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The identification of almond *GIGANTEA* gene and its expression under cold stress, variable photoperiod, and seasonal dormancy

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Abstract

Seasonal growth is characteristic for many tree species including almond. Varying conditions during the season are responsible for growth cessation, bud set, dormancy entry, cold hardening, and bud burst. Here, we report the characterization of an almond homologue of the *Arabidopsis GIGANTEA* (*AtGI*) gene (designated as *PdGI,* GenBank accession No. KJ502316). We propose a role for this gene in the transition to dormancy and cold acclimation. The complementary DNA (cDNA) sequence of *PdGI* was 4 322 bp long and contained an open reading frame of 3 512 bp. The deduced amino acid sequence of PdGI shared 76 % identity with AtGI. The expression of *PdGI* at ambient day/night temperatures of 22/20 ºC was differentially regulated under a 16-h or 12-h photoperiod, increasing during the day and decreasing after dusk. However, this diurnal regulation was disrupted when plants were transferred to cold (12 ºC) conditions. In addition, we have assessed the expression of *PdGI* and putative almond homologues of the downstream target genes *CONSTANS* (*PdCO-like*) and *FLOWERING LOCUS T* (*PdFT-like*) in flower buds and shoots from adult trees during the bud break period in autumn and early winter. Our results show a clear increase in transcript abundance towards anthesis, suggesting a role of these genes in flower development.

Additional key words: bud break, flowering, *Prunus dulcis*, seasonal development.

Introduction

In model plants such as *Arabidopsis*, the photoperiodic pathway regulating flowering transition involves *GIGANTEA (GI), CONSTANS (CO)*, and *FLOWERING LOCUS T (FT)* genes. The *CO* and *FT* are placed in a functional hierarchy with *GI* as the earliest acting gene (Kobayashi *et al.* 1999, Samach *et al.* 2000, Suárez-López *et al.* 2001, Mizoguchi *et al.* 2005). Experiments using mutants impaired in circadian clock function have demonstrated that *GI* acts between the circadian oscillator and *CO* to promote flowering by increasing *CO* and *FT* mRNA abundance (Mizoguchi *et al.* 2005). GIGANTEA is a nuclear-localized protein (Huq *et al.* 2000, Mizoguchi *et al.* 2005, Hong *et al.* 2010) of unknown biochemical function. It is identified as a key regulator in the perception of circadian rhythms and in the photoperiodic control of flowering (Araki and Komeda 1993, Park *et al.* 1999) as *Arabidopsis gi* mutants show delayed flowering under both long-day and short-day conditions (Fowler *et al.* 1999). Several reports have shown that the effect of GI in the CO/FT regulatory module may occur at multiple levels, such as protein-protein (Kim *et al.* 2007, Sawa *et al.* 2007) or protein-DNA interactions (Sawa and Kay 2011). Furthermore, Sawa and Kay (2011) showed that GI could also interact with *FT* repressor genes. Interestingly, GI was also shown to directly activate *FT* gene expression independently of *CO*, through binding to the *FT* promoter regions under short-day conditions (Sawa and Kay 2011).

The expression of *GI* is under control of the circadian clock and peaks at the end of the day. However, Cao *et al.* (2005) reported that low temperatures could also induce *GI* transcription. Moreover, the *gi-3* loss of

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Abbreviations: AtGI - *Arabidopsis GIGANTEA*; *CO* - *CONSTANS*; CRs - chilling requirements; *FT* - *FLOWERING LOCUS T*; *PdGI* - *Prunus dulcis GIGANTEA*; RT-PCR - reverse transcription polymerase chain reaction.
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function mutant line shows an increased sensitivity to freezing stress but no change in transcriptions of several cold-responsive genes from the C-Repeat Binding Factor (CBF)-dependent pathway (Cao *et al.* 2005). These authors proposed that GI positively regulates cold tolerance *via* a CBF-independent pathway.

GIGANTEA is highly conserved among seed plants including monocotyledonous, such as *Oryza sativa* (Hayama *et al.* 2002) and *Brachypodium distachyon* (Hong *et al.* 2010), and gymnosperms such as *Pinus taeda* and *Piceae abies* (Chen *et al.* 2014). Heterologous expression of the *B. distachyon GI* gene in a *gi-2* deficient *Arabidopsis* mutant rescues efficiently the late flowering phenotype. This suggested that the role of the *GI* in flower induction could be conserved in both species (Hong *et al.* 2010).

In boreal and temperate zones, continuous plant growth and development is incompatible with the perennial habit, given the variable and sometimes extreme conditions to which they can be exposed during the year. Therefore, adaptation and survival of perennial plants in these regions requires developmental transitions between active growth and dormant stages in synchrony with the surrounding environment. Some connections with the well-known flowering induction pathways from annual herbaceous models have been identified as well as other perennial-specific adaptations (Horvath 2009, Van der Schoot and Rinne 2011).

