Cold acclimation and floral development in almond bud break: insights into the regulatory pathways

Pedro M. Barros, Nuno Gonçalves, Nelson J.M. Saibo and M. Margarida Oliveira

Genomics of Plant Stress Laboratory (GPlantS), Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal and IBET, Apartado 12, 2781-901 Oeiras, Portugal

* To whom correspondence should be addressed. E-mail: mmolve@itqb.unl.pt

Received 26 February 2012; Revised 24 April 2012; Accepted 26 April 2012

Abstract

In temperate fruit trees, seasonal dormancy and cold acclimation have a major impact on blooming time and, consequently, fruit production. To gain insight into the still unclear molecular processes underlying blooming, expression of genes putatively involved in the cold response was studied in almond (Prunus dulcis Mill.), which is the earliest fruit tree in the family Rosaceae to bloom. The transcript levels of two C-repeat binding factor (PdCBF) genes and one of their putative targets, PdDehydrin1 (PdDHN1), were analysed in flower buds and shoot internodes during seasonal dormancy up to bud break. In parallel, expression of candidate genes related to flower development was also followed. In a 2-year study, PdCBF2 showed a progressive increase in transcript abundance during the autumn in close correlation with cold acclimation, while high transcript levels of PdCBF1 and PdDHN1 were already found by summer. After bud break, with temperatures still within the chilling range, both PdCBF genes and PdDHN1 were found to sharply reduce transcription in flower buds and internodes, suggesting damping of CBF-mediated cold signalling during growth resumption, in correlation with cold hardness decline. Flower bud break was also followed by a decrease in the expression of PdGA20OX, a candidate gene involved in gibberellin biosynthesis, and an increase in the expression of two homeotic genes related to floral organ development, PdMADS1 and -3. These genes may also be relevant players in the regulation of anthesis in this model Rosaceae species.

Key words: Bud break, CBF genes, deacclimation, Dehydrin1, flower development, Prunus dulcis.

Introduction

Perennial trees are able to induce dormancy of actively growing tissues in order to cope with the more extreme environmental conditions occurring during autumn and winter. The correct timing for dormancy induction and release is critical in plant adaptation and survival in perennials (Tanino et al., 2010). After an active growth period in summer, specific photoperiod and/or temperature conditions are determinant environmental cues for bud set, growth cessation, and dormancy (Nitsch, 1957; Tanino et al., 2010). Autumn/winter dormancy may be divided in two distinct and successive stages. Endodormancy is induced in early autumn as a result of physiological changes that inhibit bud development, even under the occurrence of growth-conducive conditions. Endodormancy release is only achieved after sufficient exposure to chilling temperatures, mainly during winter (Perry, 1971). At this stage, growth ability is regained, but bud growth is still prevented by unfavourable environmental conditions. This stage is known as ecodormancy and lasts until exposure to growth-promotive conditions.

Dormancy induction and maintenance in woody plants involve extensive reprogramming of transcriptional and metabolic pathways (Schrader et al., 2004; Bassett et al., 2006; Druart et al., 2007; Renaut et al., 2008; Karlberg et al., 2010), in addition to morphological and physiological changes in meristems and surrounding tissues, which prevent cell growth and proliferation (van der Schoot and Rinne, 2011). Modulation of gibberellic acid (GA) metabolism has been also correlated with short-day (SD)-
induced seasonal dormancy. In poplar, cellular GA levels were shown to be rapidly downregulated during SDs (Olsen et al., 1997), being related to growth cessation (Eriksson et al., 2000). Moreover, induction of abiotic stress-regulated genes was also observed in response to SDs, prior to cold exposure, in trees such as poplar (Druart et al., 2007; Rohde et al., 2007; Ruttink et al., 2007), Norway spruce (Asante et al., 2011), and peach (Bassett et al., 2006). This response may be explained by a decrease in water content and osmotic potential detected during dormancy induction (Rinne et al., 1998; Welling et al., 2002, 2004).

