TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2021) 45: 484-494

doi:10.3906/tar-2103-101

Crosstalk between flowering and cold tolerance genes in almonds (*Amygdalus* spp.)

Başak ÖZDEMIR[1], Fatma Yeşim OKAY[1], Gölge SARIKAMIŞ[1], Canan YÜKSEL ÖZMEN[2], Umut KİBAR[3], Ali ERGÜL[2,*]

[1]Department of Horticulture, Faculty of Agriculture, Ankara University, Ankara, Turkey

[2]Biotechnology Institute, Ankara University, Ankara, Turkey

[3]Republic of Turkey, Ministry of Agriculture and Forestry, Agriculture and Rural Development Support Institution, Ankara, Turkey

Received: 29.03.2021 • **Accepted/Published Online:** 03.06.2021 • **Final Version:** 18.08.2021

Abstract: Almond production is usually affected by late spring frosts. Late flowering is an important trait in almond production in order to avoid frost damage. Breeding for late flowering has always been an important objective in almond breeding programs. Utilising molecular approaches may guide and accelerate breeding programs. In the present study, the expressions of the *Prunus persica FLOWERING LOCUS T* (*PpFT*) and *Prunus armeniaca* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*PabSOC1*) genes known as floral integrators that promote flowering in plants were determined in almonds (*Amygdalus* spp.). Frost tolerance is another important trait in almond production. Almond accessions may vary in terms of frost tolerance. The expressions of *Prunus dulcis* C-repeat-binding factors (*PdCBF1*) and (*PdCBF2*) genes that are the major components in the cold responsive network of plants were studied in almonds. Real time PCR analysis of buds revealed the differential expression pattern of *PpFT, PabSOC1, PdCBF1, PdCBF2* genes. The expressions of *PpFT* and *PabSOC1* correlated with each other. Similarly, the expressions of *PdCBF1* and *PdCBF2* genes revealed a similar expression pattern in almonds. However, the expression of flowering genes were inversely correlated with the cold response genes in most of the almond accessions. This finding revealed the crosstalk between flowering integrator genes and cold responsive genes in controlling flowering in almonds.

Key words: Almond, *Amygdalus* spp., flowering, cold, gene expression

1. Introduction

Almond is an early flowering fruit species and usually affected by late spring frosts. Exposure of buds to cold fulfills the chilling requirement of plants and the subsequent warmer temperatures induce bud break (Erez, 2000). Flowering time vary among plants due to the differences in chilling requirements. Late flowering is one of the most important agronomical traits in *Prunus* species in order to avoid damage by spring frosts (Socias i Company et al.,1999).

Flowering is a complicated trait determined by endogenous (autonomous) and environmental factors. Genetic and molecular researches mainly carried out in the model plant *Arabidopsis* revealed that flowering is regulated by four major pathways. These are photoperiod, autonomous, vernalization, and gibberellin pathways (Simpson and Dean, 2002; Boss et al., 2004; Baurle and Dean, 2006). Studies in *Arabidopsis* have led the identification and characterization of major flowering genes involved in these major pathways. These genes are *CONSTANS* (*CO*), *FLOWERING LOCUS C* (*FLC*), *FLOWERING LOCUS T* (*FT*), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*) (Mouradov et al., 2002). *CO* gene acts as a floral activator and mediates the photoperiod pathway. *FLC* gene acts as a floral repressor and mediates the autonomous and vernalization pathways. Consequently, *CO* and *FLC* regulate the expression of downstream *FT*, *SOC1* and *LEAFY* (*LFY*) genes known as the flowering integrators that receive signals from multiple flowering pathways in *Arabidopsis* (Simpson and Dean, 2002; Parcy, 2005). Flowering time is determined by the expression level of these integrators (Moon et al.,2005). *FT* acts as a mobile flower-promoting signal that is translocated from the leaves to the apical meristem and activate floral meristem identity genes such as *APETALA 1* and *SOC1* (Turck et al., 2008). *SOC1* is regarded as a floral activator and the expression analysis in *Arabidopsis* mutants demonstrated that *SOC1* expression is regulated by *FT* (Moon et al., 2005).

Homologs of the major genes involved in flowering pathways are identified in almond (*PrdLFY, PrdMADS1 PrdTFL, PrdGA20)* and peach (*PrpAP1, PrpFT, PrpAGL2, PrpFAR1, PrpAP2* and *PrpCO)* via a candidate gene approach (Silva et al., 2005). An almond homologue (*PdGI)*

* Correspondence: ergul@ankara.edu.tr

of the *Arabidopsis GIGANTEA* (*AtGI*) gene involved in the flowering transition regulated by the photoperiodic pathway was characterized (Barros et al., 2017). In apricot, Trainin et al. (2013) studied the expression of *ParSOC1*, a distinct apricot MADS-box gene closely related to *Arabidopsis AGL20/SOC1*, in 48 apricot genotypes that vary in their chilling requirements. They have shown that variation at the *ParSOC1* locus is associated with chilling requirements of apricot cultivars. Kitamura et al. (2016) performed a synchronised expression analysis of *PmDAM6* (Dormancy-associated mads-box6) and *PmSOC1* during dormancy release in flower buds in two high-chill and low-chill Japanese apricot cultivars. They suggested that the dimer of *PmDAM6* and *PmSOC1* may play a role in the regulation of dormancy transition and blooming time in Japanese apricot flower buds.

In addition to these major pathways, flowering time is influenced by ambient temperature such that cold temperature delays, whereas warm temperature induces flowering (Blazquez et al., 2003).