Almond (*Prunus dulcis* Mill) belongs to the *Rosaceae* family and is one of the economically important and widely grown fruit tree species in temperate climates. As in other fruit species, floral initiation and development in almond is characterized by a number of distinct stages occurring during the year (Reinoso *et al.* 2002). Flower initiation occurs in late summer (the year before blooming) (Lamp *et al.* 2001), but organogenesis inside flower buds is arrested during winter. Chilling requirements (CRs) for dormancy break prevent trees from initiating growth in response to transient promotive warm temperatures occurring in early winter. The CRs have been reported as the major factor determining blooming date in almond (Egea *et al.* 2003, Sánchez-Pérez *et al.* 2011). In spite of showing a wide adaptability to different environments (Alonso *et al.* 2010), almond is the earliest *Prunus spp.* to bloom in winter/spring. Because of this earliness, short periods of low temperatures occurring after bud break can be highly damaging to the flower buds, decreasing almond production yields.

The genetic factors determining bud break in temperate fruit tree species, such as almond, are poorly understood. In our study, we report the cloning and characterization of the almond homologue of *GIGANTEA* (named *PdGI*) and unveil its expression pattern under different photoperiod and environmental conditions. Additionally, we analyzed the expression of *PdGI* together with *PdCO* and *PdFT* homologues in field-grown almond trees from mid-autumn to mid-winter.

Materials and methods

Plants and sample collection: Almond (*Prunus dulcis* Mill cv. Verdeal) shoots were obtained from axillary buds of adult trees and cultivated *in vitro* in Murashige and Skoog basal medium, supplemented with 1.33 μM N^6-benzyladenine, 0.049 μM indole-3-butyric acid, 20 g dm^{-3} sucrose, and 7 g dm^{-3} agar (Miguel *et al.* 1996). Shoots were subcultured every 3 weeks on new medium and maintained under long day (LD; 16-h photoperiod), an irradiance of 100 μmol m^{-2} s^{-1}), and day/night temperatures of 22/20 °C.

Genomic DNA was extracted as described by Martins *et al.* (2003). Expression analysis was performed on 2-week-old almond shoots grown *in vitro* at different photoperiods and temperatures. For short-day (SD) treatments, plants were previously adapted to a 12-h photoperiod at 22/20 °C for 5 d. Cold stress was applied at dawn by transferring culture flasks to new growth chambers set at 12 ºC (under SD and LD, respectively). After 1, 2, 4, 8, 12, 16, 24, and 32 h of treatment, four plantlets were pooled and frozen in liquid nitrogen. RNA was extracted using the *RNeasy® Plant Mini* kit (*Qiagen*, Valencia, CA, USA), treated with RNAse-free DNase I (*Qiagen*, Hilden, Germany) following manufacturer's instructions, and quantified using *NanoDrop* (*Thermo-Scientific*, Wilmington, DE, USA) technology.

Seasonal gene expression studies in field conditions were performed using two adult almond trees growing in Monsanto Forest Park (Lisbon, Portugal, 38° 43' 28" N, 9° 11' 36" W). Sample collection was performed in 2009/2011, from mid-autumn (November) to mid-winter (February), at approximately 15 d intervals, 3 - 4 h after dawn, to reduce variations resulting from the circadian rhythm. Developing flower buds and 1-year-old shoot internodes were collected and immediately stored on dry ice. RNA extractions were performed as above with minor modifications [1 % (m/v) polyvinylpyrrolidone (PVP-40) was added to the extraction buffer, the homogenate was incubated with 0.4 vol. of 5 M potassium acetate (pH 6.5) on ice for 15 min]. Temperature records were obtained from the nearest meteorological station (38° 44' 35" N, -9° 13' 13" W, http://www.wunderground.com). Flower bud phenology was recorded considering the following stages: swollen bud - when inner leaf scales are visible, green tip - when calix is visible, pink tip - when corolla is visible, and full bloom - when there are partially and fully opened flowers [adapted from Felipe (1977)].

Isolation of the full-length cDNA and genomic sequence of *PdGI*: Total RNA obtained from a pool of samples collected under control conditions (LD, 22/20 ºC) was used to extract mRNA according to the protocol of *PolyATtract* mRNA isolation system (*Promega*, Madison, USA). First strand cDNA was synthesized with *Transcriptor High-Fidelity* cDNA synthesis kit (*Roche Diagnostics*, Mannheim, Germany) according to the manufacturer's instructions. A short stretch of *PdGI* cDNA was cloned initially using degenerate forward 5'-TTCCTCAGCVGTTGATYTKC-3' and reverse 5'-CTCATAWGARCTRTAACTCC-3' primers designed for the NSSAVDLP and WSYSSNE amino acid motifs, respectively. Degenerated primers were designed based on the alignment of conserved amino acid sequences of GIGANTEA from several plant species, using the *CODEHOP* program (Rose *et al.* 2003). The predicted amplicon (779 bp) was purified after 1 % (m/v) agarose gel electrophoresis, cloned into pCR2.1 vector (*Invitrogen*, Carlsbad, CA, USA), and sequenced (*Macrogen*, Seoul, Korea).