After dormancy induction, the decrease in average temperatures in autumn leads to an increase in cold hardiness and freezing tolerance, reaching maximum levels during winter (Rinne et al., 1998). Low-temperature signalling pathways mediated by the C-repeat binding factor (CBF)/DREB1 family of transcription factors (TFs) are obvious candidate players in this process. The major insight into the role of CBF TFs during seasonal development in woody plants came from studies in controlled environments during short-term exposure to cold stress (e.g. Benedict et al., 2006; El Kayal et al., 2006; Welling and Palva 2008; Navarro et al., 2009; Wisniewski et al., 2011; Barros et al., 2012). In these studies, CBF genes were rapidly induced after a temperature decrease, with transcript accumulation generally peaking after 2 h and decreasing to a lower level after 24 h of exposure to a constant temperature. Additionally, transcriptional induction of CBF genes by cold stress was shown to be differentially regulated by photoperiod (El Kayal et al., 2006; Welling and Palva, 2008) or between tissues (Benedict et al., 2006). Considering that, within the same species, different CBF genes showed different levels and patterns of accumulation (Benedict et al., 2006; Welling and Palva 2008; Navarro et al., 2009; Wisniewski et al., 2011), it is easy to predict that the role of each CBF could be slightly different during specific developmental stages. However, little is known about the role of specific CBF genes during autumn and winter in response to the fluctuating environmental conditions to which field-grown trees are exposed. A major contribution to understand the role of low-temperature signalling pathways came from the study of cold-regulated (COR) genes, which play a fundamental role in stress tolerance and are deduced targets for CBF TFs. Particularly, Dehydrin-like (DHN) transcripts or corresponding protein products were shown to accumulate during endodormancy or in response to chilling (e.g. Artlip et al., 1997; Welling et al., 2004; Wisniewski et al., 2006). In poplar cambial meristems, transcription induction of COR genes, during cold-hardiness development in autumn could be grouped according to specific timing of induction (Druart et al., 2007). This supported the hypothesis of a stepwise development of cold hardiness, sequentially induced by SDs and a temperature decrease (Druart et al., 2007).

Transition from endo- to ecodormancy is achieved according to plant-specific chilling requirements. Recent evidence indicates that GA biosynthetic genes are induced by long-term chilling exposure in dormant buds, being associated with the acquisition of growth ability (Druart et al., 2007; Karlberg et al., 2010; Rinne et al., 2011). In poplar vegetative buds, GAs are further involved in a growth-promotive pathway by restoring the symplastic transport and signalling, which was previously blocked during dormancy induction (Rinne et al., 2011). In addition, dormancy-activity transitions may affect the acclimation state observed in dormant buds, as growth resumption is also often related to a decrease in cold-hardiness levels, in part associated with tissue/cellular rehydration (Kalberger et al., 2006). Differential regulation of stress-related genes has also been observed after prolonged exposure to chilling temperatures (Karlberg et al., 2010). Consistent with this, expression of specific DHN genes also appears to be downregulated in buds during dormancy break (Welling et al., 2004; Yamane et al., 2006; Yakovlev et al., 2008; Garcia-Bañuelos et al., 2009).

Although the regulation of cold acclimation and dormancy in perennial plants has received increasing attention in recent years, the role of the molecular pathways involved in low-temperature signalling is still poorly understood. In fruit tree species of the family Rosaceae, flower initiation occurs in the year before blooming, during summer, and organogenesis inside flower buds is arrested during autumn and winter. The timing of dormancy break and anthesis, as well as the maturity of reproductive organs at this stage, may be critical factors influencing fertilization and fruit production in this species (Albarguerque et al., 2002; Ruiz et al., 2010; Campoy et al., 2011). Almond is the earliest fruit tree to bloom in winter/spring, but it also shows a wide adaptability to different environments (Alonso et al., 2010; Socias i Company and Felipe, 1992). In this work, the role of two almond CBFs was investigated over the natural autumn/winter dormancy period in adult almond trees selected for their early blooming. The expression pattern of four candidate genes involved in organ identity and GA metabolism was also studied with a focus on floral development prior to anthesis. Gene expression analysis performed in shoots and flower buds in two consecutive years showed that PdCBF1, PdCBF2, and PdDHN1 were active during the cold acclimation process, reducing expression after bud break. In flower buds, this response occurred in parallel with the induction of floral homeotic genes and a putative modification in the GA biosynthetic pathway. The role of temperature variation and deacclimation in gene expression during ecodormancy is further discussed.

