1 min at 60°C for annealing and extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve after the amplification and through size estimation of the amplified product by agarose electrophoresis. We used as reference a peach actin gene amplified with specific primers (Table S1). Relative expression was measured by the relative standard curve procedure. Results were the average of two independent biological replicates repeated twice.

Chromatin immunoprecipitation (ChIP) assays

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

Chromatin immunoprecipitation was performed according to Sandoval *et al.* (2004) and Ferres-Maso *et al.* (2009). The sonicated chromatin was centrifuged at 13 800 ***g*** for 10 min, and the supernatant diluted 10-fold with dilution buffer (16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 0.01% (w : v) SDS, 1.1% (v : v) Triton X-100, 1.2 mM EDTA, 0.5% (v : v) PIC). Aliquots of 600 μl of diluted chromatin were incubated overnight at 4°C on a rotating platform with Dynabeads-Protein G (Invitrogen) previously washed with PBS buffer (1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl, 5 mg ml^{-1} BSA) and saturated with 2 μg of the different antibodies. The antibodies used were anti-trimethyl-histone H3 (Lys4) (07-473), anti-trimethyl-histone H3 (Lys27) (07-449), and anti-acetyl-histone H3 (06-599) from Millipore, and anti-histone H3 (ab1791) from Abcam (Cambridge, UK). Immunocomplexes were recovered using a DynaMag-2 magnetic particle concentrator (Invitrogen). Samples were washed twice with cold low-salt buffer (50 mM Hepes pH 8.0, 140 mM NaCl, 1% (v : v) Triton X-100, 0.1% (w : v)

sodium deoxycholate, 1 mM EDTA), twice with cold high-salt buffer (50 mM Hepes pH 8.0, 500 mM NaCl, 1% (v : v) Triton X-100, 0.1% (w : v) sodium deoxycholate, 1 mM EDTA), twice with cold LiCl buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% (v : v) Nonidet P40, 0.5% (w : v) sodium deoxycholate, 1 mM EDTA), and finally once with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Washings were performed at 4°C for 4 min, under rotation. After discarding TE buffer, the immunoprecipitated chromatin was eluted from the Dynabead-protein G by adding 50 μl of elution buffer (98 mM $NaHCO_3$, 1% (w : v) SDS), vortexing and incubating for 10 min at 65°C. A second elution step with additional 50 μl of elution buffer was performed. Formaldehyde cross-linking was reversed by incubating overnight at 65°C in the presence of proteinase K (0.4 mg ml^{-1}). DNA was purified with High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) and resuspended in 100 μl of 10 mM Tris-HCl pH 8.0. Chromatin cross-linking and ChIP was made on two independent samples per condition.

Two microlitres of DNA was used for each amplification reaction in a final volume of 20 μl. Quantitative real-time PCR was performed on a StepOnePlus Real-Time PCR System using PerfeCTa SYBR Green SuperMix ROX (Quanta Biosciences, Gaithersburg, MD, USA) and primers shown in Table S1. Cycling protocol consisted of 10 min at 95°C, followed by 45 cycles of 15 s at 95°C, and 1 min at 60°C. The specificity of the PCR reaction was assessed as described earlier. The relative standard curve procedure was used for quantification. Results were made relative to the anti-histone H3 reaction. Results were the average of three PCR reactions for each sample.

Results

Dormancy-related behaviour of 10 different peach cultivars

The particular response to climatic and environmental factors affecting bud dormancy in 10 peach cultivars was studied by three related methods. The flowering time method integrated complex intrinsic and extrinsic inputs, such as cultivar-specific chilling and heat requirements for endodormancy and ecodormancy releases, and the influence of light and meteorological conditions. However, the measurement of chilling requirement did not account, in principle, for the ecodormancy-related factors and was thus expected to better respond to particular mechanisms overcoming winter. Finally, the percentage of bud break method, measured in samples exposed to 400 CH, allowed an additional estimation of cultivar-specific depth of dormancy in a fixed-time condition. The peach phenological stages more relevant to this study are shown in Fig. 1, depicting dormant buds, opening buds showing the green sepals used for chilling requirement and bud break estimation, and fully open flowers employed for flowering time determination.

The three methods gave essentially different measurements, generating three distinct grading of cultivars according to their dormancy behaviour (Table 1). However, comparative analysis of flowering time, chilling requirement and percentage of bud break variables supported an overall classification of cultivars into three major groups. The earliest cultivars 'Red Candem', 'Flor Red', 'May Glo', '86-6', 'Precocinho' and 'Sunraycer' were not consistently separated by the three methods and showed similar responses to dormancy releasing factors. 'Rose Diamond' and 'Big Top' were clearly later than the other cultivars. Finally, a third group containing 'Carolina' and 'Crimson Baby' had an intermediate behaviour between the early and late groups. This broad classification was useful for a general overview of the plant material studied but the different nature of the three methods precluded their fusion in a unique joint source of data. Therefore, the three sets of measurements were used independently.

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

To search for genes responding to bud dormancy stage in peach, we compared gene expression in flower buds excised from the different cultivars at a fixed date, using a custom microarray. Buds were collected after 400 CH, which was supposed to be an intermediate value among the chilling requirements of the cultivars studied. The corresponding percentage of bud break after 10 d ranged from 0% ('Rose Diamond' and 'Big Top') to 86.1% ('May Glo'), a nearly uniform distribution that ensured the utilization of flower buds with diverse degrees of dormancy (Table 1). RNA obtained from these buds was labelled and hybridized to a microarray slide containing a set of dormancy-related cDNAs obtained by suppression subtractive hybridization (SSH) in Leida *et al.* (2010).

In order to determine whether the overall gene expression was conditioned by cultivar-specific factors, a principal component analysis was performed; this showed that > 60% of the variability in the original data was explained by two principal components. Component two divided the varieties according to their dormancy stage, conferring positive values to the group of six early cultivars and negative values to the others (Fig. 2). In addition, the intermediate 'Carolina' and 'Crimson Baby', and the relatively late 'Rose

Fig. 1 Flower developmental changes during dormancy progression and bud break in peach (*Prunus persica*). (a) Two dormant flower buds flanking a vegetative bud; (b) several swollen flower buds are opening and starting to show the green sepals on their tips; (c) a fully open flower.