Full-length *PdGI* cDNA was cloned by following Rapid Amplification of cDNA Ends (RACE) protocol (Frohman *et al.* 1988) with modifications as described in Barros *et al*. (2012): the 3'-RACE was performed using a gene specific primer 3GSP (5'-GAATACTAG CCATTTTGGAGGC-3') designed from the previously identified *PdGI* fragment and 3'-RACE adaptor primer. For the 5'-RACE, first strand cDNA synthesis was primed by using a gene specific primer 5GSP1 (5'-GTT GCCCAAATCTGAAGCATC-3'). Given the predicted size of the unknown 5' coding sequence, 5'RACE was conducted in two stages. Thus, after oligo-dC tailing of cDNA, PCR was conducted using a forward degenerate primer (5'-GATGGACTGCAATTCTCTTCT-3' (for DGLQFSSLFWPP conserved motif) and a gene specific reverse primer 5GSP2 (5'-TGGCAACAATGATCTCAG GAAG-3') to amplify the remaining 5' coding sequence, which yielded a 1 500 bp fragment. The 5' UTR sequence was further amplified using a reverse primer (5'-GCT TCACCTCCGATGGATAA-3') designed for the previously cloned fragment and the 5'RACE adaptor primer and yielded a 700 bp fragment. All these fragments were overlapped to build the full-length *PdGI* cDNA. The genomic sequence of *PdGI* was obtained using several primer pairs designed for several overlapping fragments, which were amplified by PCR using genomic DNA (data not shown). All PCR products were gel purified, cloned to pCR2.1 vector (*Invitrogen*), and sequenced (*Macrogen*).

A phylogenetic tree was constructed based on the alignment of *PdGI* predicted peptide sequence with GI homologues from *Prunus persica*, *Malus × domestica*, *Pisum sativum*, *Glycine max*, *Ricinus communis*, *Arabidopsis thaliana*, *Lemna gibba*, *Allium cepa*, *Oryza sativa*, var. *japonica*, *Lolium perenne*, and *Picea abies* (used as outgroup). Multiple alignment was performed using *Clustal W* algorithm (Larkin *et al*. 2007) and a phylogenetic tree was constructed using the neighbour-joining method with *MEGA7* software (Kumar *et al*. 2016) with default parameters.

Southern-blot analysis was carried out following the method of Sambrook *et al.* (1989) using 10 μg of total DNA, digested with the enzymes *N*deI, *Eco*RI, and *Eco*RV. A 0.4 kb fragment from the 3'- region of the PdGI cDNA was used as a probe, containing no recognition sites for the previously mentioned enzymes. The probe was generated from cDNA by PCR using primers 5'-AAAGCCTGCCAAAGAAAATG-3' and 5'-TGCCTCAAGTGTGCTTCCAATGG-3'. Probe labeling by digoxigenin, hybridization, and detection were conducted according to the manufacturer's instructions (*Roche Diagnostics*).

Expression analysis of *PdGI*, *PdCO*, and *PdFT* by reverse transcription (RT)-PCR: Synthesis of cDNA was performed using 2 μg of purified total RNA from all the collected time points with *Transcriptor High-Fidelity* cDNA synthesis kit (*Roche Diagnostics*), according to the manufacturer's instructions. A set of primers (Table 1 Suppl.) designed from the 3'-end of the cDNA that includes an intronic area were used for expression studies with *PdGI*, *PdCO*, and *PdFT*. Specific primers for *PdCO-like* and *PdFT-like* genes were designed based on the corresponding sequences (acc. Nos. EMJ16712.1 and AEO72030.1) obtained from the peach genome database (http://www.rosaceae.org/species/prunus_persica/genome_v1.0). Predicted amplicons were cloned and sequenced for confirmation (data not shown). Sample of each cDNA (1 mm^3) was used as template for PCR, using gene-specific primers and 1 U of *GoTaq*® DNA polymerase (*Promega*), according to the manufacturer's instructions. PCR was performed as follows: incubation at 95 °C for 5 min, followed by 25 cycles at 94 °C for 45 s, at the appropriate annealing temperature (Table 1 Suppl.) for 30 s, and at 72 °C for 30 s, with a final extension step at 72 °C for 10 min. Total reaction volumes were analyzed by electrophoresis in a 1.2 % agarose gel stained with ethidium bromide. Images were captured using the *ChemiDoc XRS* System (*Bio-Rad*, Hercules, CA, USA). Expression of housekeeping genes *PdActin* (AM491134) and *Pdα Tubulin* (X67162) was also analyzed and representative results are shown for *PdActin*.