Materials and methods

Plant material
Seasonal gene expression studies were performed using three adult almond trees growing in the Lisbon area (Monsanto Forest Park, Portugal, 38° 43' 28.34" N, 9° 11' 35.55" W). Sample collection was performed from late summer (September) to mid-winter (February), at approximately 15-d intervals, from 9 a.m. to 11 a.m. over 2 consecutive years – 2009/2010 (year I) and 2010/2011 (year II). Developing flower buds and 1-year-old shoot internodes were collected and immediately frozen on dry ice. To improve RNA yield and quality, brown scale leaves were removed from flower buds prior to freezing. Phenological stages after flower bud break occurring at the time of collection were recorded as: swollen bud (inner leaf scales visible), green tip (sepals visible), pink tip (petals visible), full bloom (partially and fully opened flowers) and petal fall (adapted from University of California Integrated Pest Management Program, 1985). RNA was extracted according to the method of Brunner et al. (2004) with minor modifications, treated with DNase I (Ambion® Turbo DNA-free™ kit; Applied Biosystems, Carlsbad, CA, USA) and quantified using Nanodrop (Thermo Scientific, Wilmington, DE, USA). Temperature records were obtained from the nearest meteorological station (38° 44' 35" N, 9° 13' 13" W; http://www.wunderground.com).
Semi-quantitative RT-PCR
Two micrograms of total RNA was used for cDNA synthesis with a Transcriptor High-Fidelity cDNA Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) in 10 \( \mu l \) total volume. cDNA samples were diluted 1:3 in sterile Milli-Q water and used as template for PCR in 20 \( \mu l \) total volume, using gene-specific primers (Table 1) and 1 U GoTaq \(^\text{®} \) DNA polymerase (Promega Corp., Madison, WI, USA), according to the manufacturer’s instructions. \( PdCBF \) genes were amplified using 2 \( \mu l \) cDNA template, and 1 \( \mu l \) was used for the remaining genes under study. PCR was performed as follows: 5 min incubation at 95 °C, followed by 24–35 cycles of 30 s at 95 °C, 30–40 s at the appropriate annealing temperature (Table 1) and 40–60 s at 72 °C, with a final extension step for 5 min at 72 °C. Total reaction volumes were analysed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. Images were captured using the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Technical PCR replicates were repeated at least once for each gene. Amplicon intensities were quantified using ImageJ (http://imagej.nih.gov/ij/). The relative expression of each target gene was normalized for amplification of \( PdTubulin \) and \( PdActin \), which were reference genes for the flower buds and shoot internodes, respectively. \( PdTubulin \) was initially selected as the reference gene for both tissues, according to previous studies in peach (Li et al., 2009). However, this gene showed some variation between replicate trees in shoot internodes, which was not observed with \( PdActin \).

Results
Seasonal development of almond trees in two consecutive years
To assess the putative role of \( PdCBF \) genes and other candidate genes during natural dormancy and the cold acclimation period, three almond trees growing naturally in Monsanto forest were selected to study flower development over two consecutive years. These trees, although growing in a feral state, showed similar phenological development and early flowering habit. According to the temperature records, the collection period in year II (2010/2011) was on average colder than in year I (2009/2010), particularly during autumn. In fact, chilling accumulation (number of hours below 7.2 °C; Weinberger, 1950) started earlier in year II, and was closely followed by that of year I, throughout the sample collection period (Supplementary Fig. S1 at JXB online). Timing of bud break in vegetative and flower buds was determined by macroscopic observation of bud morphology. In year I, leaves started to emerge from vegetative buds mainly in late January, while in year II this occurred approximately 2 weeks earlier (Fig. 1). In both years, flower bud break (occurrence of swollen buds, up to the emergence of inner green scales) was detected by December (Table 2; Fig. 1). During late December to early January, the phenological stages observed were closely similar in both years.

Table 1. Primer sequences used for gene expression analysis by semi-quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene (accession no.)</th>
<th>Sequence (5'→3') Annealing (ºC) PCR cycles Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( PdCBF1 ) (JQ317157)</td>
<td>F: CCGCCTAAATGACAGTCCCTCTTCTCA 58 32 580</td>
</tr>
<tr>
<td>( PdCBF2 ) (JQ317158)</td>
<td>R: TTTCTTCTCTTATCCTTCTCTCA 58 32 540</td>
</tr>
<tr>
<td>( PdDHN1 ) (JQ317156)</td>
<td>F: TGGTGCTTTCTTCTTGCTCTTCTTC 62 23 329</td>
</tr>
<tr>
<td>( PdGA20OX ) (JQ412172)</td>
<td>R: TAGTGATTTTTCTGTTGTCACTGT 58 28 559</td>
</tr>
<tr>
<td>( PdGA20OX ) (ppa084144m)*</td>
<td>F: AAGGAGGGCGACACTTCCTCTCTCTTCTTC 58 28 466</td>
</tr>
<tr>
<td>( PdMADS1 ) (AY947462)</td>
<td>R: TGATCAGGTTGACGGCTGAAAT 58 28 737</td>
</tr>
<tr>
<td>( PdMADS3 ) (AY947464)</td>
<td>F: CCGAGGAACTGAAAGTAAAGGT 58 24 800</td>
</tr>
<tr>
<td>( PdActin1 ) (AM91134)</td>
<td>R: TTYCAGGCTTGCTCTCTGCTCTCC 58 24 385</td>
</tr>
<tr>
<td>( PdTubulin ) (X67162)</td>
<td>F: ATTCAGGGCCACCATTCACAC 58 24 428</td>
</tr>
</tbody>
</table>

