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Monitoring Dormancy Transition in Almond [*Prunus Dulcis* (Miller) Webb] during Cold and Warm Mediterranean Seasons through the Analysis of a *DAM* (Dormancy-Associated MADS-Box) Gene

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Abstract: For fruit tree (*Prunus*) species, flower bud dormancy completion determines the quality of bud break and the flowering time. In the present climate change and global warming context, the relationship between dormancy and flowering processes is a fundamental goal in molecular biology of these species. In almond [*P. dulcis* (Miller) Webb], flowering time is a trait of great interest in the development of new cultivars adapted to different climatic areas. Late flowering is related to a long dormancy period due to high chilling requirements of the cultivar. It is considered a quantitative and highly heritable character but a dominant gene (*Late bloom*, *Lb*) was also described. A major QTL (quantitative trait loci) in the linkage group (LG) 4 was associated with *Lb*, together with other three QTLs in LG1 and LG7. In addition, *DAM* (Dormancy-Associated MADS-Box) genes located in LG1 have been largely described as a gene family involved in bud dormancy in different *Prunus* species including peach [*P. persica* (L.) Batsch] and Japanese apricot (*P. mume* Sieb. et Zucc.). In this work, a *DAM* transcript was cloned and its expression was analysed by qPCR (quantitative Polymerase Chain Reaction) in almond flower buds during the dormancy release. For this purpose two almond cultivars ('Desmayo Largueta' and 'Penta') with different chilling requirements and flowering time were used, and the study was performed along two years. The complete coding sequence, designated *PdDAM6* (*Prunus dulcis* *DAM6*), was subjected to a phylogenetic analysis with homologous sequences from other *Prunus* species. Finally, expression dynamics analysed by using qPCR showed a continuous decrease in transcript levels for both cultivars and years during the period analysed. Monitoring almond flower bud dormancy through *DAM* expression should be used to improve almond production in different climate conditions.

Keywords: flowering; breeding; chilling requirements; qPCR; transcription; cloning

1. Introduction

During autumn temperate fruit trees (*Prunus*) activate a survival strategy called endodormancy, to protect against unfavourable chill conditions. Trees cease growth and form structures called buds in order to protect meristems from unfavourable environmental conditions, including low temperature and desiccation [1]. Chill accumulation allows the progression from flower bud endodormancy stage to flower bud ecodormancy which is regulated by heat accumulation [2]. Flowering time in almond [*Prunus dulcis* (Miller) Webb] is mainly dependent on chilling requirements to overcome this endodormancy stage [3]. These chilling requirements are considered a cultivar-dependent trait, correlated with species or cultivar origin [4,5].

Warm winter temperatures affect cold accumulation and if chilling requirement is not fully satisfied such a condition could lead to irregular and insufficient flowering with a loss of production [4,6]. Due to its economic importance, dormancy release is being studied in different species, although knowledge is still scarce and no common mechanism has been described. Thus, expression analysis of candidate genes may be a useful tool for the interannual monitoring of endodormancy progression within the flower bud. This is especially interesting for commercial fruit tree cultivars displaying a wide range of flowering and ripening time phenotypes, as in case of almond [6]. Adaptation to climatic conditions largely depends on an adequate flowering time, and it is one of the most important agronomic traits in almond breeding programs, as it determines whether the pollination period will occur in favourable climatic conditions [4]. In this context, the development of new extra-late flowering cultivars to avoid the spring frosts is one of the main objectives of almond breeding programs [7].