C-repeat-binding factors (*CBF*s) are reported as the central components in the cold responsive network (Gilmour et al., 2004; Vogel et al., 2005; Park et al., 2015; Shi et al., 2015). *CBF* genes bind to the promoters of downstream cold responsive (*COR*) genes and promote their transcription resulting in increased cold tolerance in plants (Gilmour et al., 2004; Vogel et al., 2005; Park et al., 2015; Shi et al., 2015). The molecular basis of cold tolerance was first determined by the identification of the *Arabidopsis CBF*s (Gilmour et al., 1998; Medina et al., 1999). These are also known as dehydration-responsive element-binding factors (*CBF1/DREB1B,CBF2/DREB1C* and *CBF3/DREB1A*). According to research findings in *Arabidopsis*, *CBF* genes are regulated by upstream transcription factors, such as *ICE*1 (inducer of *CBF* expression 1). The *ICE1-CBF* transcriptional cascade is reported to have an important role in the cold tolerance of plants (Chinnusamy et al., 2003, 2007; Ito et al., 2006; Medina et al., 2011).

PdCBF1 and *PdCBF2* genes were characterized based on the nucleotide sequence of sweet cherry *PaCBF1* (Barros et al., 2012 a), and the transcription of these two genes in almond was rapidly induced by low temperature, suggesting their involvement in cold acclimation. This finding was further evidenced by demonstrating higher levels of the expression of *PdCBF2* with cold acclimation on flower buds and shoot internodes in almond (Barros et al., 2012b). Seo et al. (2009) revealed the crosstalk between cold response and flowering in *Arabidopsis*. They reported that over expression of *CBFs* delay flowering through increased regulation of *FLC* that acts as a floral repressor. Contrarily, the floral activator *SOC1* involved in vernalization, autonomous, gibberellin dependent pathways represses the expression of cold responsive *CBF* genes. In the light of these findings in *Arabidopsis*, they suggested that *SOC1* functions as the negative regulator of cold responsive *CBF* genes (Seo et al., 2009).

In the present study, the expressions of *Prunus persica* FLOWERING LOCUS T (*PpFT*) and *Prunus armeniaca* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*PabSOC1*) known as the flowering integrators were determined in almond buds. At the same time, the expressions of *Prunus dulcis CBF1* (*PdCBF1)* and *Prunus dulcis CBF2* (*PdCBF2)* involved in the cold responsive network of plants were determined in cold (–2 °C) treated almond buds. The objective was to determine the expression profiles of the major genes involved in the flowering and cold tolerance of almonds including wild almond species (*Amygdalus arabica*, *Amygdalus orientalis*) local and commercial almond cultivars that vary in terms of flowering time.

2. Materials and methods

2.1. Plant material

Almond accessions used in the present study were obtained from the almond germplasm collection of Gaziantep Pistachio Research Institute of the Turkish Republic Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policy. The collection included wild species (*Amygdalus arabica*, *Amygdalus orientalis*), 3 local almond (*Prunus dulcis*) cultivars (Akbadem, Gülcan 2 and 17-4) and 4 commercial cultivars (Bertina, Ferraduel, Ferragnes, Garrigues) (Yılmaz, 2017). The local almond cultivars Akbadem, Gülcan 2, 17-4 are prominent cultivars that are obtained via selective breeding, and they have been used in almond production for many years. The commercial cultivars Bertina, Ferraduel, Ferragnes, Garrigues are the cultivars that are widely used in almond production worldwide. For each accession, almond shoots carrying 20–30 unopened floral buds were collected from the collection site (Gaziantep) in 2016 and 2017. The shoots were transferred to Ankara University Biotechnology Institute for molecular assays. The shoots were collected and 20 cm cuttings carrying the unopened floral buds were prepared. Cuttings were placed in 1/10 Hoagland's solution (Hoagland and Arnon,1950) containing macronutrients (K_2SO_4, KH_2PO_4, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$ and KCl) and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4.5H_2O$, NH_4Mo, $ZnSO_4.7H_2O$) with a final concentration of the following ions: 2 mM Ca, 10^{-6} M Mn, 4 mM NO_3, 2.10^{-7} M Cu, 1 mM Mg, 10^{-8} M NH4, 2 mM K, 10^{-6} M Zn, 0.2 mM P, 10^{-6}M B, and 10^{-4} M Fe and maintained in the climate controlled growth chamber. They were monitored on a daily basis until reached pink bud stage at 25 °C and a 16-h photoperiod of cool-white fluorescent light. The samples were collected as control (day 0) and unopen buds at pink bud stage from each accession. For *CBF* analysis, buds were exposed to freezing temperature (–2 °C) for 1, 5, 3, 6, 12 h. Buds that were not exposed to cold were used as

control. Preliminary experiments were performed to test the response of *PdCBF1* and *PdCBF2* genes at different time points in Bertina and Ferragnes that were used as the late flowering reference cultivars in this study. The highest expression of both genes was determined in flower buds exposed to -2 °C for 6 h. Therefore, this time point was selected for further analysis in almond accessions. The samples were placed in 15 mL falcon tubes at –80 °C until RNA extraction. Untreated samples were included as the control treatments for each accession during gene expression analysis. The experiment was conducted as three biological replicates.

2.2. RNA isolation and qRT-PCR

RNA extraction was performed using the Promega SV Total RNA Isolation System kit (Madison, USA). Extracted total RNAs were visualised on 1% (w/v) agarose gel and checked with Nanodrop ND-1000 spectrophotometer for quality and quantity assessment. For the cDNA synthesis, Eurx NG Dart RT kit (Cat no:E0801) was used. cDNA samples were visualised on 1% (w/v) agarose gel and checked with Nanodrop ND–1000 spectrophotometer prior to use in qRT-PCR analysis.