* International Peach Genome Initiative (www.rosaceae.org).

Corresponding GenBank accession numbers, annealing temperatures, number of PCR cycles, and amplicon sizes are shown for each gene. F, Forward primer; R, reverse primer.

Fig. 1. Representative developmental stages of vegetative and reproductive buds observed from December to early February in years I and II. Arrows indicate vegetative buds.
Table 2. Flower bud developmental stages occurring after bud break in both years in all trees sampled

<table>
<thead>
<tr>
<th>Stage</th>
<th>Year I</th>
<th>Date</th>
<th>Year II</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>23 Dec</td>
<td>08 Jan</td>
<td>06 Jan</td>
</tr>
<tr>
<td>b</td>
<td>21 Jan</td>
<td>03 Feb</td>
<td>20 Jan</td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>20 Dec</td>
<td>04 Feb</td>
</tr>
<tr>
<td>d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Phenological development was divided as follows: (a) swollen bud; (b) green tip (visible calyx); (c) pink tip (visible corolla); (d) blooming (open flower); (e) petal fall. Black squares represent the predominant stage (>50%) and grey represent the other stages scored (<50%).

(65x763)

(Table 2). However, blooming (considered here as >50% anthesis) occurred earlier in year II compared with year I (Table 2). The early fulfilment of chilling requirements and the mild temperature periods occurring during late December to early January in year II (Supplementary Fig. S2 at JXB online) are suggested as promoters of this advanced development.

Expression pattern of PdCBF genes in young shoots during natural cold acclimation

Under controlled environmental conditions, PdCBF1 and PdCBF2 genes have been shown to be induced following low-temperature exposure (below 12 °C) (Barros et al., 2012). To assess the putative role of PdCBF genes during natural cold acclimation, their seasonal transcript expression was studied in adult almond trees. One-year-old shoot internodes were collected from late summer to mid-winter at regular intervals. The mean gene expression was compared with the daily minimum temperature, which occurred 3–4 h prior to sample collection. During year I, PdCBF1, PdCBF2, and PdDHN transcripts showed increased levels during mid-autumn stages (after 5 November), in agreement with the decrease in minimum temperatures to levels below 15 °C (Fig. 2A). After 8 January, and up to the end of sample collection, the expression levels of PdCBF1, PdCBF2 and PdDHN1 appeared to decrease, although temperatures were still around 10 °C (Fig. 2A). However, these observations were not validated by statistical analysis due to the variability in expression occurring between trees (Supplementary Fig. S3A). Nevertheless, replicate expression analysis conducted during year II using two of the trees used in year I again showed a putative induction of at least PdCBF2 during early autumn (Fig. 2B). In addition, PdCBF2 transcript accumulation during the autumn showed an interesting variation, which agreed with the temperature variation recorded for specific dates, particularly from November to December (Fig. 2B). Moreover, the transcript accumulation of PdCBF1, PdCBF2, and PdDHN1 was also shown to decrease in the late collection stages in January. In both years, the decrease in gene expression during the winter stages agreed with the timing of vegetative bud break.