Late flowering is considered a quantitative and highly heritable character but a major gene (*Late bloom*, *Lb*) was also described. A QTL (quantitative trait loci) explaining 57% of the observed variance in the Linkage Group (LG) 4 was associated with *Lb*, together with other three QTLs (explaining 20, 12, and 8% of the variance) in LG1 and LG7 [8,9]. In addition, bud endodormancy has a set of genetic controls which may be characterized through examination of gene expression in bud tissues over time. Prior studies showed the *Dormancy Associated MADS-Box* (*DAM*) gene family is a group of transcription factors that regulated peach [*P. persica* (L.) Batsch] dormancy [10]. This gene family was discovered in the *Evergrowing* (*evg*) mutant of peach. The mutation consisted of a deletion in the *EVG* (*EVERGROWING*) locus affecting up to four genes which prevents terminal buds from entering the endodormancy stage [11]. The map-based cloning analyses of *EVG* locus revealed that it included six tandemly arrayed genes [11,12]. Moreover, *DAM5* expression was analysed in different cultivars of peach [13] and *PpDAM6* was postulated as one of the main factors involved in the regulation of dormancy in different *Prunus* species including peach [14–16] and Japanese apricot (*P. mume* Sieb. et Zucc.) [17,18].

In this work, a candidate *DAM* transcript was cloned and expression from endodormancy to ecodormancy stages in two almond cultivars with different chilling requirements and flowering time: ‘Desmayo Largueta’ and ‘Penta’ was determined.

2. Materials and Methods

2.1. Plant Material

‘Desmayo Largueta’ is a traditional Spanish almond cultivar with very low chilling requirements and extra-early flowering time, and ‘Penta’, a cultivar released from the Almond Breeding Program of CEBAS-CSIC (Murcia, South-East Spain) with high chilling requirements and extra-late flowering time, were used [6]. The plant material consisted of flower buds sampled weekly between stages A (dormancy phase) and B (after dormancy release) referenced to the phenological stages described by Felipe [19] (Figure 1).

2.2. Chilling Requirements Evaluation

Experiments for the evaluation of chilling and heat requirements were conducted in the experimental field of CEBAS-CSIC, in Murcia (South-East Spain), during two seasons of study: 2015–2016 and 2016–2017. Temperatures were recorded hourly with a data logger (HOBO® UX100-003 Temp/Relative Humidity, Madrid, Spain) from November to February during both seasons. Three branches (40 cm in length and 5 mm in diameter) were collected weekly from the same tree in the field, and placed in a growth chamber in controlled conditions (25 ± 1 °C, RH $40 \pm 3.5\%$ during a 16 h light photoperiod and 20 ± 1 °C, RH 60% during the dark period). Almond branches were placed in the growth chamber, in a 5% sucrose solution and 1% aluminium sulphate, making a fresh cut in the base of the branches. After 10 days, the development state of the flower buds was recorded.

The date of dormancy breakage was established when, after 10 days in the growth chamber, 50% of the flower buds were in the B-C state [20]. The calculation of chilling accumulation in field conditions was calculated as the chill contributions in the field necessary for breaking of dormancy (transition from stage A to stage B, see Figure 1) in chill units (CUs) according to the model described by Richardson et al. [16] with an initial date for chilling accumulation when consistent chilling accumulation occurred and temperatures producing a negative effect (chilling negation) were rare [21]. These CUs were calculated as hours below 7 °C. In addition, chilling accumulation was calculated in chill portions (CPs) according to the dynamic model [22,23] with an initial chilling accumulation of 0. The model is based on the assumption that dormancy completion may be estimated as a dynamic two-stage process controlling an accumulated bud break factor. The model is “dynamic” in the sense that relatively high temperatures, typically 19 °C and above, effectively negate earlier chilling; alternatively, moderate temperatures, typically around 13–14 °C effectively enhance moderate earlier chilling temperatures.