PpFT (*Prunus persica* FT, BU044758.1) associated with flowering time in peach (Silva et al., 2005) and *PabSOC1 g*ene associated with flowering time in apricot (*Prunus armeniaca SOC1*, FJ472817.1) (Trainin et al., 2013), *PdCBF1* (*Prunus dulcis CBF1,* gene bank accessions code:JQ317157.1) and *PdCBF2* (*Prunus dulcis CBF2,* gene bank accessions code:JQ317158.1) (Barros et al., 2012a) were used. Primers were designed from these putative genes using the Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primers were tested on reference almond cultivars (Bertina and Ferragnes). The amplicons of expected size (bp) were then gel purified and homolog regions were sequenced by the Sanger dideoxy method (BM Labosis Ltd. Co., Ankara, Turkey). Specific qRT-PCR primers were designed from the sequence information using the Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences primarily recommended by the design tool is used in the present study for qRT-PCR (Table 1). *Prunus dulcisActin* (*PdAct)* gene (LOC117630898) was selected as the housekeeping gene for qRT-PCR analysis.

The qRT-PCR amplifications were performed using Light Cycler 480, Roche and all reactions run three times according to İbrahime et al. (2019). The reaction mixture of 10µL composed of 1–2 µL (500 ng/µl) of cDNA, 10 pmol forward and reverse primer, 5µL of LightCycler 480 SYBR Green I Master (Roche) and ddH_2O. The standard curve was prepared from six serial dilutions (i.e. 1/10 to 1/100000) of control cDNAs. qRT-PCR conditions were 2 min pre-incubation, followed by 40 cycles at 95 °C for 15 s, 56–57–50–52–58 °C 1 min for annealing (*PabSOC1*, *PpFT*, *PdCBF1*, *PdCBF2*, *PdAct* respectively) 72 °C for 1 min. The specificity of the qRT-PCR amplification was checked with a melting curve analysis following the final cycle of the PCR. qRT-PCR conditions were optimized for high amplification efficiency >95% for all primer pairs used. In melting curve analyses, overlapping single peak images were obtained, and contamination was not detected. Cycle threshold (Ct) values were established by using the amplicon peak profiles.

2.3. Statistical analysis

The experimental setup was designed according to completely randomised design as three replicates each of which containing 4–5 floral buds. According to the Ct values, the relative expression levels were calculated by using the $2^{-\Delta\Delta CT}$ (the delta-delta-Ct or ddCt) algorithm (Livak and Schmittgen, 2001). Reaction efficiency (RE) has been considered as 1 (one). The expression of *PpFT, PabSOC1, PdCBF1*, *PdCBF2* genes was normalized according to *PdAct* housekeeping gene.

Table 1. Primer sequences designed for gene expression analysis by qRT-PCR.

Gene	Sequence (5'→3')	Annealing (°C)	Amplicon (bp)
PabSOC1	5'-TGAACTGGCCGCACATTTTG	56	127
	5'-ATTCTTGCTTCAGGTGCTGC		
PpFT	5'-TAACTGCCAAAGGGAGAGCG	57	284
	5'-TGGGGACGTAGTGTCGTACT		
PdCBF1	F: GCTCGGGACTTATCCGACG	52	93
	R: GGGAAGTTGAGGCAGGCAAG		
PdCBF2	F: AGGATATGGCTCGGGACGTTA	50	83
	R: GCTTCCCTCTAAACGCCAATC		
PdAct	F: AGCGGGAAATTGTCCGTGAT	58	172
	R: AAGAGAACTTCTGGGCACCG		

3. Results

3.1. Expression analysis of *PpFT* and *PabSOC1* genes associated with flowering

The qRT-PCR analysis of *PpFT* and *PabSOC1* genes was determined in almonds buds. The *PpFT* gene showed differential expression pattern among almond accessions. The expression presented as fold change of *PpFT* transcripts was significantly high in some breeding lines and commercial cultivars. In wild almonds, the expression of *A. arabica* (9.79-fold) were upregulated, whereas, in *A. orientalis,* the expression was significantly downregulated (–27.29-fold). Among commercial almond cultivars, the expression of *PpFT* gene was significantly increased in the late flowering cultivars Ferraduel (56.94-fold) and early flowering Garrigues (26.29-fold). Contrarily, *PpFT* expression was decreased in the late flowering reference cultivars Ferragnes (–44.93-fold) and Bertina (–16.35-fold) (Figure 1).

In terms of local cultivars, the highest increase in *PpFT* was determined in the the early flowering Akbadem (20.37-fold), late flowering cultivar Gülcan 2 (17.90-fold) and early flowering cultivar 17–4 (2.51-fold) (Figure 1).

The expression of *PabSOC1* gene showed differential expression pattern among almond accessions. Wild almond *A. arabica* (5.64-fold) revealed an increased gene expression profile, while the expression was significantly decreased in *A.orientalis* (–39.91-fold). In commercial almond cultivars, the highest expression was determined in Ferraduel (54.74-fold) and Garrigues (26.53-fold). The decrease in *PabSOC1* was determined in the late flowering reference cultivars Bertina (–39.66-fold) and Ferragnes (–27.49-fold). In local cultivars, the expression was upregulated in Akbadem (19.04-fold), Gülcan 2 (13.97-fold) and 17-4 (7.85-fold) ($p \leq 0.05$) (Figure 2).