Results

PdGI full-length cDNA was isolated following 3' and 5'-RACE. *PdGI* cDNA had a coding region of 3513 bp flanked by a 5'- untranslated region of 530 bp and a 284 bp 3'-untranslated region. The protein sequence of

this gene had 1 170 amino acid residues corresponding to 128.52 kDa polypeptide with an isoelectric point of 6.18. Alignment using *CLUSTAL W* showed that PdGI shared 76 % identity to the amino acid sequence of *Arabidopsis*. Genomic sequence analysis revealed the gene structure and showed that there were 14 exons and 14 introns (one in the 5'-UTR) in *PdGI* covering a total length of approximately 11.3 kb (Fig. 1).

A phylogenetic analysis was carried out to establish the relationship between PdGI and GI protein sequences from other plant species. PdGI clustered with the GI homologues from peach and apple, both from *Rosaceae* family (Fig. 2). Southern blot analysis showed two copies of PdGI in almond (Fig. 1 Suppl.) Considering that

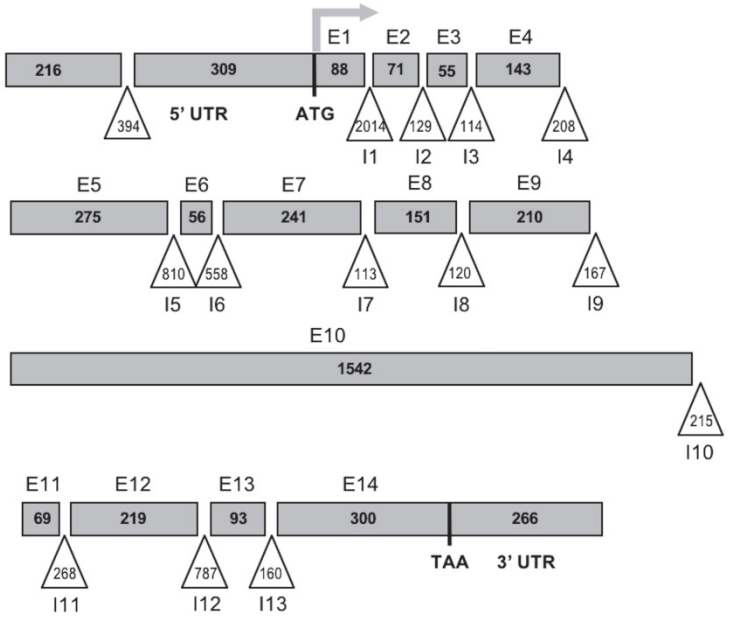

Fig. 1. Schematic representation of the *PdGI* gene. Introns (I) are represented by *triangles*, exons (E) are represented as *rectangles*, and the *arrow* represents the translation start site. The corresponding size [bp] of each intron and exon is shown.

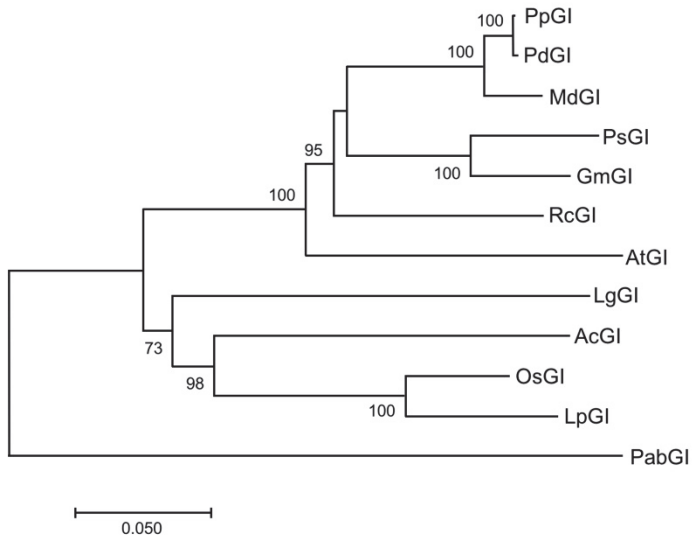

Fig. 2. Neighbour-joining (NJ) tree showing the evolutionary relationships (based on amino acid sequences) between GI homologues. The tree was constructed based on *Clustal W* alignment, using *Picea abies* GI as outgroup. Bootstrap values (higher than 70 %) obtained for 1 000 replications are shown on the branches and *lower bar* indicates 0.05 substitutions per site. Accession numbers: PdGI (*Prunus dulcis*): KJ502316; PpGI (*Prunus persica*): XP_007199688.1; MdGI (*Malus* × *domestica*): XP_008381855; PsGI (*Pisum sativum*): ABP81863; GmGI (*Glycine max*): BAJ22595; RcGI (*Ricinus communis*) XP_002524341; AtGI (*Arabidopsis thaliana*): AAT80910; LgGI (*Lemna gibba*) BAD 97869; AcGI (*Allium cepa*): ACT22764; OsGI (*Oryza sativa*, *var. japonica*): NP_001042220; LpGI (*Lolium perenne*): CAY26028; PabGI (*Picea abies*): AGH20049.1