Table 1 Dormancy variables measured for peach (*Prunus persica*) cultivars

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

[a]Measured in chilling hours (CH). [b]Measured after 10 d incubation of 400 CH shoots. [c]Measured in days after 'May Glo'.

Diamond' and 'Big Top' varieties were placed closely, reproducing properly the three-group classification described earlier. This result suggested that the dormancy stage of collected buds had a wide influence on gene expression, which validates the use of this transcriptomic approach for identification of dormancy related genes.

A correlation analysis of hybridization signals and the three dormancy variables shown in Table 1 was performed using a false discovery rate of 0.05 (Table S3). Both positive and negative correlations were obtained for ESTs, that were either more or less abundant in dormant tissues. Following the guidelines outlined in the Materials and Methods section, 242 ESTs were finally identified (Table S2).

A Venn diagram representing the number of ESTs whose expression level correlated with each of the three dormancy-related variables is shown in Fig. 3(a). Both positive and negative correlations were accounted for. The results showed the high degree of overlap between them, with 59 ESTs (from a total of 242) common to chilling requirement, percentage of bud break

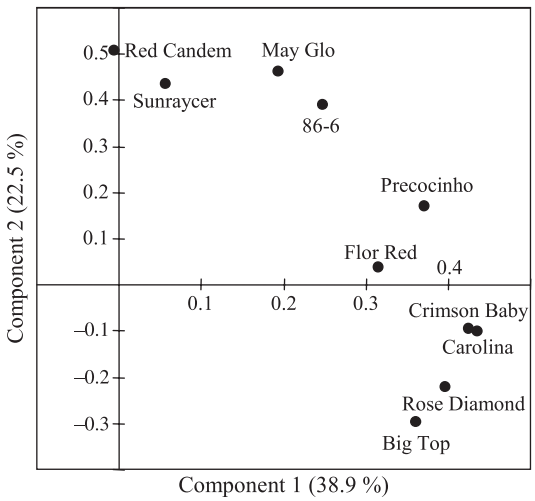


Fig. 2 Principal component analysis of expressed sequence tags (ESTs) signal ratio for the different peach (*Prunus persica*) cultivars assayed. The percentage of the explained variance is shown in parentheses.

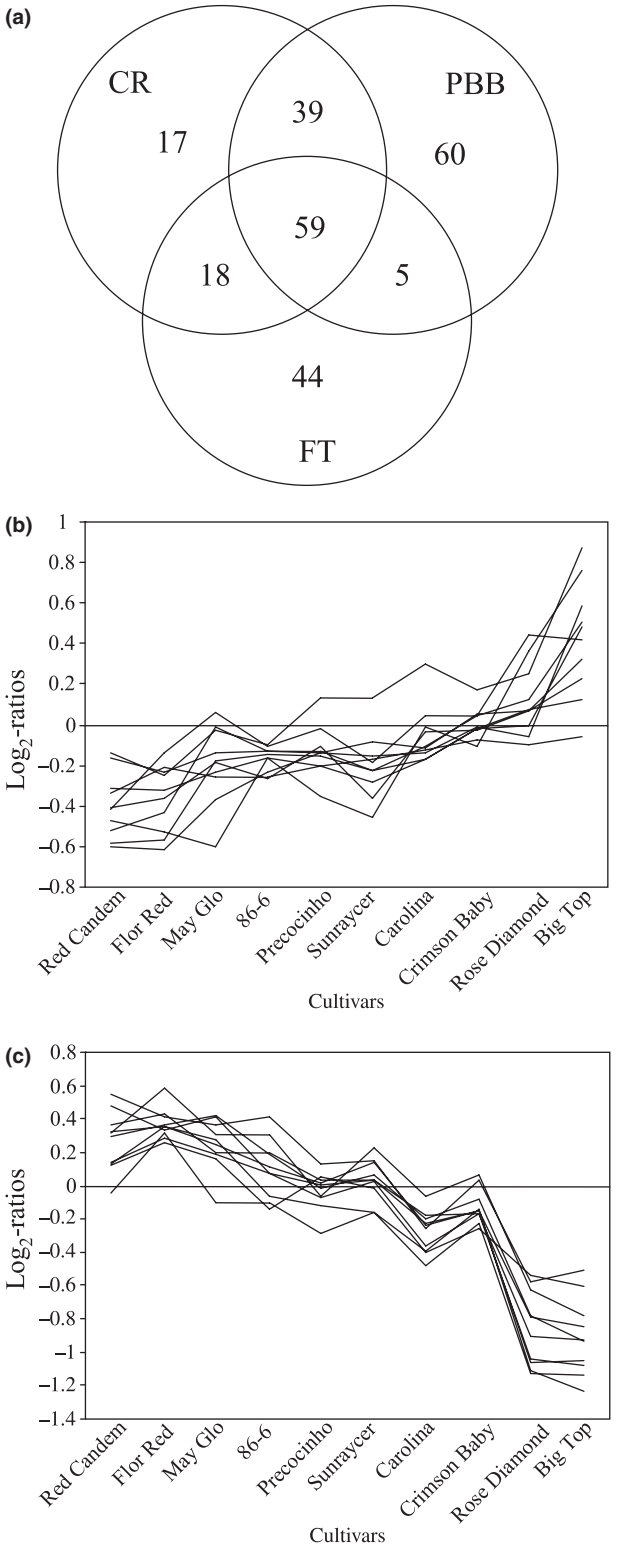


Fig. 3 Analysis of expressed sequence tags (ESTs) correlating with bud dormancy variables in peach (*Prunus persica*). A Venn diagram showing the number of ESTs found under a false discovery rate of 0.05 for chilling requirement (CR), percentage of bud break (PBB) and time to flowering (FT) parameters (a). The normalized $\log_2$ signal ratios of the 10 ESTs having better positive (b) and negative (c) correlation values for chilling requirement are plotted.

and flowering time variables, and 39 coincident ESTs in chilling requirement and percentage of bud break analyses. In conclusion, the three variables offered similar results with certain particularities that should be taken into consideration. The hybridization signals of 10 ESTs showing better positive and negative correlation with the chilling requirement were plotted in Fig. 3(b,c) to illustrate their overall cultivar dependence.