Analysis of the expression patterns of *PpFT* and *PabSOC1* genes among almond germplasm collection revealed a positive correlation between the two genes (Figure 3). Within these accessions, *PpFT* and *PabSOC1* genes were expressed in a similar pattern in the majority of accessions. Both of the genes were up regulated in 6 of the accessions, whereas they were downregulated in 3 almond accessions (Table 2).

3.2. Expression of *PdCBF1* and *PdCBF2* genes

CBF-mediated cold response of almonds was determined. According to the findings, the *PdCBF1* expression was increased in *A. orientalis* (2.15-fold), while the expression was significantly decreased in *A.arabica* (-13.89-fold) ($p \leq 0.05$). The most significant increase in *PdCBF1* was determined in the late flowering reference cultivars, Bertina (99.63-fold) and Ferragnes (95.08-fold). On the contrary, the expression of *PdCBF1* was decreased in some cultivars, including Ferraduel (–59.30-fold) and Garrigues (–8.11-fold) ($p \leq 0.05$) (Figure 4). The local almond cultivar 48-1 revealed a significant increase (12.62-fold)

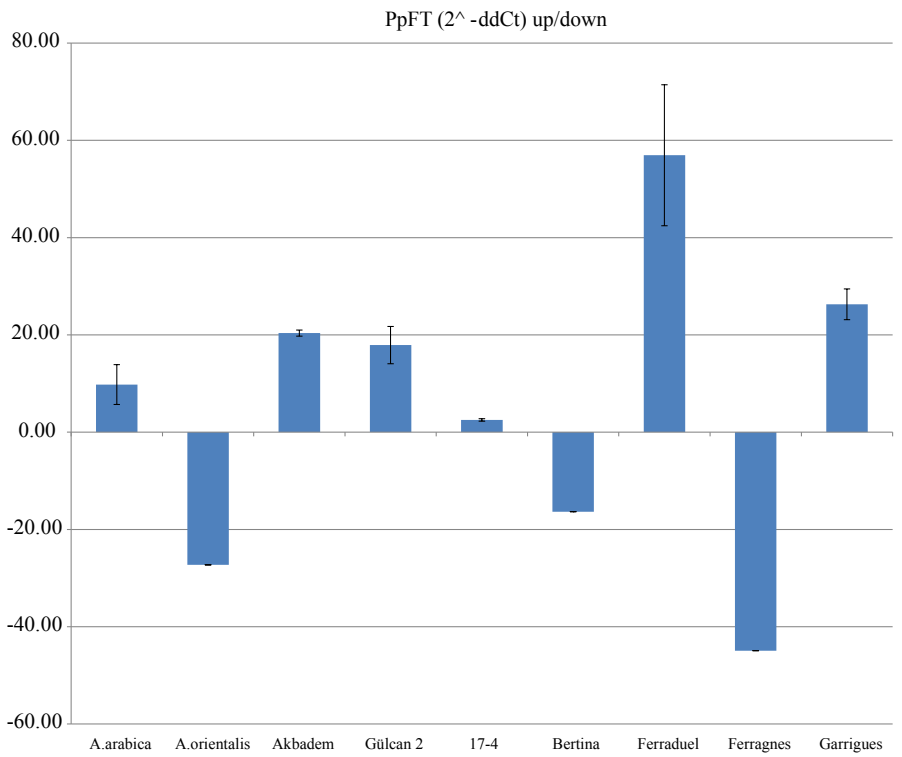

Figure 1. The relative expression of *PpFT* gene among almond accessions at the pink bud stage.

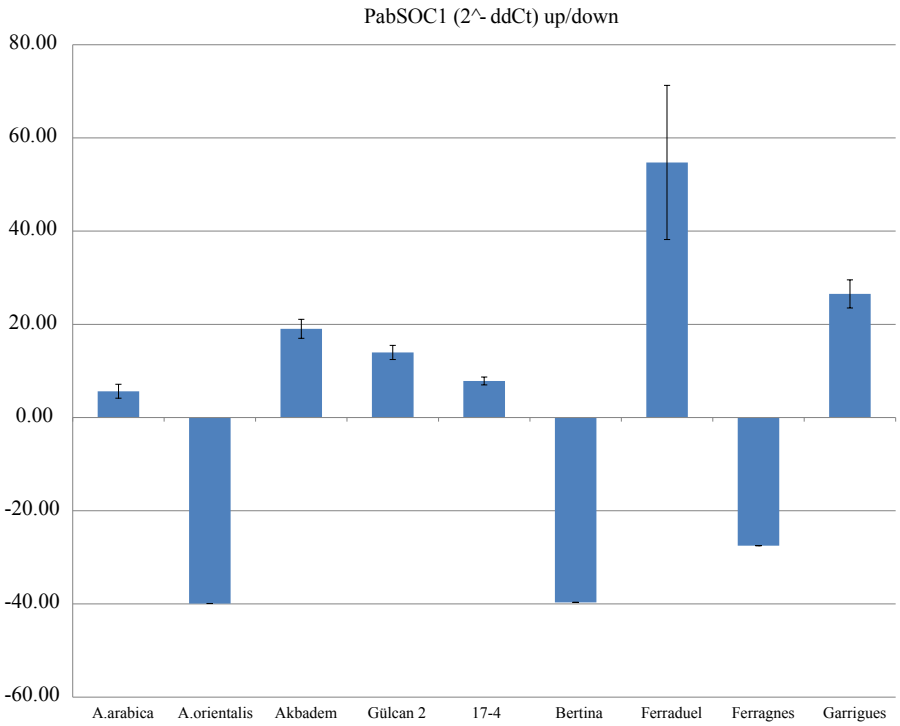

Figure 2. The relative expression of *PabSOC1* gene among almond accessions at the pink bud stage.