PdCBFs expression is repressed after flower bud break

Gene expression in flower buds was determined from November to February. In year I, from November to early December, expression of PdCBF1, PdCBF2, and PdDHN1 was somewhat constant in the stages prior to bud swelling (Fig. 3A). In year II, expression of PdCBF1 and PdCBF2 showed a higher variation between the three time points analysed during the same period (Fig. 3B), which closely reflected differences in minimum temperatures recorded between collection dates. In fact, the expression pattern of PdCBF2 in flower buds was very similar to that observed in shoot internodes during the same collection trial (Fig. 2B). These results seemed to reinforce the active environmental regulation of PdCBF genes, especially PdCBF2. During the initial stages of bud break, occurring in year I from late December to early January, PdCBF2 and PdDHN1 transcript levels were similar to previous stages but decreased significantly when petals started to emerge from the calyx (Fig. 3A, Table 2). A similar decrease was detected 2 weeks earlier in year II (Fig. 3B), in agreement with the advanced phenological development observed in this year (Table 2). Thus, downregulation of PdCBF2 and PdDHN1 seems to anticipate the onset of anthesis. In turn, expression of PdCBF1 was not consistent between years, although it was also low prior to and/or after full-bloom stages (Fig. 3). In both years, the last time point (early February) was characterized by a sharp decrease in minimum temperatures. However, the transcript levels determined for PdCBF1, PdCBF2, and PdDHN1 were lower than those observed during cold acclimation stages in autumn. This observation suggests that cold-signalling pathways mediated by both PdCBF genes (which may include regulation of PdDHN1) are repressed after flower bud break. Interestingly, this repression agrees to some extent with the expression pattern of PdCBF genes and PdDHN1 determined for shoot internodes (Fig. 2).

Expression of two MADS-box genes is induced after bud break

To correlate the expression pattern of both PdCBF genes with developmental changes occurring in flower buds during dormancy/activity cycles, the expression of two homeotic MADS-box genes was also studied. PdMADS1 is a D-type MADS-box gene, expressed in carpels (Silva et al., 2007), homologous to the peach PPERSTK gene (99% amino acid identity; data not shown) (Tani et al., 2009) and the Arabidopsis thaliana SEEDSTICK (STK) gene (Rounsley et al., 1995), both specifically expressed during ovule development. PdMADS3 belongs to the E-type subgroup of MADS-box genes, which are expressed specifically in flowers in all the whorls (Silva et al., 2007). In both years, PdMADS1 tran-
Script accumulation was only detected after bud break (Fig. 4A, B), mainly from the green-tip stage (Table 2). This late-stage expression pattern may correlate with the beginning of ovule development. In turn, *PdMADS3* expression was detected at low levels from November to early December but was gradually upregulated during bud break up to the onset of blooming (Fig. 4A, B). These results revealed that both genes could be candidate markers of specific changes in tissue differentiation and specialization occurring after ecodormancy break up to anthesis.

Two genes involved in GA metabolism are differentially regulated during bud break

In peach, endogenous GA levels are fine-tuned during dormancy/activity transitions in flower buds (Reinoso et al., 2002a). To elucidate the role of GAs in almond flower bud development, the expression of two genes for GA metabolism, *PdGA20OX* and *PdGA2OX*, was followed from autumn to winter, up to anthesis. *PdGA20OX* is a close homologue of the GA 20-oxidase family in *Arabidopsis*, involved in the late steps of GA biosynthesis (Olszewski et al., 2002). In both years, *PdGA20OX* expression was found prior to, and shortly after, bud break (Fig. 4A, B). However, in the later stages, mainly after the transition from green-tip to pink-tip stages (Table 2), *PdGA20OX* transcript accumulation was reduced or not detected. As this transition occurred later in year I, *PdGA20OX* transcription was still detected in early January (Fig. 4A), while in year II expression levels were already reduced at this stage (Fig. 4B). Bioactive GAs are regulated by both the rate of their synthesis and the conversion into inactive forms, a reaction catalysed by GA 2-oxidases (Olszewski et al., 2002). Expression of a *PdGA2OX* candidate gene was observed in all the flower bud stages sampled in this study (Fig. 4A, B). Transcript levels showed an increase but the trend was not statistically significant, probably due to the variability in expression observed between trees (Supplementary Fig. S5 at JXB online). Nevertheless, the distinct expression pattern of both *PdGA20OX* and *PdGA2OX* suggested that, prior to anthesis, there is a genetic reprogramming of the GA metabolic pathway.

Discussion

The involvement of the CBF TFs during winter dormancy has received some attention in perennial plants, particularly in temperate fruit trees. However, there is still little information regarding the role of these TFs over extended periods of time or
During dormancy/activity transitions in perennials. In this study, the expression of *PdCBF1* and *PdCBF2* was analysed in tissue samples collected from adult almond trees growing under natural conditions, from late summer to mid-winter.