almond is highly heterozygous (Arús *et al.* 2009), this pattern may correspond to two putative allelic forms.

The expressions of *PdGI* and its putative downstream targets *PdCO* and *PdFT*, were assessed in *in vitro* propagated almond shoots along a 24 h period under LD or SD at 22 ºC. The *PdGI* showed a peak expression around Zeitgeber time (ZT; hours after light onset) 8 to 12 in LDs and ZT 8 in SDs, decreasing towards the end of the day (Fig. 3). The *PdCO* expression also followed a similar trend under LD, increasing along the day and showing a peak at ZT 12. Under SD conditions, *PdCO* showed a stable and high expression until (at least) ZT4 with a declining trend towards the night. When plants were transferred to cold conditions (12 ºC), *PdGI* expression showed an increase again along the day period under SD and LD photoperiods (Fig. 3). However, under both conditions, high *PdGI* expressions were still detected during the night period suggesting a disruption of the circadian regulation imposed by cold. This response was also observed on *PdCO* transcript accumulation. These results were consistent in replicate analysis.

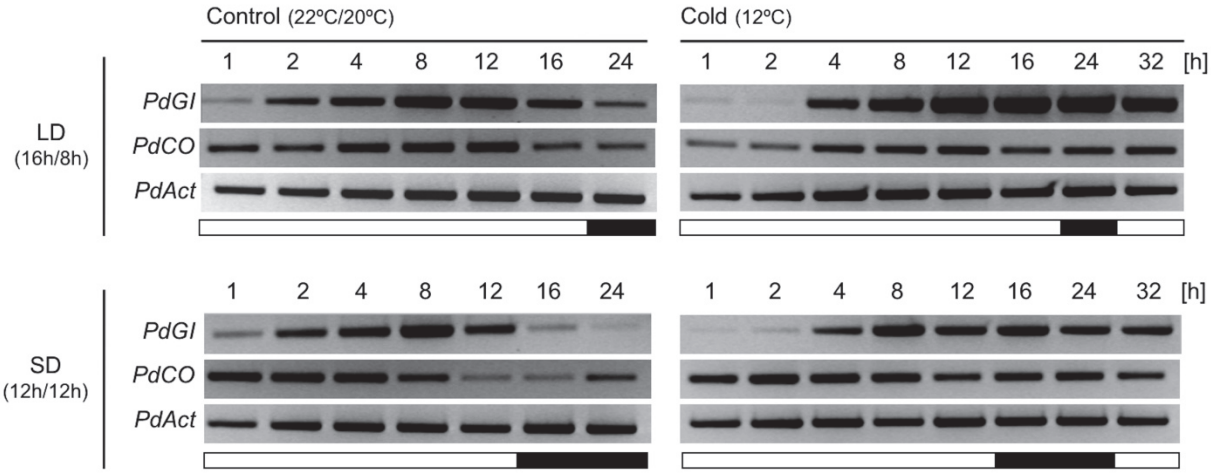

Fig. 3. Analysis of *PdGI* and *PdCO* gene expressions in *in vitro* grown almond shoots maintained at day/night temperatures of 22/20 ºC or exposed to cold stress (12 ºC). Plants were grown under 16-h photoperiod (LD, upper panel) or 12- photoperiod (SD, lower panel). Cold stress was imposed at dawn. The *white* and *black bars* represent day and night period, respectively. Expression of *PdActin* was used as housekeeping gene, and PCR analysis was repeated at least once for each gene.

Expressions of *PdGI*, *PdCO*, and *PdFT* were assessed in RNA samples obtained from flower buds and current-year shoot internodes collected from wild almond trees under natural climatic conditions. The daily photoperiod and temperatures experienced by these plants were recorded for the sampling period (Fig. 2 Suppl.). Flower bud break was detected in late December and blooming (50 % anthesis) was observed for both trees in early February. Yet, in January 21, anthesis was already observed in flower buds from tree I, while in tree II, flower buds were mostly at the pink tip stage (Fig. 4*A*).