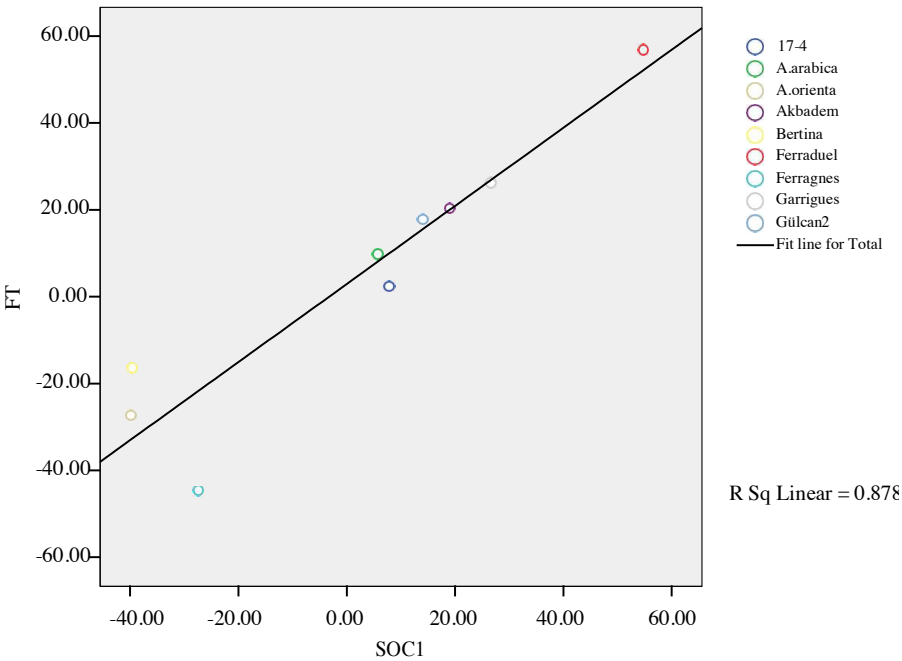

Figure 3. The correlation graph showing the relative expression of *PpFT* and *PabSOC1*.

in *PdCBF1* expression. The *PdCBF1* expression of local cultivars Akbadem (–20.78-fold) and 17-4 (–20.73-fold) and Gülcan 2 (–2.40-fold) was significantly decreased ($p \leq 0.05$) (Figure 4).

The expression of *PdCBF2* gene was significantly increased in *A. orientalis* (18.84-fold) and decreased in *A. arabica* (–6.20-fold). The highest increase was determined in the reference cultivars Bertina (78.62-fold)

Table 2. The expression pattern of *PpFT*, *PabSOC1*, *PdCBF1* and *PdCBF2* in wild and cultivated almonds (*Amygdalus* spp).

Almond accessions	*PpFT*	*PabSOC1*	*PdCBF1*	*PdCBF2*	*PpFT*	*PabSOC1*	*PdCBF1*	*PdCBF2*
A.arabica	9.79	5.64	–13.89	–6.20	up	up	down	down
A.orientalis	–27.29	–39.91	2.15	18.84	down	down	up	up
Akbadem	20.37	19.04	–20.78	–12.59	up	up	down	down
Gülcan 2	17.90	13.97	–2.40	–5.22	up	up	down	down
17–4	2.51	7.85	–20.73	–24.77	up	up	down	down
Bertina	–16.35	–39.66	99.63	78.62	down	down	up	up
Ferraduel	56.94	54.74	–59.30	–77.70	up	up	down	down
Ferragnes	–44.93	–27.49	95.08	35.70	down	down	up	up
Garrigues	26.29	26.53	–8.11	–27.10	up	up	down	down

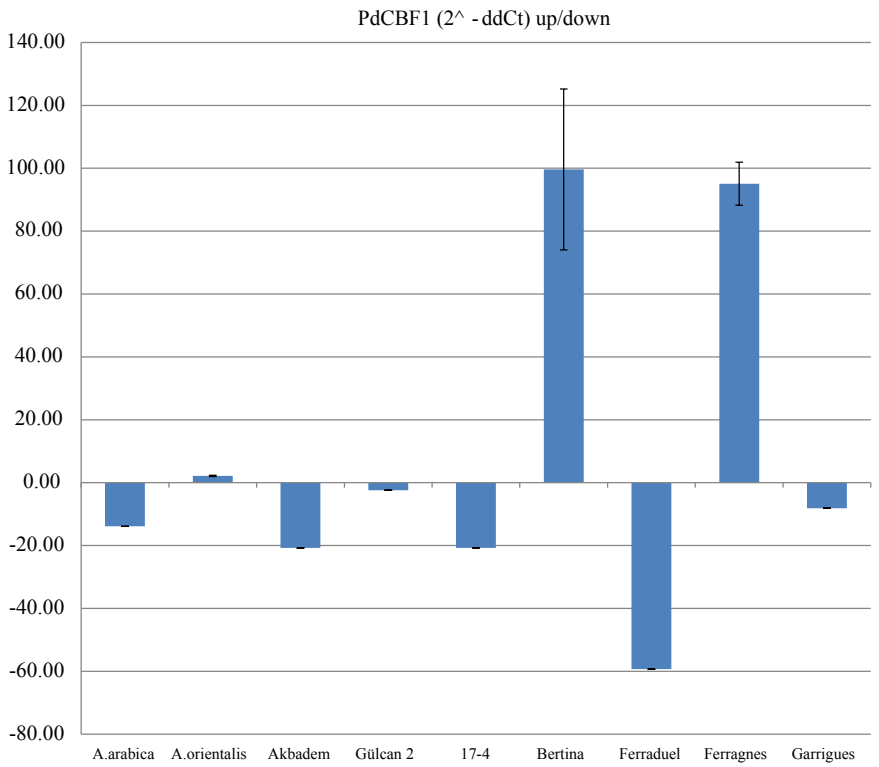

Figure 4. The relative expression of *PdCBF1* gene among almond accessions exposed to –2 °C for 6 h at the pink bud stage.

and Ferragnes (35.70-fold). The expression of *PdCBF2* was significantly decreased in some cultivars, namely Ferraduel (–77.70-fold) and Garrigues (–27.10-fold) (Figure 5).