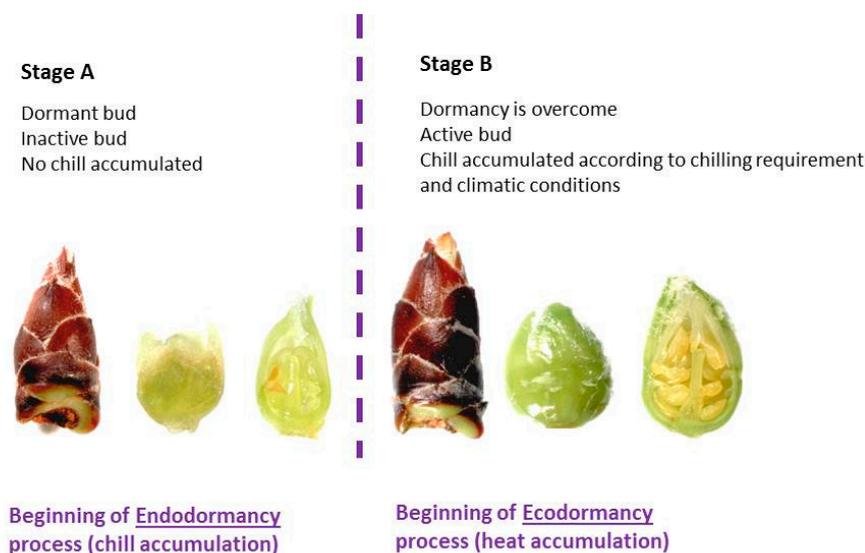


Figure 1. Plant material assayed from the almond cultivar ‘Desmayo Largueta’. Flower buds in the dormant stage (A) and after dormancy release (B).

2.3. cDNA Isolation and Cloning

Almond samples assayed include flower buds in state A (completely dormant bud), state B (when 40–50% of the chilling requirement of each cultivar are satisfied) and state B (when the flower bud has broken its dormancy) (Figure 1). Total RNA was extracted from almond flower buds [24] and treated with DNaseI (Ambion). cDNA was synthesized using SSIII Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA, USA). The full-length cDNA was isolated from cDNA of flower buds of ‘Desmayo Largueta’ and ‘Penta’ cultivars using 3’-RACE strategy and specific primers from *Prunus persica* available sequences in databases. High-fidelity PCR (Polymerase Chain Reaction) was performed using KOD (from Archaeon *Thermococcus kodakaraensis*) Hot Start DNA polymerase (Novagen, Berlin, Germany) and the product was cloned into *E. coli* using Zero Blunt Topo PCR Cloning Kit (Life Technologies, Carlsbad, CA, USA) for sequencing.

2.4. Phylogenetic Analysis

A BLAST (Basic Local Alignment Search Tool) search was performed with the full-length PdDAM6 cDNA (*Prunus Dulcis* DORMANCY-ASSOCIATED MADS-BOX 6), in order to confirm the identity of the sequence and to collect homologous proteins from the *Prunus* genus with a high percentage identity. A pPutative PdDAM6 protein sequence was obtained using the ExPASy translate tool (<http://web.expasy.org/translate/>). A phylogenetic tree was created using Phylogeny.fr (<http://phylogeny.lirmm.fr/phylo.cgi/index.cgi>).

2.5. Gene Expression Analysis

To investigate the expression pattern of PdDAM6 during bud dormancy progression, real time qPCR experiments were executed with a One Step Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Specific primers were designed based on an almond PdDAM6 sequence using Primer3 software (Forward Primer: 5' AGGAAATACTGGACCTGCGT-3'; Reverse Primer: 5'-GGTGGAGGTGGCAATTATGG-3'). Reaction efficiency was checked by the standard curve method. For all real-time qPCR reactions, a 10 μ L mix was made including: 5 μ L Power SYBR®Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 20 ng of cDNA, and 0.5 μ L of each primer (5 μ M). High-fidelity PCR was performed using KOD Hot Start DNA polymerase (Novagen, Berlin, Germany), and the product was cloned into *E. coli* using a Zero Blunt Topo PCR Cloning Kit (Life Technologies, Carlsbad, CA, USA) for sequencing. PCR was performed in a 30 μ L mix according to the manufacturer's instructions with 150 ng of cDNA from each almond cultivar and 10 μ M primers. The PCR reaction was incubated at 94 °C for 2' for the initial denaturalisation step, followed by 35 cycles of 94 °C for 30", 62 °C for 1' and 68 °C for 1'. A final extension step at 68 °C was set for 10'. Each biological sample was implemented in duplicate. RPII was used as reference gene for data normalization using primers designed by Tong et al. [25] (Forward Primer: 5'-TGAAGCATACACCTATGATGATGAAG; Reverse primer: 5' CTTTGACAGCACCAGTAGATTCC-3') and the levels of relative expression were calculated by the 2- $\Delta\Delta$ Ct method [26] taking Ct value from November the 10th samples as the reference expression level.