Using RT-PCR we could detect *PdGI* transcription in all collection stages for both tissues (Fig. 4*B*,*C*) although transcript accumulation in flower buds showed a clear increase through autumn, reaching a peak in January. Such increase was less clear in shoots, particularly for tree II. The shoots from tree I also showed low *PdGI* expression by early November. The expression pattern of *PdCO* followed closely that of *PdGI* in all tissues. In flower buds high expressions were detected after December 17 up to January 21. It is noteworthy that the average minimum temperature recorded from December 27 to January 21 was close to 10 ºC. *PdFT* transcription was detected in flower buds since early collection stages, but showed increase along the collection dates up to December 17 in tree I and January 8 in tree II (Fig. 4*B*). In contrast, expression of *PdFT* was not detected in shoots up to December 17, being induced in January up to the last collection point of February. This induction occurred after visible signs of bud break were detected, *i.e.*, bud swelling and emergence of inner leaf scales (green-tip stage, Fig. 4*A*). The expressions of target genes were also assessed in the following year and, although some changes were observed, the global pattern of expression was maintained (Fig. 3 Suppl.). The changes observed were likely associated with the different environmental conditions detected, namely lower average temperatures during autumn (data not shown).

Discussion

The genetic network controlling flowering in *Arabidopsis* has *GI*, *CO*, and *FT* genes as key players in the photoperiodic pathway and is broadly conserved across different plant species (Kobayashi *et al.* 1999, Park *et al.*

1999, Griffiths *et al.* 2003, Lifschitz and Eshed 2006, Lin *et al.* 2007, Tamaki *et al.* 2007, Taylor *et al.* 2010). However, flowering is a much more complex process in tree species as compared to herbaceous plants because of additional phenomena such as a juvenile phase (which lasts for several years during which no flowering/fruiting occurs) or seasonal dormancy. This work was designed to identify an almond homolog of *Arabidopsis GI* (*PdGI*) and to explore its role, as well as that of its putative target genes *PdCO* and *PdFT*, during seasonal development.