Among local almond cultivars, the expression of *PdCBF2* was significantly decreased in the almond cultivars 17-4 (–24.77-fold), Akbadem (–12.59-fold) and Gülcan 2 (–5.22-fold) ($p \leq 0.05$) (Figure 5).

Analysis of the expression patterns of *PdCBF1* and *PdCBF2* genes revealed a positive correlation among almonds (Figure 6). Both of the genes were downregulated in 6 accessions whereas upregulated in 3 accessions (Table 2).

Expression pattern of *SOC1* and *CBF* genes suggested a negative association among almonds (Figures 7, 8).

4. Discussion

The expression profiles of the *PpFT* and *PabSOC1* genes associated with flowering time revealed a differential expression pattern within almond accesssions including wild and cultivated almonds. *FT* acts as a mobile

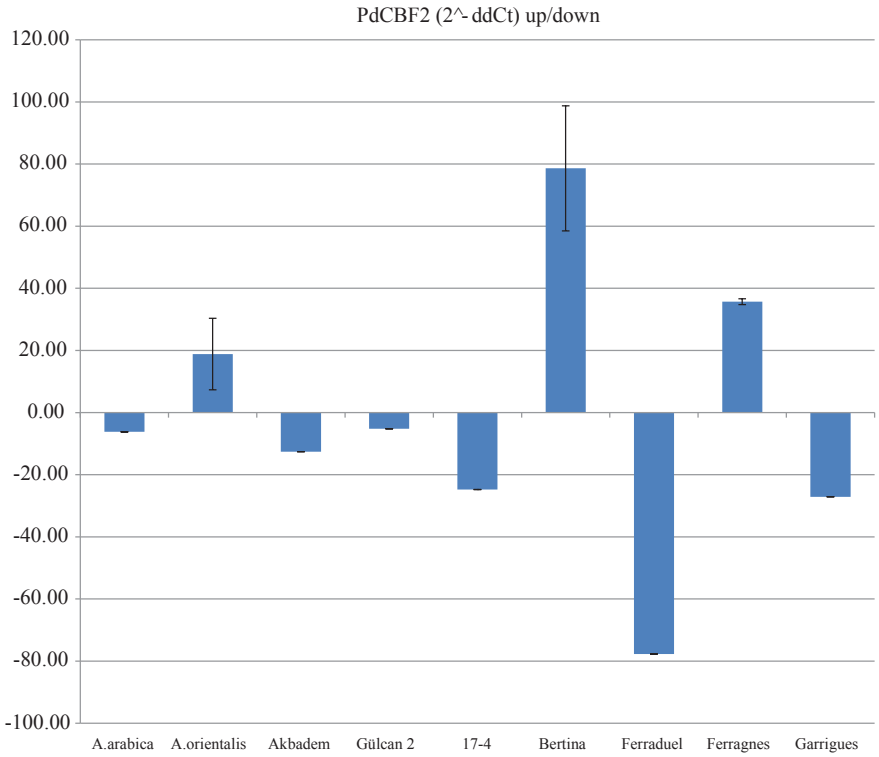

Figure 5. The relative expression of *PdCBF2* gene among almond accessions exposed to –2 °C for 6 h at the pink bud stage.

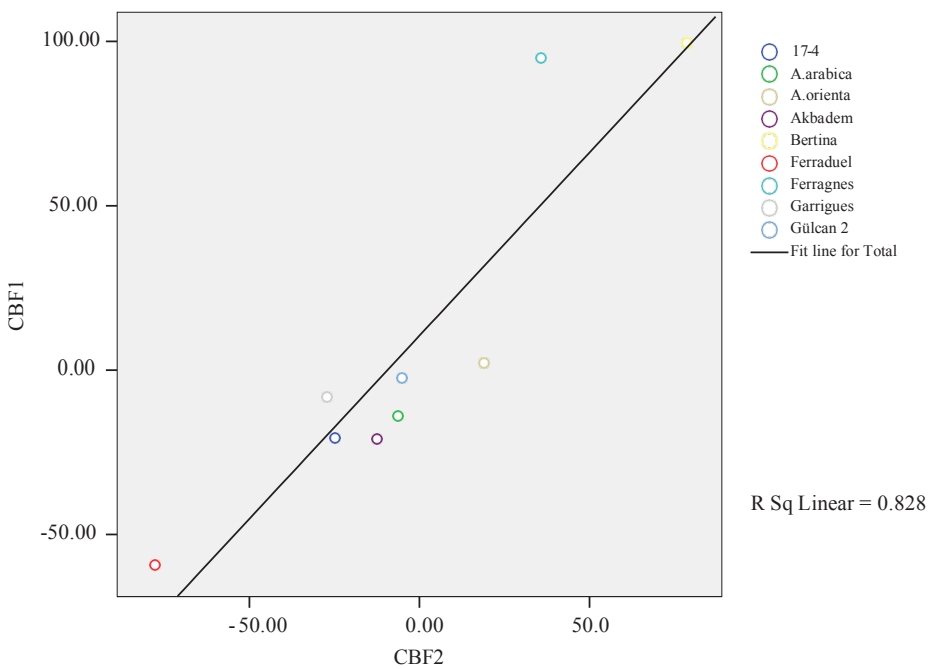

Figure 6. The correlation graph showing the relative expression of *PdCBF1* and *PdCBF2*.