3. Results

3.1. Chilling Requirements Evaluation

Chilling accumulation in field conditions during the two seasons of study (2015–2016 and 2016–2017) calculated as chill units (CUs) according to the Richardson model and in chill portions (CPs) according to the dynamic model is shown in Figure 2.

During the first year of the study (2015–2016), an important reduction of chill accumulation was observed, mainly in terms of chill units (CUs). As shown in Table 1, the dormancy release date was observed earlier during the 2016–2017 season, when a higher amount of chill units accumulated. Regarding flowering time, an important advance was observed in the late cultivar 'Penta'.

Table 1. Chill accumulated percentage (chill portions) in field conditions during the seasons 2015–2016 and 2016–2017.

Season	Stage A		Stage B		Flowering Date	
	Date	Chill Accumulation	Date	Chill Accumulation		
'Desmayo	2015/2016	November 10	0	December 21	16	January 28
Largueta'	2016/2017	November 10	0	December 15	24	January 27
'Penta'	2015/2016	November 10	0	February 10	41	March 25
	2016/2017	November 10	0	February 2	54	March 12

3.2. cDNA Isolation, Cloning and Phylogenetic Analysis

Phylogenetic analysis of PdDAM6 protein sequence from 'Desmayo Largueta' and 'Penta' sequences confirmed that PdDAM6 is indeed a member of the DAM family transcription factors. In addition, the phylogenetic tree showed that PdDAM6 branch (including sequences from 'Desmayo Largueta' and 'Penta' cultivars) is closer to *Prunus persica* DAM6 (PpDAM6) and *Prunus pseudocerasus* (PpsDAM6) rather than to DAM5 protein group (Figure 3).

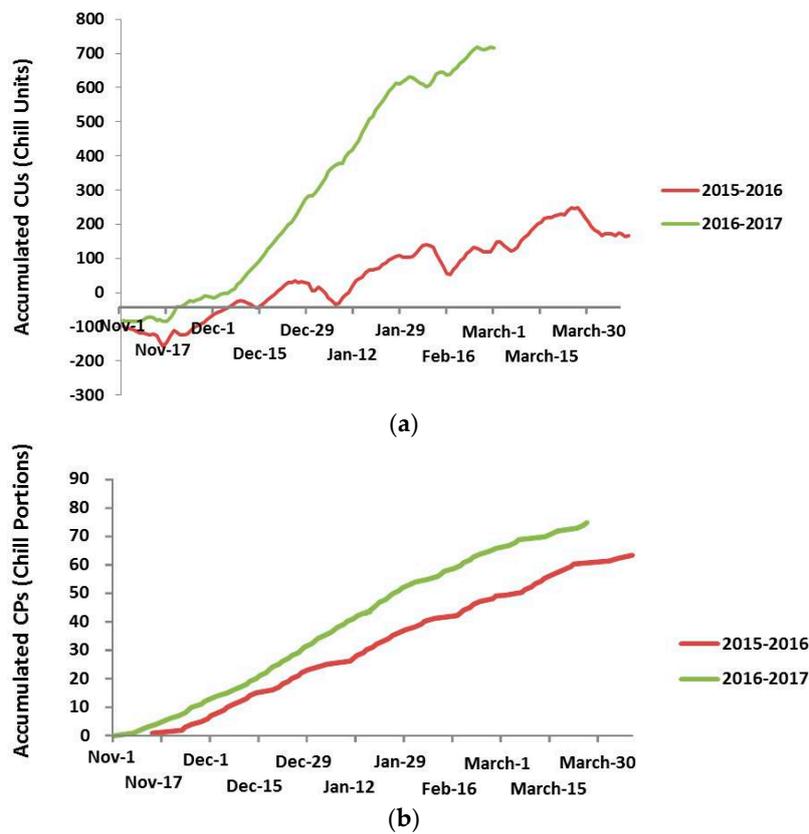


Figure 2. (a) Chilling accumulation in field conditions during the two seasons of study (2015–2016 and 2016–2017) calculated as chill units (CUs) according to the Richardson model; (b) calculated in chill portions (CPs) according to the dynamic model.