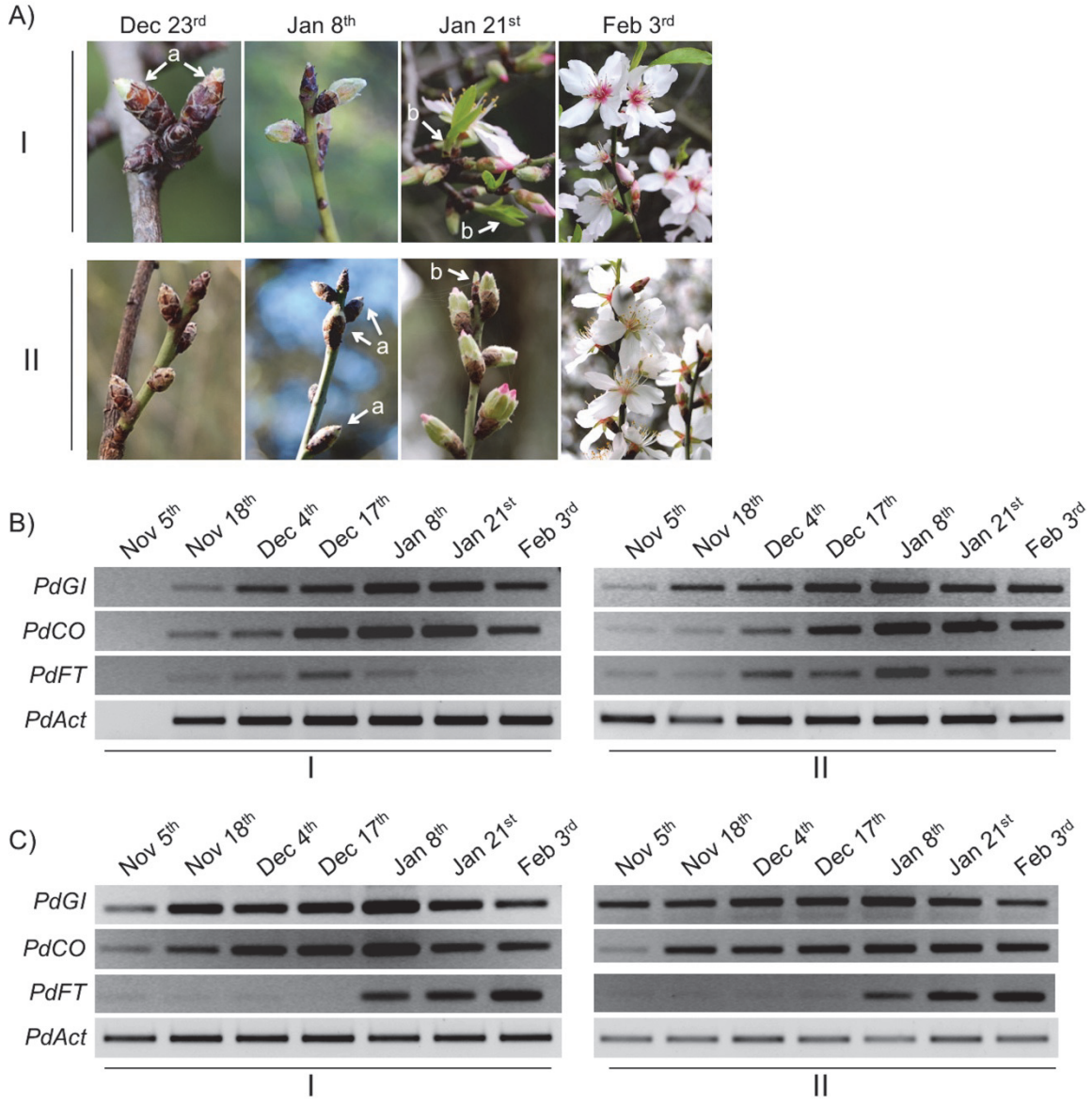

Fig. 4. *A* - Developmental stages of floral buds from two representative almond trees (I and II) sampled in this study. Flower buds at the green-tip stage are indicated by *a* + *arrows*; vegetative bud breaks are indicated by *b* + *arrows*. *B* and *C* - Seasonal gene expression patterns of *PdGI*, *PdCO*, and *PdFT* determined by semi-quantitative RT-PCR, in flower buds (*B*) and current-year shoot internodes (*C*), collected from two representative almond trees (I and II) from mid-autumn to early-winter. Expression of *PdActin* was used as housekeeping gene and PCR analysis was repeated at least once for each gene.

Results on the overexpression of *PdGI* under LD and SD at room temperature (22 °C), using plant material *in vitro*, reflected a similar trend as shown for *Arabidopsis GI*, which peaks at ZT 10 in LD (16/8 h) and at ZT 8 in SD (10/14 h) (Fowler *et al.* 1999). *PdCO* expression also followed a similar pattern with a peak towards the end of the photoperiod (ZT 12) in LD (Fig. 3), highlighting its diurnal expression pattern. Still, further confirmation of circadian regulation would be required in expression studies under constant irradiance. It was reported that *CO* expression rises towards the end of the day in LD conditions, but after dusk in SD (Suárez-

López *et al.* 2001, Imaizumi *et al.* 2003, Böhlenius *et al.* 2006) and that irradiance stabilizes the CO protein, which is degraded in the dark (Valverde *et al.* 2004). Apart from the circadian control of *CO* transcription, the role of *GI* on *CO* expressions should also be taken into account. In *Arabidopsis*, GI has been shown to form a complex with *FKF1* leading to the degradation of *CDF1*, a transcriptional repressor of *CO*. This happens later on in the day resulting in increased *CO* expression particularly under LD (Sawa *et al.* 2007). Under SD conditions, however, unlike the reported increase in *CO* expression at the end of the day in *Arabidopsis* (Suárez-López *et al.* 2001), our results show that *PdCO* expression during the day remained stable up to ZT 4 (Fig 3). Still, this expression pattern is similar to that observed in other tree species. In *Picea abies* two *CO*-like genes, *PaCOL1* and *PaCOL2,* were shown to be regulated by irradiance with increasing and decreasing levels after dawn and during night, respectively (Holefors *et al.* 2009). In poplar (*Populus tremula* × *Populus tremuloides*)*, PttCO2* expression shows a biphasic pattern under SD (12/12 h), peaking at ZT 24/0 and ZT 8 to ZT 12, while peak expression of *PttGI* occurs at ZT 12 (Ibáñez *et al.* 2010). A different study performed with field grown *Populus deltoids* also reported a peak of expression of two CO homologues at ZT 24/0, declining during the day (Hsu *et al.* 2012).

The effect of low temperature (12 °C) on *PdGI* and *PdCO* expressions were also analyzed in *in vitro* plants. In contrast to what we observed at room temperature, the expressions of *PdGI* and *PdCO* remained high at the end of day and during the night, up to the following day (ZT 32). The positive regulation of *PdGI* in response to cold shock agrees with what was observed for *AtGI* (Cao *et al.* 2005), suggesting a role of *PdGI* in cold stress response. The up-regulation of *CO* transcription under cold is also observed in *Arabidopsis* (Jung *et al.* 2012), although it is associated with a decline in CO protein abundance. This could be explained by the increase in CO degradation mediated by cold-activated E3 ubiquitin ligase HOS1 (High Expression of Osmotically Responsive genes 1) (Jung *et al.* 2012, Lazaro *et al.* 2012). The increase in CO transcription could reflect a feedback regulation mechanism to compensate for the reduced CO protein content. A similar mechanism could actually explain the increase in *PdCO* in response to cold.