flower promoting signal and the expression analysis in *Arabidopsis* mutants demonstrated that *SOC1* expression is regulated by *FT* (Moon et al., 2005). This is consistent with our findings demonstrating that both genes acted in a similar pattern such that their transcripts were either increased or decreased simultaneously in most almond

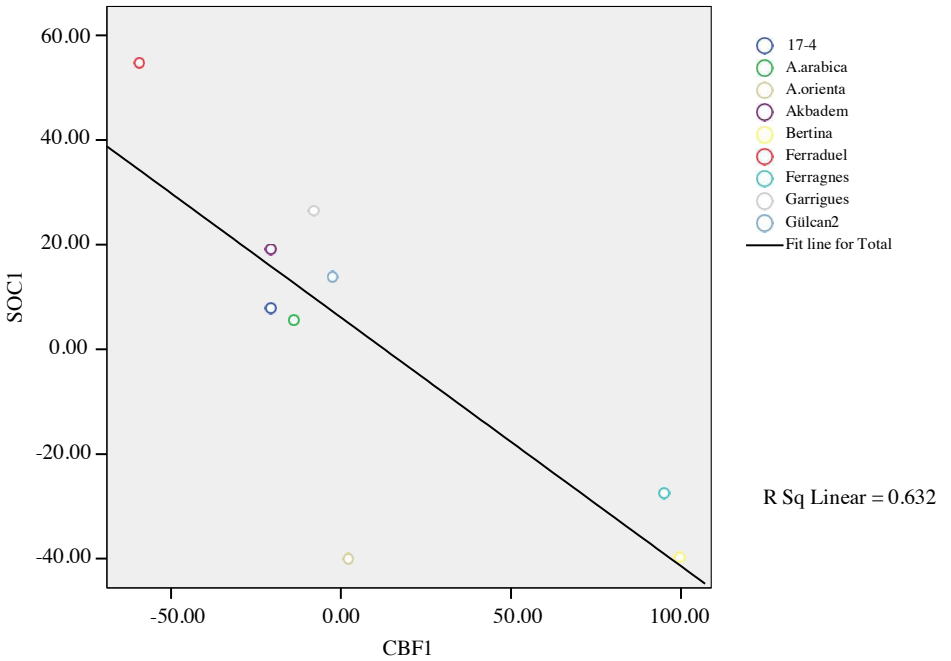

Figure 7. The correlation graph showing the relative expression of *PdCBF1* and *PabSOC1*.

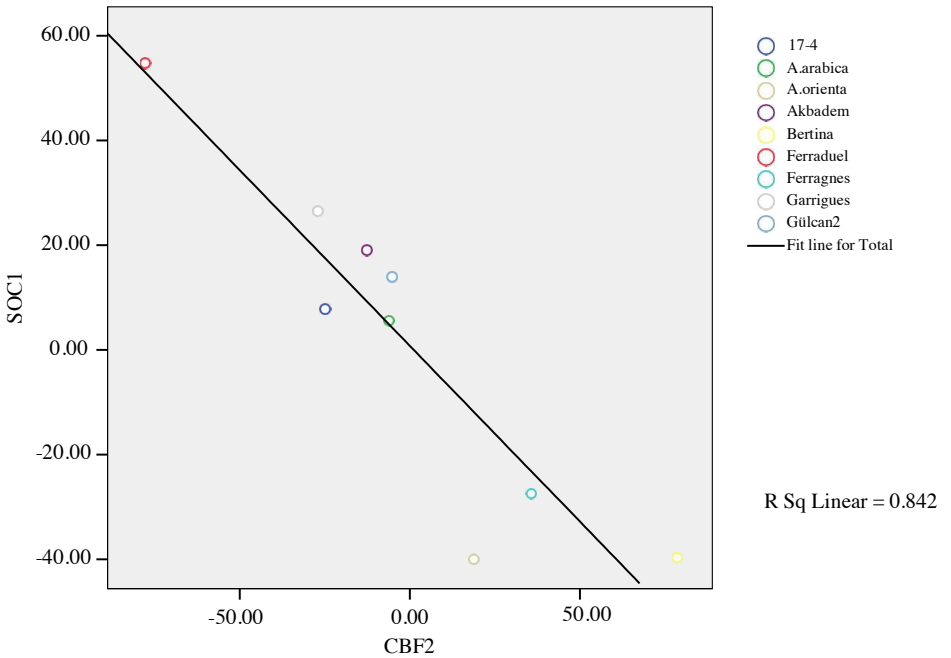

Figure 8. The correlation graph showing the relative expression of *PdCBF2* and *PabSOC1*.

accessions (Figure 3). In the present study, the floral integrator genes *PpFT* and *PabSOC1* were downregulated in the late flowering reference cultivars Ferragnes (–44.93-fold, –27.49-fold) and Bertina (–16.35-fold,

–39.66-fold), respectively. Downregulation of *FT* in the floral meristem were associated with delayed flowering in *Arabidopsis* (Agliassa et al., 2018). *SOC1* is a MADS domain–containing transcription factor acting as a promoter of flowering (Lee et al., 2004). The regulation of *SOC1* is by the floral activator *CONSTANS* (*CO*) through *FT* or floral repressor *FLC*, and the expression of *SOC1* promotes early flowering (Lee and Lee, 2010), whereas

recessive loss of function alleles of this gene causes late flowering (Onouchi et al.,2000). The introduction of citrus *SOC1* like genes into the *SOC1* mutant resulted in earlier flowering in citrus (Tan and Swain, 2007).