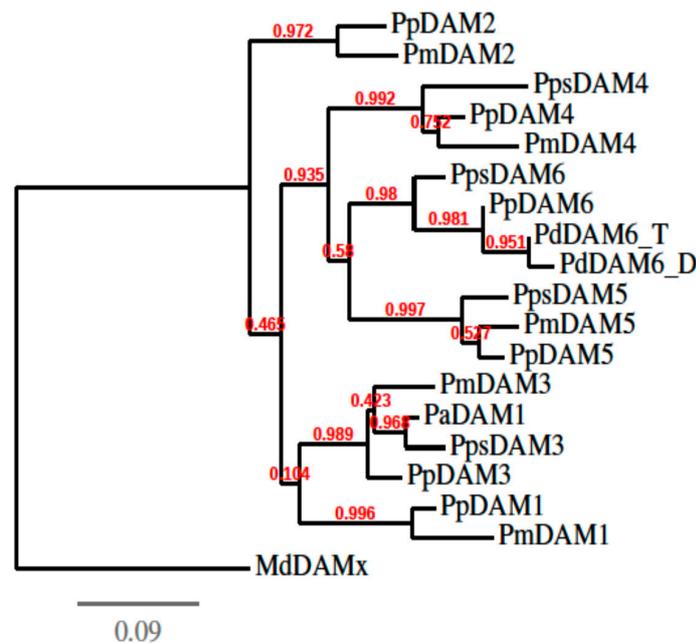


Figure 3. Phylogenetic tree showing relationships between homologous proteins to ‘Desmayo Largueta’ (PdDAM6_D) and ‘Penta’ (PdDAM6_P) sequence. *Prunus persica* (Pp), *Prunus mume* (Pm), *Prunus pseudocerasus* (Pps) and *Prunus avium* (Pa). aLRT (approximate likelihood ratio) values are indicated in red. *Malus domestica* DAM protein sequence (MdDAMx) was used as outgroup to root the phylogenetic tree.

3.3. Expression Analysis

Expression analysis of *PdDAM6* showed a progressive decrease in mRNA levels until the dormancy period was completely overcome, in both ‘Desmayo Langueta’ and ‘Penta’ cultivar samples (Figure 4).