In *Rosaceae* fruit trees, as in other temperate fruit trees, flower initiation occurs the year before blooming, and organogenesis inside flower buds is arrested during autumn/winter dormancy. In early autumn, buds of most perennial plants become endodormant as a consequence of reducing day-length and temperature. During this stage, repression of growth and development persists, even under environmental conditions that, in a different context, would favour growth. Endodormancy is maintained by endogenous factors in a straight correlation with specific chilling requirements, which later contributes to restore growth ability. However, growth reactivation is only possible after the onset of growth-promotive conditions, which often relate to warmer temperatures in spring. Therefore, this quiescent stage following endodormancy is referred to as ecodormancy and it is maintained under low temperatures (Horvath 2009). In the present paper we determined the expression patterns of *PdGI*, *PdCO*, and *PdFT* in shoot and flower bud tissues, mostly during the ecodormancy break up to full anthesis.

Expression of *PdGI* and *PdCO* showed increase throughout the ordered collection points particularly in flower buds, reaching a peak after bud break (early January). In chestnut circadian regulation it was shown to be disrupted during winter dormancy (Ramos *et al.* 2005) and our results using *in vitro* almond shoots showed that transcription of *PdGI* and *PdCO* was upregulated by low temperature. Considering that sample collection in the field was performed shortly after the night minimal temperatures, we suggest that the high expression assessed in January could be a response to the low temperature to which plants were exposed.

The expression pattern of *PdFT* showed clear tissue-specific patterns. In flower buds an increase in *PdFT* transcription was detected along the collection dates up to mid-December and early-January in trees I and II, respectively. Interestingly, buds in tree I developed faster than in tree II, showing the full-bloom stage already by late-January, while most flower buds from tree II were still at the pink tip stage (Fig. 4*A*). In poplar, the overexpressions of two *FT* orthologs (*PtFT1* and *PdFT2*), not only induce early flowering (Böhlenius *et al.* 2006, Hsu *et al.* 2006) but also inhibit SD-induced growth cessation (Böhlenius *et al.* 2006). In fact *PtFT1* show to be down-regulated under SDs leading to seasonal growth arrest (Böhlenius *et al.* 2006, Ruonala *et al.* 2008). These results suggest a dual role of *FT* in perennials, namely by controlling flower induction as well as vegetative growth (Van der Schoot and Rinne 2011). However, Rinne *et al.* (2011) reported that *PtFT1* transcription can be induced in dormant vegetative buds after exposure to chilling conditions (5 ºC). These authors proposed that, within the dormant bud, chilling exposure would induce *PtFT1* expression in embryonic leaves but the PtFT1 protein would only move to the shoot apex after the reestablishment of plasmodesmata functionality under growth-promoting temperatures.

There are six genes in the *Arabidopsis FT/TFL1* family (Turck *et al.* 2008) and corresponding orthologs have been found in peach, except for *TSF* (*TWIN SISTER OF FT*) (Chen *et al*. 2013). Phylogenetic analysis identified five distinct clades that corresponded to each of the family members, grouping *PpFT* with two other FT orthologs from apple and strawberry. In our work we observe that *PpFT* shared 98 % nucleotide similarity with *PdFT*-like. Considering that in almond, flower induction occurs during late summer, we may correlate the

induction of *PdFT* expression in winter to the reestablishment of growth ability in flower buds. During early stages of flower bud development, vascular connections to the branch are also under development, and the complete establishment of these connections marks the transition to the swollen bud stages (Reinoso *et al.* 2002). In our study, this transition was observed by late December and after this time, *PdFT* transcripts were observed in shoots. Although we did not study gene expression in vegetative buds, our results suggest the flower buds as potential sites of *PdFT* transcription. Considering the role of FT mRNA in systemic signalling (Li *et al.* 2011), we may hypothesize that after the establishment of the vascular vessels, *PdFT* could be transported along shoots to promote vegetative growth in apical vegetative buds, such as it usually occurs after flower bud break in this species (Oliveira *et al.* 2008).

Conclusions

A major challenge for genomic studies in fruit trees is to understand the function of candidate genes and to use this knowledge to improve economically important agronomic traits such as flowering time. In this study we performed the characterization of the almond homologue of *GIGANTEA* and unveiled its expression pattern in *in vitro*-cultured shoots subjected to different photoperiod and environmental (cold) conditions. We also followed the expression of *PdGI* and the putative downstream targets *PdCO* and *PdFT* in field-grown trees along seasonal development. Our results show that *PdGI* and *PdFT* may be important regulators of dormancy-activity transitions in almond, with *PdGI* playing a role in cold acclimation and *PdFT* being associated with growth resumption after dormancy, in both flower buds and vegetative buds.

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