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

Table 2 lists transcript models and ESTs with increased expression in dormant buds of the late cultivars. A gene coding for a putative late embryogenesis abundant protein (LEA) was previously reported to depend on the dormancy stage (Leida *et al.*, 2010); it was also identified in a transcriptomic approach defining peach mesocarp genes affected by chilling (Ogundiwin *et al.*, 2008). Other genes associated with dormancy in peach are: ppa005514m, coding for a dehydrin from bark tissue, which has been described to have a restricted expression pattern in dormancy-defective genotypes of peach (Artlip *et al.*, 1997), and *DAM4-6* genes, part of a six-member gene family of MADS-box transcription factors that have been postulated to cause dormancy-related alterations in the *evg* mutant of peach (Bielenberg *et al.*, 2008). The presence of seven additional genes coding for putative transcription factors (ppa003017m, ppa005713m, ppa007606m, ppa008311m, ppa008979m, ppa009498m and ppa012329m) suggests that multiple regulation pathways are involved in dormancy maintenance and bud development processes.

Transcripts accumulated in buds after dormancy release are shown in Table 3. The transcript models of ppa020321m and ppa008309m, encoding peroxidase-like proteins, were the most represented genes in the experiment, with 69 and 27 ESTs, respectively. Upregulation of these and other related peroxidase genes has been reported in nondormant buds of peach (Leida *et al.*, 2010), coincidently with bud-break induced by chilling accumulation in *Vitis riparia* (Mathiason *et al.*, 2009), and during dormancy release in leafy spurge (Jia *et al.*, 2006). Peroxidases have been proposed to counteract the production of H_2O_2, a signal molecule exerting a dormancy breaking effect in grapevine buds and *A. thaliana* seeds (Pérez *et al.*, 2008; Liu *et al.*, 2010). Other transcripts related to dormancy release in Table 3 coded for putative lipid-transfer proteins (ppa020886m, ppa018509m, ppa025857 and ppa021109m), peptidases (ppa017856m, ppa014645m and ppa010924m), and dehydration-responsive proteins (ppa005535m, ppa005767m, ppa006739m and ppa020936m).

Real-time PCR validation of microarray data

Several genes selected from Tables 2, 3 were analysed by quantitative real-time RT-PCR using bud samples after 400 CH (Fig. 4). Genes from the tandemly repeated family of MADS box transcription factors *DAM4*, *5* and *6* showed a pattern of higher expression in 'Rose Diamond' and 'Big Top', and lower expression in early cultivars, with maximum differences of *c.* 100-fold. Genes ppa008651m, ppa012373m, ppa006974m, ppa007606m, ppa009498m and ppa012188m, listed in Table 2, showed an expression profile similar to the *DAM* genes, but with much lower differences between cultivars. Five of these genes showed a slightly higher expression level in 'Flor Red' and 'Precocinho' compared with other cultivars with proximate dormancy behaviour.

The most striking feature of transcripts associated with dormancy release in Table 3 is their almost null expression in the late varieties 'Rose Diamond' and 'Big Top' (Fig. 4). Interestingly, three of these genes (ppa020886m, ppa018509m and ppa008548m) showed a specific pattern of overexpression in 'Red Candem', '86-6' and 'Sunraycer', resembling cultivar-specific alterations described earlier for five dormancy-related genes, which supports involvement of common transcription regulatory mechanisms in case of a subset of genes.

DAM6 expression decreases concomitantly with dormancy release

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

We measured *DAM6* expression in two representative cultivars of early ('Red Candem') and relatively late ('Big Top') cultivars at different dates during bud dormancy release. In both cultivars, *DAM6* transcript level was strongly reduced coincidently with a rise in growth competence of flower buds measured in the bud break assay (Fig. 5a,b). Owing to their distinct behaviour with respect to dormancy, such bud growth competence occurred after very different periods of chilling accumulation, 276 CH for 'Red Candem' and 684 CH for 'Big Top'. Thus, in agreement with previous works, *DAM6* expression was correlating well with the dormancy release stage of two different cultivars. An expression peak was observed in 'Red Candem' after 50 CH, which could be a result of the combination of light and chilling effects, following a peaked pattern similar to semiquantitative measurements of *DAM6* expression in Li *et al.* (2009).

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

We conducted a ChIP assay in order to define histone modifications of *DAM6* chromatin during dormancy release. Three genomic fragments corresponding to *DAM6* promoter ('PR'), the translation start site ('ST'), and a region of the second large