Expression of *PdCBF1* was detected in the late summer stages, while *PdCBF2* expression was detected mainly during autumn, in agreement with a decrease in average temperatures and the onset of chilling. In turn, expression of the predicted target of the *PdCBF* genes, *PdDHN1* (Barros et al., 2012), was also detected at high levels during late summer. In poplar, SD-induced growth cessation and dormancy was enough to activate the expression of several cold-regulated genes and increase cold hardiness, without prior exposure to cold temperatures (Welling et al., 2002; Druart et al., 2007; Rohde et al., 2007; Ruttink et al., 2007). In several *Prunus* species, the induction of growth cessation and dormancy by SD photoperiod is highly dependent on species/cultivar-specific low-temperature perception (Heide, 2008). In peach, continuous growth was observed at a 23/17 °C day/night temperature in a 16 h photoperiod, while at the same temperatures, but with a photoperiod of ≤13 h, they ceased growth and entered dormancy (Went, 1957, in Heide, 2008). In the current study, during the first half of September, the day length was approximately 12.5 h, with average temperatures ranging from 29 °C (maximum) to 18 °C (minimum) (data not shown). These conditions may have been enough to trigger dormancy development in this species, but further confirmation is required. In peach, expression of the *PdDHN1* homologue was also observed during late summer in bark and root tissues from field-grown trees (Artlip et al., 1997; Wisniewski et al., 2006; Bassett et al., 2009), but this gene was not responsive during dormancy induction under controlled conditions (SD treatment at 25 °C; Wisniewski et al., 2006). These authors suggested that the lack of transcriptional activation of *PpDHN1* could be related to an insufficient decrease in water content under the tested conditions (Wisniewski et al., 2006). In turn, the involvement of a peach CBF gene (*PpCBF1*) in SD-dependent dormancy induction was recently proposed (Wisniewski et al., 2011), but BLAST analysis on the peach genome sequence database.

![Figure 3](JXBonline.png)

**Fig. 3.** Seasonal gene expression patterns obtained for *PdCBF1*, *PdCBF2*, and *PdDHN1* in flower bud samples, collected from adult almond trees during autumn and early winter development in (A) year I and (B) year II. The transcription level for each gene was calculated by quantification of band intensities obtained from semi-quantitative RT-PCR and normalized against *PdTubulin*. Data represent means ± standard deviation (n=2 in Nov/05; n=3 in the remaining collection dates). Different letters indicate statistical significant differences based on Student’s t-test (P <0.05) between time points with n=3 replicates. Minimum temperatures determined for each date are indicated as open triangles. Representative gels obtained for each tree are shown in Supplementary Fig. S4 at *JXB* online.
showed that PdCBF1 and PpCBF1 are not direct homologues (Supplementary Fig. S6 at *JXB* online; Barros et al., 2012). Therefore, while the effect on dormancy induction may not yet be discarded, the expression of PdCBF1 and PdDHN1 during late summer could be related to other stress conditions, such as drought or oxidative stress.

During the autumn (October–December), PdCBF2 transcript accumulation in both shoot internodes and flower buds showed an association with the minimum temperatures at each time point assessed, with higher levels being detected when minimum temperatures were close to or below 12 °C. This observation was particularly obvious in year II, in which minimum temperatures were more variable during that period. Under controlled conditions, CBF genes are rapidly upregulated by cold, returning to lower/basal levels within 24 h of exposure to a constant temperature (Benedict et al., 2006; El Kayal et al., 2006; Welling and Palva, 2008; Navarro et al., 2009; Wisniewski et al., 2011; Barros et al., 2012). In the present study, samples were collected 3–4 h after dawn (shortly after the night minimal temperatures). However, under field conditions, it is difficult to conclude whether the measured transcript levels of PdCBF2 resulted from the minimum temperature reached shortly prior sample collection or from the temperature decline verified during the previous night. Further expression studies using potted trees grown under different

![Fig. 4.](http://www.rosaceae.org/peach/genome) Seasonal gene expression analysis of genes related to floral organ development and GA metabolism in flower buds, collected from adult almond trees during autumn and early winter development in (A) year I and (B) year II. PdMADS1, PdMADS3, PdGA20OX, and PdGA2OX transcript levels were determined by quantification of band intensities obtained from semi-quantitative RT-PCR, and normalized against PdTubulin. Data represent means ± standard deviation (*n* = 2 in Nov/05; *n* = 3 in the remaining time points). Different letters indicate statistical significant differences based on Student’s t-test (*P* < 0.05) between time points with *n* = 3 replicates. Representative gels obtained for each tree are shown in Supplementary Fig. S5.
temperature conditions may help to clarify this issue. Nevertheless, *PdCBF2* appears to be an important player in temperature signaling in almond during the cold acclimation initiated in autumn.