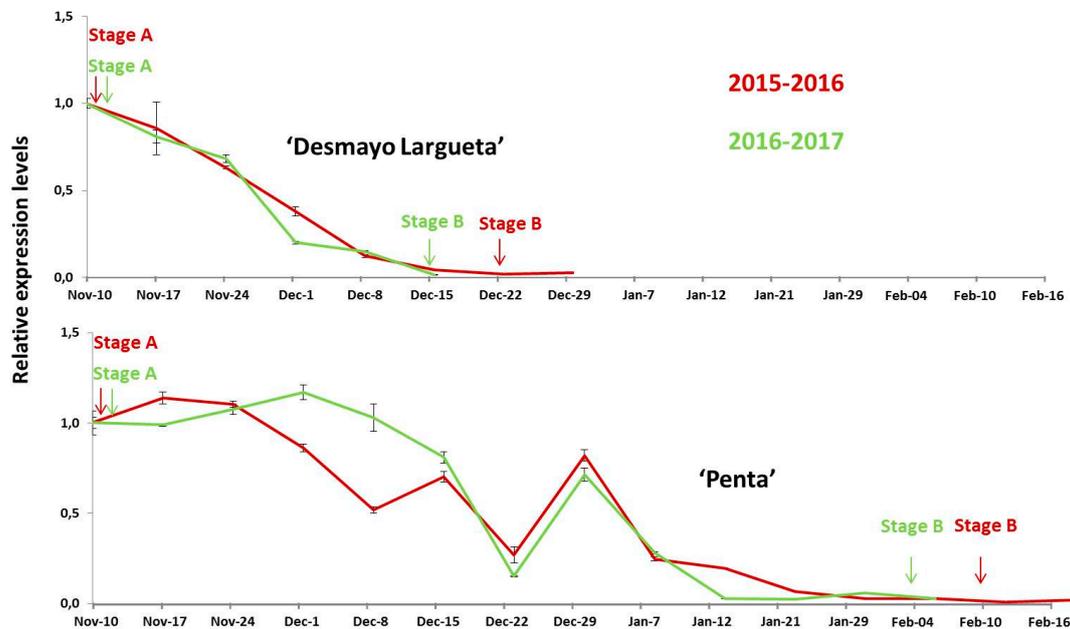


Figure 4. Relative gene expression of *PdDAM6* gene evaluated by qPCR (quantitative Polymerase Chain Reaction) ‘Desmayo Langueta’ and ‘Penta’ almond cultivars during the seasons 2015–2016 and 2016–2017. Standard deviations are indicated with vertical bars.

4. Discussion

As shown by Prudencio et al. [6], the estimation of chill accumulation under different climatic conditions showed that the dynamic model presents less variation than the Richardson model. As expected, the chilling requirements of the almond cultivars were related to their flowering time. However, in general, these values were lower than in previous evaluations performed by our group [3,8] mainly in the case of the warmer year.

The first full-length cDNA from the DAM gene family was obtained for almond. The clone designated *PdDAM6* (*Prunus dulcis DAM6*) was obtained from ‘Desmayo Langueta’ and ‘Penta’ almond cultivars, which display different phenotypes regarding chilling requirement and flowering time. Phylogenetic and expression analysis was performed to further characterize the sequences and to study the biological role of DAM proteins during flower bud dormancy progression in almond.

Our results clearly indicated that the level of expression of *DAM6* in both almond cultivars with different chilling requirements and flowering time decreased concomitantly with chill accumulation and dormancy progression, although for the late cultivar ‘Penta’, a relative increase was observed prior to dormancy release. These results supported that obtained by Leida et al. [14] and Jiménez et al. [15], highlighting the role of this gene in flower bud dormancy maintenance. In addition, a down-regulation of *DORMANCY-ASSOCIATED MADS-box6* has been observed in Japanese apricot [17,18] during dormancy release.

Monitoring bud transition from endodormancy to ecodormancy should be of great interest in terms of the use and optimization of biostimulants to promote flowering in fruit tree species [27,28] in the present climate change and warming context. The moment of application of these biostimulants is critical for success and depends on the endodormancy stage of the bud and its transition to ecodormancy [29] or the forcing strategies [30]. Treatments with these biostimulants should be

applied at the optimum time for breaking bud dormancy, as they can be null or even toxic depending on the stage of the bud [29]. Monitoring almond flower bud dormancy through *DAM* expression could be used to determine the suitable moment to apply these biostimulants.

5. Conclusions

The estimation of chill accumulation using different models showed that the 2015–2016 season was warmer than the 2016–2017 season, and this was reflected in the dormancy release date of the cultivars. This illustrates the risk of growing extra-late cultivars in warm-winter areas, as production could be negatively affected if chilling requirement is not satisfied. The endodormancy to ecodormancy transition involves a transcriptional reprogramming in which genes acting on dormancy maintenance would be downregulated. This seems to be the case of *PdDAM6* for almond.

Author Contributions: A.S.P. and P.M.-G. participated in the design and coordination of the study. F.D. and P.M.-G. collaborated in the fieldwork. A.S.P. carried out the qPCR and cloning protocols. A.S.P., F.D. and P.M.-G. carried out data analysis. A.S.P., F.D. and P.M.-G. participated in the manuscript elaboration and discussion.

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Conflicts of Interest: The authors declare no conflicts of interest.

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