Table 2 Genes showing higher expression in dormant buds

Transcript name	Representative EST	Number of ESTs	BLASTP hit	BLASTP hit annotation	*E* value	Variable[a]
ppa008651m	GR410432	4	1601521B	LEA D-29 gene	6×10^{-36}	FT
ppa010714m	JK006283	4	ABJ96360	DAM6 (*Prunus persica*)	2×10^{-132}	FT
ppa014312m	JK006309	4	ACG24938	Hypothetical protein (*Zea mays*)	9×10^{-25}	CR, FT
ppa010822m	GR410442	3	ABJ96359	DAM5 (*P. persica*)	2×10^{-128}	CR, PBB, FT
Not found	GR410720	2				PBB, FT
ppa005514m	JK006287	2	AAC49658	Dehydrin (*P. persica*)	3×10^{-169}	CR, FT
ppa009007m	GR410685	2	ABQ45405	Sorbitol-6-phosphate dehydrogenase (*P. persica*)	0	FT
ppa010086m	JK006300	2	EEF35690	Ferritin, putative (*Ricinus communis*)	2×10^{-95}	CR
ppa011123m	GR410688	2	ABJ96358	DAM4 (*P. persica*)	2×10^{-122}	CR, PBB
ppa011831m	JK006295	2	CAB85625	Putative ripening-related protein (*Vitis vinifera*)	8×10^{-67}	CR
ppa012373m	GR410435	2	ABI31653	Zinc finger protein (*Camellia sinensis*)	2×10^{-62}	CR, FT
ppa001989m	JK006292	1	AAL91171	Low-temperature-induced 65 kDa protein (*Arabidopsis thaliana*)	2×10^{-28}	PBB, FT
ppa002102m	JK006378	1	AAQ23899	RSH2 (*Nicotiana tabacum*)	0	PBB, FT
ppa003017m	JK006373	1	NP_179869	AtGRF1 (GROWTH-REGULATING FACTOR 1) (*A. thaliana*)	2×10^{-97}	PBB
ppa003327m	JK006380	1	CAA48630	4-α-glucanotransferase precursor (*Solanum tuberosum*)	0	PBB
ppa005713m	JK006285	1	AAK96816	Putative B-box zinc finger protein (*A. thaliana*)	7×10^{-75}	FT
ppa005802m	JK006375	1	NP_194274	ZFWD1 (zinc finger WD40 repeat protein 1) (*A. thaliana*)	7×10^{-124}	CR
ppa006008m	JK006366	1	NP_564673	Peptidoglycan-binding LysM domain-containing protein (*A. thaliana*)	8×10^{-13}	CR, PBB
ppa006974m	JK006374	1	NP_564956	AFP (ABI FIVE BINDING PROTEIN) (*A. thaliana*)	2×10^{-62}	CR, PBB
ppa007137m	JK006313	1	AAG01381	Alcohol dehydrogenase 1 (*V. vinifera*)	0	CR, PBB, FT
ppa007415m	JK006372	1	XP_002266388	Similar to B2 protein (*V. vinifera*)	6×10^{-118}	PBB
ppa007606m	JK006297	1	ADE41131	AP2 domain class transcription factor (*Malus* × *domestica*)	2×10^{-103}	CR, FT
ppa007666m	JK006369	1	EEF30918	Palmitoyl-protein thioesterase 1 precursor, putative (*R. communis*)	7×10^{-124}	CR, PBB
ppa008311m	JK006299	1	ABI34650	bZIP transcription factor bZIP68 (*Glycine max*)	4×10^{-44}	CR
ppa008849m	JK006284	1	ACF06448	Annexin (*Elaeis guineensis*)	1×10^{-129}	FT
ppa008859m	JK006379	1	ACM45713	Class I chitinase (*Pyrus pyrifolia*)	2×10^{-148}	FT
ppa008979m	JK006331	1	EEF52342	R2R3-MYB transcription factor, putative (*R. communis*)	8×10^{-92}	FT
ppa009032m	JK006286	1	EEF52567	2-hydroxyacid dehydrogenase, putative (*R. communis*)	6×10^{-109}	CR, FT
ppa009498m	JK006370	1	EEF42166	Homeobox protein, putative (*R. communis*)	4×10^{-136}	CR, PBB
ppa010299m	JK006382	1	BAG09366	Peroxisomal short-chain dehydrogenase/reductase family protein (*Glycine max*)	1×10^{-118}	CR, PBB, FT
ppa010931m	JK006367	1	ABN08437	Ribosomal protein L10 (*Medicago truncatula*)	6×10^{-98}	FT
ppa011776m	JK006357	1	EEF50502	Remorin, putative (*R. communis*)	2×10^{-60}	CR
ppa012188m	JK006293	1	NP_563710	AWPM-19-like membrane family protein (*A. thaliana*)	1×10^{-72}	FT
ppa012329m	JK006304	1	EEF35031	Transcription initiation factor iia (tfiia), gamma chain, putative (*R. communis*)	4×10^{-55}	FT
ppa012578m	JK006359	1	EEF30224	Conserved hypothetical protein (*R. communis*)	3×10^{-46}	CR
ppa012801m	JK006290	1	NP_195570	ATFP6 (FARNESYLATED PROTEIN 6) (*A. thaliana*)	8×10^{-69}	CR
ppa012915m	JK006288	1	CBY94070	Early responsive to dehydration (*Fagus sylvatica*)	5×10^{-41}	CR, PBB
ppa013063m	JK006291	1	NP_197518	Ribosomal protein L36 family protein (*A. thaliana*)	7×10^{-28}	CR, PBB
ppa013625m	JK006296	1	NP_568818	Eukaryotic translation initiation factor SUI1, putative (*A. thaliana*)	2×10^{-49}	CR
ppa013723m	JK006360	1	EEF34837	Small nuclear ribonucleoprotein sm d2, putative (*R. communis*)	3×10^{-51}	CR, PBB, FT
ppa014118m	JK006303	1	AAK73280	Drought-induced protein (*Retama raetam*)	1×10^{-3}	FT
ppa014358m	JK006376	1	EEF30268	Conserved hypothetical protein (*R. communis*)	3×10^{-24}	FT
ppa015914m	JK006294	1	ABK94181	Unknown (*Populus trichocarpa*)	1×10^{-07}	FT
ppa017425m	JK006335	1	NP_193292	MAA3 (MAGATAMA 3) (*A. thaliana*)	0	FT
ppa020191m	JK006368	1	BAG80556	UDP-glucose:glucosyltransferase (*Lycium barbarum*)	4×10^{-149}	CR, PBB, FT
ppa024188m	JK006365	1	NP_564673	Peptidoglycan-binding LysM domain-containing protein (*A. thaliana*)	3×10^{-36}	CR, PBB
Not found	JK006298	1				CR, PBB
Not found	JK006306	1				CR

[a]Variables correlating with the expression of at least one expressed sequence tag (EST) of the gene. CR, chilling requirement; FT, time to flowering; PBB, percentage of bud break.