Bud break and growth resumption in vegetative and reproductive buds occurred very early in the almond trees under study, confirming their reduced temperature requirements for dormancy break. Flower bud break was characterized by the transcriptional induction of two floral homeotic genes, *PdMADS1* and *PdMADS3*. These genes are candidate members of the flower *MADS-box* gene family, which includes well-known players regulating meristem identity and floral organ development in model plants, organized according to the ABCDE model (Immink et al., 2010). *PdMADS1* is a carpel-specific expressed gene (Silva et al., 2007), a homologue of *PPERSTK*, which belongs to the ovule specific D-class of *MADS-box* genes in peach (Tani et al., 2009). In *Arabidopsis*, the homolog *STK* gene is required for normal development of the funiculus, during the emergence of ovule primordia (Pinyopich et al., 2003). In turn, *PdMADS3* belongs to the E-class, also known as *SEPALATA* (*SEP*) genes, which are necessary for the function of A-, B-, and C-class genes (Pelaz et al., 2000; Honma and Goto, 2001; Ditta et al., 2004), which are expressed in all floral whorls (Silva et al., 2007). According to Reinoso et al. (2002b), peach flower buds show continuous development during late autumn and winter. Sterile whorls differentiate rapidly in late summer through to early autumn, arresting their development in autumn/winter dormancy, while the reproductive organs differentiate and develop throughout winter (Reinoso et al., 2002b). Assuming a similar developmental pattern in almond, these morphological changes could relate to the residual but increasing expression levels of *PdMADS3* observed during November and early December. The high transcript levels observed after bud break may support the rapid development of sepalas and petals, leading to anthesis. In *Prunus* spp., meagorphogenesis and further ovule maturation is often observed close to, or during, anthesis (Alburquerque et al., 2002; Ruiz et al., 2010). Thus, different stages of ovule maturity at anthesis are often observed in *Prunus* spp. cultivars (Pimienta and Polito, 1983; Egea and Burgos, 2000; Alburquerque et al., 2002; Ruiz et al., 2010), which may impact on fertilization and fruit set. In the present study, *PdMADS1* transcription was only detected in the final stages of development, when flower buds were in the green-tip stage. This suggests that *PdMADS1* could be an interesting marker of ovule emergence.

Exogenous applications of GAs often induce dormancy break in a wide variety of woody angiosperms (Looney, 1997). However, GA application in flower buds may have contrasting effects (Jiménez et al., 2010; Yamane et al., 2011). Thus, it would be interesting to investigate further whether downregulation of both *PdCBF* genes in winter is also caused after prolonged chilling exposure.
Expression of PdDHN1 in autumn/winter development closely resembles that observed for PpDHN1 in peach bark tissues (Artlip et al., 1997; Wisniewski et al., 2006). In this species, distinct patterns of expression were observed between sibling evergrowing (non-dormant) and deciduous genotypes, as, in the former, the induction of PpDHN1 was delayed in autumn and its accumulation declined earlier in winter (Artlip et al., 1997). This pattern was associated with the absence of terminal growth cessation and with the reduced levels of cold hardiness observed in the evergrowing plants (Arora et al., 1992; Artlip et al., 1997). The almond trees analysed in the present study showed terminal and axillary bud set and growth cessation, but PdDHN1 expression was also shown to decline in early winter. Although the levels of cold hardness of the studied trees during seasonal dormancy were not assessed, according to temperature records, the decline in PdCBF1, PdCBF2, and PdDHN1 expression after bud break followed, in both years, a period in which temperatures reached deep chilling, increasing thereafter (Supplementary Fig. S2). As reviewed by Kalberger et al. (2006), resistance to deacclimation tends to decrease during dormancy/activity transitions, and loss of cold hardness is usually related to growth resumption. The negative effects of growth and development in cold hardness are related to changes in subcellular structure caused, for example, by the increase in water content and increased vacuolar volume that accompanies cell expansion (Kalberger et al., 2006). These changes will greatly increase frost sensitivity and, as active growth may also compete for energy resources, the plant’s ability to reinduce cold acclimation is further compromised. Thus, the negative regulation of stress-responsive genes (including CBF regulons), which are usually involved in defining the chilling response, is also likely to occur during growth initiation.