Table 3 Genes showing higher expression in nondormant buds

Transcript name	Representative EST	Number of ESTs	BLASTP hit	BLASTP hit annotation	*E* value	Variable[a]
ppa020321m	JK006332	69	EEF52630	Peroxidase 9 precursor, putative (*Ricinus communis*)	3×10^{-146}	CR, PBB, FT
ppa008309m	GR410503	27	ABW82528	Class III peroxidase (*Gossypium hirsutum*)	9×10^{-119}	CR, PBB, FT
ppa020886m	GR410508	26	EEF51430	MEN-8 protein precursor, putative (*R. communis*)	5×10^{-21}	CR, PBB
ppa018509m	GR410669	9	EEF49202	Lipid binding protein, putative (*R. communis*)	3×10^{-32}	CR, PBB
ppa008548m	GR410674	5	EEF38791	Cinnamoyl-CoA reductase, putative (*R. communis*)	4×10^{-153}	PBB
ppa017856m	GR410555	5	ACG41003	Carboxyl-terminal peptidase (*Zea mays*)	8×10^{-86}	CR, PBB, FT
ppa005535m	JK006364	4	AAL26909	Dehydration-responsive protein RD22 (*Prunus persica*)	1×10^{-81}	CR, PBB, FT
ppa005767m	JK006334	4	AAL26909	Dehydration-responsive protein RD22 (*P. persica*)	6×10^{-82}	CR, PBB, FT
ppa006739m	GR410750	3	EEF45922	Dehydration-responsive protein RD22 precursor, putative (*R. communis*)	1×10^{-78}	CR, PBB, FT
ppa014645m	GR410516	3	ACG41003	Carboxyl-terminal peptidase (*Z. mays*)	1×10^{-84}	CR, PBB, FT
ppa025857m	GR410576	3	NP_177530	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (*Arabidopsis thaliana*)	1×10^{-20}	PBB, FT
Not found	JK006315	3				CR, PBB
ppa009789m	GR410684	2	NP_196821	SAG29 (SENESCENCE-ASSOCIATED PROTEIN 29) (*A. thaliana*)	2×10^{-85}	PBB
ppa021109m	JK006325	2	EEF51426	Nonspecific lipid-transfer protein precursor, putative (*R. communis*)	9×10^{-32}	PBB
ppa003039m	JK006342	1	EEF48818	Proteasome-activating nucleotidase, putative (*R. communis*)	0	CR, PBB
ppa003411m	JK006371	1	EEF32187	L-ascorbate oxidase, putative (*R. communis*)	0	CR, PBB, FT
ppa003797m	GR410504	1	EEE82643	Acyl:coa ligase (*Populus trichocarpa*)	0	FT
ppa004872m	JK006302	1	XP_002268893	Hypothetical protein (*Vitis vinifera*)	0	CR, PBB, FT
ppa006506m	GR410648	1	AAO42227	Putative strictosidine synthase (*A. thaliana*)	0	CR
ppa006852m	JK006362	1	EEE85993	Chs-like protein (*P. trichocarpa*)	0	CR
ppa010924m	JK006328	1	NP_181525	Microsomal signal peptidase 25 kDa subunit, putative (SPC25) (*A. thaliana*)	1×10^{-71}	CR, FT
ppa020936m	JK006318	1	AAL26909	Dehydration-responsive protein RD22 (*P. persica*)	5×10^{-94}	FT
ppa025137m	JK006336	1	XP_002277756	Hypothetical protein (*V. vinifera*)	2×10^{-22}	CR, PBB, FT
ppb012876m	GR410653	1	EEF42354	Conserved hypothetical protein (*R. communis*)	3×10^{-38}	CR, PBB
Not found	JK006311	1				PBB

[a]Variables correlating with the expression of at least one expressed sequence tag (EST) of the gene. CR, chilling requirement; FT, time to flowering; PBB, percentage of bud break.

intron ('IN') were selected for ChIP analysis (Fig. 5c) using 'Big Top' and 'Red Candem' bud samples shown in Fig. 5(a,b). Real-time quantitative PCR data were standardized to histone H3 levels to calculate the relative ratio of modified H3 shown in Fig. 5(d,e).

H3K4me3 was, in general, more abundant in the 'ST' region than the upstream promoter and downstream intron in both cultivars, suggesting that the zone around the translational start could be susceptible to regulation (Fig. 5d,e). Indeed, H3K4me3 was reduced in 'Big Top' buds in parallel to dormancy release in two independent experiments, reproducing accurately the *DAM6* downregulation shown in Fig. 5(a). A significantly lower rate of H3K4me3 in 'Red Candem' after 276 CH (RC4; Fig. 5e) was also coincident with a lower expression level of the gene and the concomitant end of bud dormancy measured as bud break competence (Fig. 5b). The rise in *DAM6* expression observed after 50 CH (RC2) was, however, not accompanied by a corresponding increase in H3K4me3. This discrepancy could be arise because of the presence of an as yet unknown chromatin modification contributing to gene expression, the binding of a transcriptional activator, or alternatively could have a post-transcriptional origin, as a differential rate of mRNA degradation.

Similar results were observed when measuring the relative level of H3 acetylation (H3ac). H3ac was not consistently regulated during bud development in PR and IN fragments, but a significant decrease in H3ac was found around the ST region during dormancy progression of 'Big Top' and 'Red Candem' samples (Fig. 5d,e). In 'Big Top' a significant reduction of H3ac level occurred in BT3, a dormant sample before dormancy release, but no events timing could be established in 'Red Candem' because of certain discrepancies in the decreasing pattern observed between independent experiments.

H3K27me3 increases along *DAM6* gene after dormancy release

H3K27me3 followed an opposite pattern to H3K4me3 and H3ac modifications. A relevant accumulation of H3K27me3 was only detected in the last 'Big Top' sample (BT5), when buds had already passed the dormancy period (Fig. 5d). Interestingly, H3K27me3 occurred to a similar extent in the three genomic regions of *DAM6*, which supports overall gene modification rather than modulation of a short regulatory element. An increase in H3K27me3 was noted in the nondormant RC4 sample of

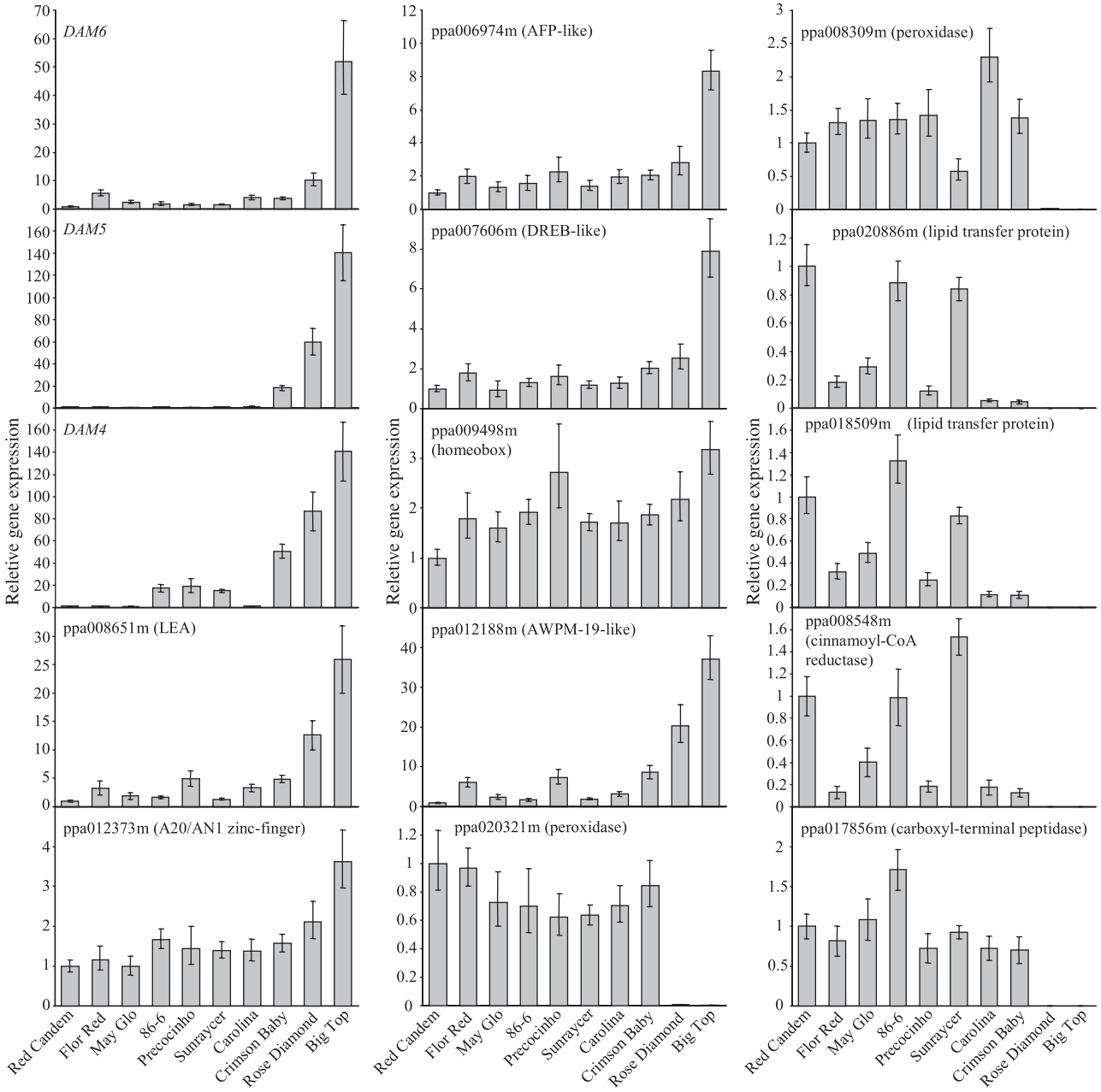


Fig. 4 Real-time reverse-transcription polymerase chain reaction (RT-PCR) of selected genes. RNA samples were obtained from peach (*Prunus persica*) flower buds harvested after 400 chilling hours (CH). The name of the gene or transcript model is shown in the upper left corner of the graph. Expression levels are relative to actin. An expression value of one is assigned to the 'Red Candem' sample. Data are means from two biological replicates, with error bars representing ± SD.

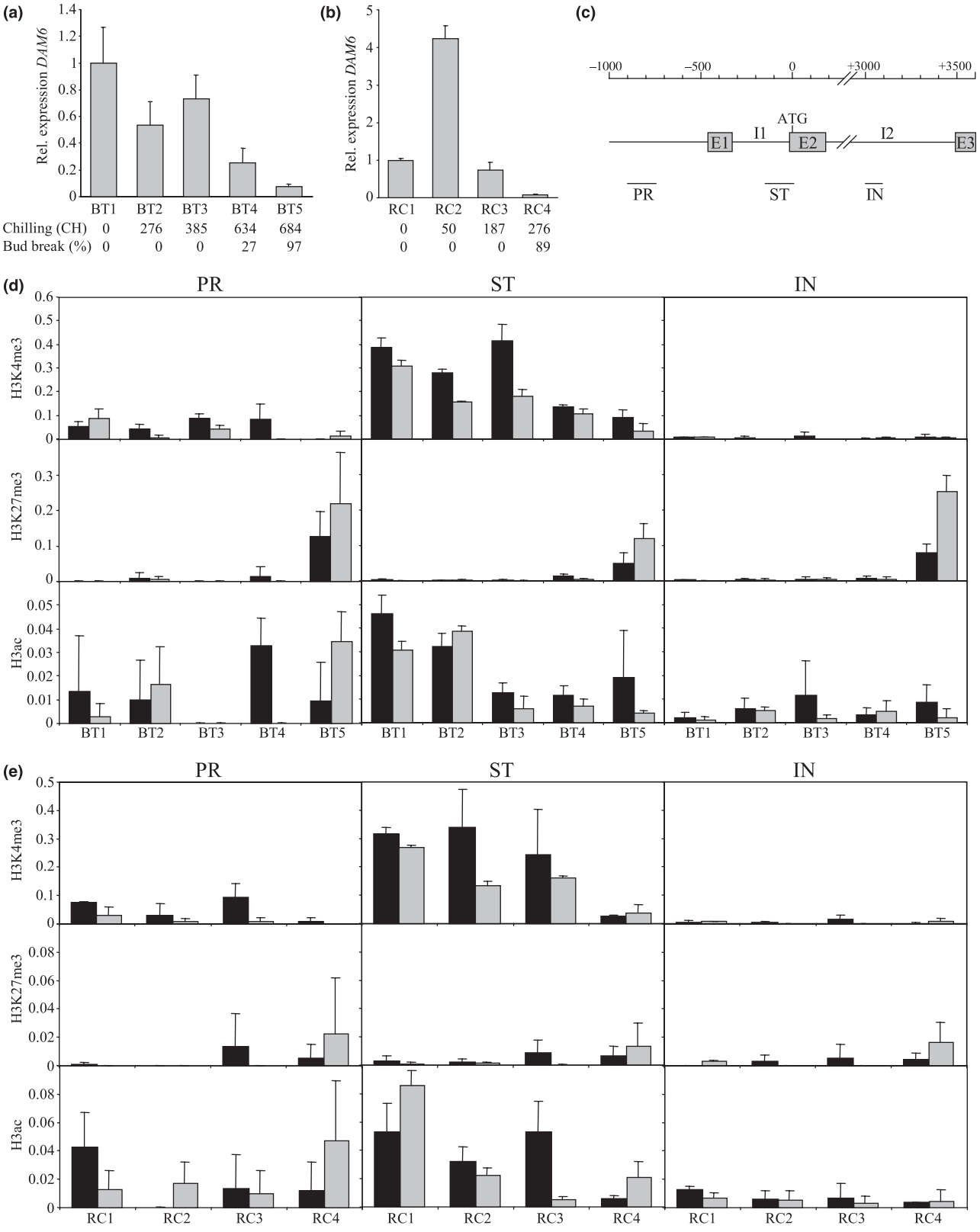
'Red Candem', but this alteration was not significant and *c.* 10-fold lower than in BT5 (Fig. 5e). Consequently, H3K27me3 modification, unlike H3K4me3 and H3ac, correlated positively with bud break competence following the dormancy period in a cultivar-dependent manner and showed a wide distribution along the *DAM6* gene.