The present study provides the first molecular evidence for the role of CBF-mediated low-temperature signalling in fruit tree flower buds, also indicating putative markers to study the complex network of events occurring prior to anthesis. The results are summarized in a comprehensive scheme in Fig. 5. During the cold acclimation stage in autumn, which will overlap sequentially with endo- and ecodormancy periods, PdCBF2 may play an active role in temperature signalling, responding to the temperature variations occurring during these stages, and probably contributing to maintain the active expression of PdDHN1 (and other members of the regulon). In turn, the role of PdCBF1 remains inconclusive, as it may be regulated by additional factors. During ecodormancy, while chilling exposure represses growth, the occurrence of promotive temperatures induces growth and leads to a decrease in cold hardness, which may be greatly due to a decrease in the expression of cold-regulated genes (or at least the PdCBF gene regulon). The timing of bud break and growth resumption is also pinpointed by the specific upregulation of PdMADS1 and PdMADS3, related to floral organ development, and by the downregulation of PdGA20OX, which could be a prerequisite for anthesis. At this stage, reacclimation capacity also seems to be hampered due to active growth (Kalberger et al., 2006), while reinduction of PdCBF1 and PdCBF2 in response to chilling may be repressed.

![Fig. 5. Schematic representation of the control of cold acclimation and dormancy break in almond flower buds and shoots. PdCBF2 may be involved in cold acclimation after endodormancy induction, playing a role in environmental signalling, mainly through low temperatures. Chilling temperatures (CHILL) play a role in determining the timing of endodormancy break and re-establishment of growth ability, according to plant-specific chilling requirements. During the following ecodormancy stage, chilling represses growth until warm promoting temperatures (HEAT) occur. When temperature requirements for ecodormancy break are met, growth resumption occurs leading to bud break. At this stage, the plant’s ability to maintain cold hardness decreases (Kalberger et al., 2006), which is probably induced by the downregulation of PdCBF2 and PdDHN1 expression, among other putative players. After this stage, chilling temperatures may occur, but this may only have a small effect on the induction of PdCBF2 and PdDHN1 (dashed arrows). In flower buds, bud break is characterized by an increase in PdMADS3 expression. At later stages, prior to anthesis, PdMADS1 is also induced, in agreement with the predicted timing of ovule emergence. During ecodormancy and bud break stages, there are also changes in GA levels (arrow boxes, Luna et al., 1998), which was supported by the decreased expression of PdGA20OX, a candidate gene in GA biosynthesis. The putative induction of PdGA20OX expression (related to GA catabolism) after bud break requires further confirmation.](image-url)
Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1 Cumulative hours below 7.2°C (chill hours) measured from November to February, in year-I and year-II.

Supplementary Fig. S2 Maximum and minimum temperatures (°C) recorded from November to February, in year-I and year-II.

Supplementary Fig. S3 Seasonal gene expression patterns of PdCBF1, PdCBF2 and PdDHN1 in one-year-old shoots obtained from each replicate tree in year-I and year-II.

Supplementary Fig. S4 Seasonal gene expression patterns obtained for PdCBF1, PdCBF2 and PdDHN1 in flower bud samples from each replicate tree in year-I and year II.

Supplementary Figure S5. Expression analysis of genes related to floral organ development and GA metabolism in flower buds from each replicate tree.

Supplementary Fig. S6. Phylogenetic tree obtained for peach and almond CBFs already identified and the five CBF-like proteins predicted from the peach genome database.

Acknowledgments

This work was supported by a PhD fellowship (Ref. SFRH/BD/31594/2006) awarded to P.M.B., from the Fundação para a Ciência e Tecnologia (FCT). N.J.M.S. was supported by Programa Ciência 2007, financed by POPH (QREN). This work was also supported by FCT through grant # PEst-OE/EQB/LA0004/2011. We gratefully acknowledge Dr Ana Paula Farinha for the valuable suggestions that helped to improve the manuscript.

References


Alonso JM, Espiau MT, Socías i Company R. 2010. Increase in the chill and heat requirements for blooming of the new almond cultivars. Options Méditerranéennes (Series A) 94, 65–69.


Regulation of floral bud break in almond | 4595


Tanino KK, Kalcksl S, Silim S, Kendall E, Gray GR. 2010. Temperature-driven plasticity in growth cessation and dormancy development in deciduous woody plants: a working hypothesis suggesting how molecular and cellular function is affected by
temperature during dormancy induction. Plant Molecular Biology 73, 49–65.