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

Discussion

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

The aim of this work was to identify peach genes whose expression in flower buds at a single developmental stage (400 CH) correlated with the chilling requirement and other dormancy variables of 10 different cultivars. Previous studies employed the SSH procedure for the isolation of bud dormancy-related genes in peach. Jiménez *et al.* (2010a) used SSH to compare wild-type



and *evg* mutant gene expression after bud growth cessation mediated by transfer to short-day conditions. From 23 genes found by Jiménez and coworkers, only one (*DAM6*) has been also obtained in this work, most likely because of differences in the experimental design. Whereas Jiménez and colleagues examined entry to dormancy by modulating photoperiodic conditions, our

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

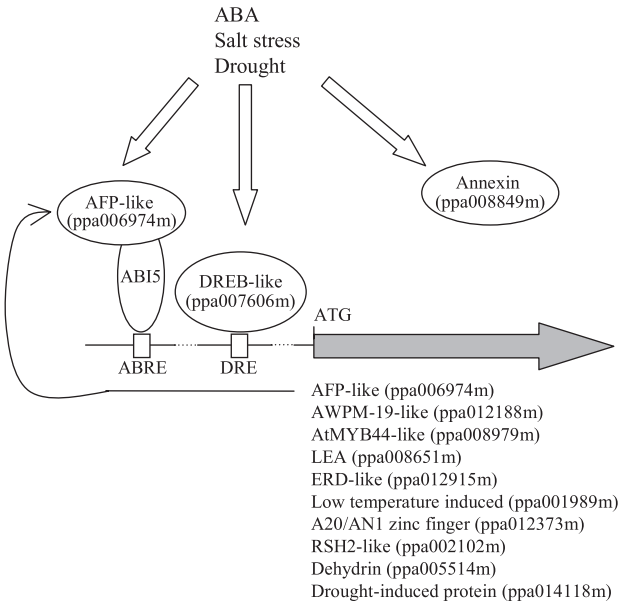


Fig. 6 Abscisic acid and drought related proteins. Open arrows symbolize the transduction of ABA and abiotic stress signals. Open boxes represent ABA-responsive element (ABRE) and dehydration-responsive element (DRE) on the promoter of ABA and drought responsive genes. Proteins coded by genes responding to these signals are located below the tinted arrow. The transcript models of genes described in this work are shown in parentheses.

work is mostly devoted to dormancy break processes based on cultivar diversity. Thus, the identification of *DAM6* in both studies confers increasing interest to this gene.

By contrast, Leida *et al.* (2010) employed SSH to identify genes associated with bud dormancy release processes, which served to design the microarray used in this work. Despite the experimental differences between both approaches, the number of coincidences with our work is higher in this case (25). The set of common genes includes three members of the *DAM* family (*DAM4, DAM5* and *DAM6*) and genes coding for a LEA, a Zn-finger protein, peroxidases, lipid transfer proteins and peptidases, among others. The percentage of cDNAs correlating with cultivar-specific differences may seem unexpectedly low if we consider that microarray probes were obtained by subtractive hybridization of dormant vs nondormant samples; however, previous results show that the ratio of genuine differentially expressed clones after SSH may be very low, and a further validation step is required in most cases (Yamane *et al.*, 2008; Leida *et al.*, 2010).

The role of DAM transcription factors in regulating bud dormancy entrance and release in peach is not only supported by transcriptomic analyses, but also by expression data (Jiménez *et al.*, 2010b; Yamane *et al.*, 2011), and genetic studies. Deletion of several *DAM* genes was tightly linked to the nondormant and defective terminal bud formation phenotype of the *evg* mutant (Bielenberg *et al.*, 2008). In addition, quantitative trait loci (QTL) mapping of chilling requirement, heat requirement and bloom date in peach led to a major QTL in linkage group 1, overlapping with the *EVG* locus containing *DAM* genes (Fan *et al.*, 2010). Thus, in agreement with our results DAM factors are therefore considered the main candidates to control bud dormancy and meristem growth cessation in peach and other perennial plants, through regulation of gene expression. *DAM4–DAM6* and other genes found in this work could be used as expression markers for comparing the chilling requirements and dormancy aptitudes of different cultivars. In our opinion, a single-time RT-PCR assay using few genes could facilitate the phenotypic evaluation of large collections of individuals as the segregating population of a cross for plant breeding purposes.

ABA and drought-responding genes are dormancy regulated

It is remarkable that many genes expressed in dormant buds correspond to ABA and drought-related genes in other species. Fig. 6 lists some proteins identified in this work with homologues described as inducible by abiotic stresses or ABA. One of these proteins encoded by ppa006974m is similar to ABA-INSENSITIVE5 (ABI5) binding protein (AFP), involved in ABA signal transduction in *A. thaliana*. AFP binds to and promotes proteolytic degradation of ABI5, a basic leucine zipper (bZIP) transcription factor that regulates ABA-dependent genes by binding to the ABA-responsive element, ABRE (Lopez-Molina *et al.*, 2003). In addition to ABRE, abiotic stresses affect gene expression through the dehydration-responsive element (DRE) and their respective DRE-binding proteins (DREB; Liu *et al.*, 1998). The transcript model ppa007606m found in this work encodes a DREB-like factor that could contribute to the dormancy-specific expression of ABA and drought-responsive genes. Conversely, calcium-binding annexins related to the product of ppa008849m have been found to be involved in ABA and osmotic stress signal transduction in *A. thaliana* (Lee *et al.*, 2004).

These observations are in agreement with recent findings by Jiménez *et al.* (2010a), showing that genes encoding a LEA and

KEEP ON GOING (KEG)-like proteins are misregulated in *evg* mutant during short-day induction of bud dormancy. KEG is an E3 ligase that regulates ABI5 abundance by means of its ubiquitination and subsequent proteasome-mediated degradation in *A. thaliana* (Stone *et al.*, 2006).

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

Some of the proteins shown in Fig. 6 could contribute to cold hardening processes, improving frost tolerance of buds. Interestingly, overexpression of *AtMYB44* (Jung *et al.*, 2008), rice A20/AN1 zinc-finger protein (Mukhopadhyay *et al.*, 2004) and *A. thaliana* DREB2C gene, similar to ppa007606m (Lee *et al.*, 2010), conferred tolerance to cold or freezing temperatures in transgenic plants. In addition, dehydrins and AWPM-19-like plasma membrane proteins have been associated with cold tolerance in peach and wheat, respectively (Artlip *et al.*, 1997; Koike *et al.*, 1997). In conclusion, our results support a role of ABA and drought responses in bud dormancy and cold acclimatization processes, and contribute to identify several genes encoding putative regulatory factors of these pathways in peach.

DAM6 is regulated at the chromatin level

The chromatin modifications H3K4me3, H3ac and H3K27me3 had a different timing for 'Big Top' and 'Red Candem', coinciding with their specific patterns of gene repression and increase in bud break competence. This argues for a potential role of chromatin modification in modulating *DAM6* expression and subsequently the state of bud dormancy. Nevertheless, H3K27me3 was not significantly increased in the 'Red Candem' buds just after dormancy release (RC4), which suggests a sequential chain of molecular events affecting the local state of *DAM6* chromatin, as outlined in Fig. 7. In dormant buds (BT1 and RC1 samples), a transcriptionally active *DAM6* gene would contain H3K4me3 and H3ac in a short chromatin region around its ATG (Fig. 7a). Following demethylation of H3K4 and deacetylation of H3, *DAM6* repression would contribute to release dormancy in samples BT4/BT5 and RC4 (Fig. 7b). Finally, H3K27me3 in a region of at least 4 kb including promoter, coding sequence and introns, would mediate stable epigenetic repression of the gene through subsequent cell cycles (Fig. 7c). This last stage corresponds to the BT5 sample, but does not have a 'Red Candem' counterpart in this work, which confirms the temporal separation of H3K4me3 and H3ac events from H3K27me3. Two scenarios

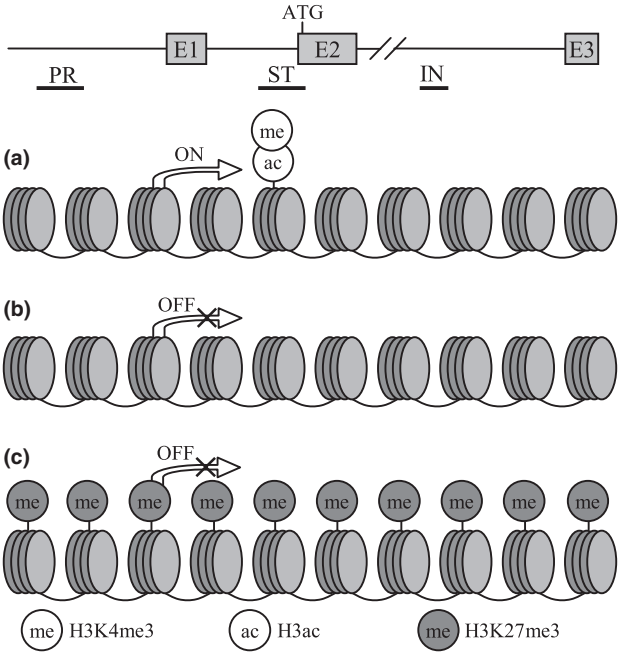


Fig. 7 Possible sequence of *DAM6* chromatin events during dormancy release. In dormant buds, transcriptionally active *DAM6* has H3K4me3 and H3ac modifications around the translation start region (a) (PR, promoter; ST, translation start; IN, second large intron). Buds undergoing dormancy release lose H3K4me3 and H3ac modifications concomitantly with gene repression (b), as observed in peach (*Prunus persica*) cv Red Candem. Finally, overall H3K27me3 modification could facilitate long-term gene inactivation (c).

are possible in 'Red Candem' after the induction stage detected in RC4: *DAM6* chromatin is not trimethylated on H3K27, which could contribute to cultivar-specific differences in dormancy response; H3K27 trimethylation occurs in a subsequent step, and thus phenotypic differences between cultivars could simply depend on the distinct chilling time required for triggering the whole process.

Changes in the methylation state of H3K27 and H3K4 have been recently observed in the promoter of *DAM1* gene of leafy spurge when comparing buds in different dormancy stages (Horvath *et al.*, 2010). These chromatin modifications have been related to the downregulation of *DAM1*, as observed for *DAM6* in this work. Both genes encode MIKC^c-type MADS-box proteins belonging to the SVP/StMADS11 clade (Becker & Theißen, 2003; Jiménez *et al.*, 2009; Horvath *et al.*, 2010), but are not strictly orthologous. Leafy spurge *DAM1* is more similar to the peach transcript ppa022274m, which has not been related to dormancy processes. They may have been originated in an ancestral duplication event followed by functional diversification.

Modification of *DAM6* chromatin in a similar way to *FLC* and *VRN1*, as a consequence of the prolonged exposure to low temperatures (Bastow *et al.*, 2004; Sung & Amasino, 2004; Oliver *et al.*, 2009), confirms the existence of mechanistic similarities between vernalization and bud dormancy processes. The coincidence of H3K27me3 and gene repression in these three models suggests the common concurrence of methyl-transferase activities associated with related PcG multiprotein complexes in *FLC*,

VRN1 and *DAM6*. Thus, PcG complexes are potentially responsible for the stable repression of *DAM6* at the end of the seasonal dormancy until the next period of bud formation, which would require as yet unknown mechanisms of *DAM6* activation for the initiation of a new dormancy cycle. Additional genetic and biochemical approaches are required to identify these and other regulatory elements implicated in DAM-dependent pathway.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Primers used in this work

Table S2 Accession numbers of expressed sequences tags (ESTs)

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

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