Previous knowledge on the floral integrator genes reporting delayed flowering may explain the downregulation of *PpFT* and *PabSOC1* in late flowering Bertina and Ferragnes.

In terms of wild almonds, *A. orientalis* is known as a late flowering species and used for cold resistance and self-fertile cultivar breeding of almonds (Bayazit et al., 2011). In another work, *A.orientalis* is classified as mid flowering (Sorkeh et al., 2007). The expressions of *PpFT* and *PabSOC1* were downregulated in *A.orientalis* (–27.29-fold,-39.91-fold), respectively. Increased *PpFT* and *PabSOC1* expression is associated with flowering. The upregulation of *FT* and *SOC1* in early flowering cultivar Garrigues, local cultivars Akbadem and 17-4 may indicate the onset of flowering in these cultivars (Table 2).

Frost tolerance is an important trait in almond production affecting yield and productivity. The role of *CBF* genes has been reported in many studies as being induced upon cold exposure as an indication of cold tolerance in plants (Benedict et al., 2006; Barros et al., 2012 a, 2012b). Research in transgenic *Arabidopsis* plants revealed that the overexpression of *CBF1, CBF2*, or *CBF3* genes results in an increase in cold tolerance (Gilmour et al., 2004), whereas the downregulation of the *CBF* pathway results in a decrease in cold tolerance (Novilla et al., 2007). We have demonstrated the cold response of almond buds exposed to –2 °C for 6 h based on *PdCBF1* and *PdCBF2* gene expression profiles.

In wild almonds, both cold related genes were upregulated in *A. orientalis* (2.15-fold, 18.84-fold), with a marked increase in *PdCBF2* expression. Bayazit et al. (2011) suggested that *A. orientalis* was used for the cold resistance and self-fertile cultivar breeding of almonds. Contrarily, both *PdCBF1* and *PdCBF2* genes were downregulated in *A. arabica* (–13.89-fold, –6.20-fold).

The highest increase in terms of both genes were determined in some commercial almond cultivars, including the almond cultivar Ferragnes. Information regarding the frost tolerance of most almond cultivars is scarce. However, Imani and Mahamadkhani (2011) reported that the damage to the buds of Ferragnes almond cultivar was 25% and 100% when exposed to –4 °C at the popcorn stage and during anthesis, respectively. In terms of local cultivars, the increase in *PdCBF1* and *PdCBF2* expressions was lower compared to the commercial cultivars. However, the decrease in the expression of both genes was most evident in early flowering 17-4 and Akbadem cultivars.

Analysis of the expression patterns of almond accessions revealed a positive correlation between *PdCBF1* and *PdCBF2* genes such that their transcipts were either increased or decreased simultaneously in each almond accession (Figure 6).

The overexpression of *CBFs* in plants is reported to result in enhanced cold tolerance (Gilmour et al., 2000). Although many other factors might also influence cold tolerance in plants, *CBF*s are revealed as the major components in the complex cold responsive network through the regulation of COR genes.

Alisoltani et al. (2015) reported the expression profiles of cold-related genes, including *PdCBF* of almond accessions consisting of a frost tolerant (G19) and a frost sensitive (M3) genotype. They reported that *PdCBF* showed a steady over-expression pattern from 0 to –2 °C treatments across all accessions. In frost sensitive M3, the expression of *PdCBF* decreased after 2 h at –2 °C. They reported that the expression of frost tolerant G19 was higher than frost-sensitive M3. The authors suggested that the over-expression of cold-related genes, including *CBF* resulted in an increase in the plant's tolerance to frost stress. In the light of these findings, our findings demonstrated the frost tolerance of almonds.

In general, the increased expression of *CBF*s is usually reported after a temperature decrease. However, Barros et al. (2012b) reported a differential expression of *PdCBF1* and *PdCBF2* genes during the developmental stages of the plant. They demonstrated that a sharp decrease occured after bud break and during growth resumption in flower buds and internodes when temperatures were still low. These findings may also explain the decreases in the expression of *PdCBF* genes in some almond accessions in response to cold treatments.

In this work, we have demonstrated a positive correlation between *FT* and *SOC1* genes (Figure 3) and between *CBF1* and *CBF2* (Figure 6). Seo et al. (2009) suggested that *SOC1* functions as the negative regulator of cold responsive *CBF* genes in *Arabidopsis*. Chew and Halliday (2010) suggested that *CBF* and vernalization pathways are linked such that *CBF*s are activated before vernalization to suppress flowering by influencing *FLC*. Contrarily, at the onset of reproductive growth, *SOC1* negatively regulates *CBF*s to initiate flowering in *Arabidopsis*. According to these authors, this crosstalk is a natural mechanism to avoid flowering during cold conditions while enable flowering during warmer temperatures. In this work, we have demonstrated a negative association between *SOC1* and *CBF*s in almonds that include wild species and prominent cultivars (Figures 7, 8). Our findings demonstrated the crosstalk between the flowering and cold tolerance genes in mediating flowering for the first time in almonds.

Acknowledgment

This research was supported by Ankara University Scientific Research Projects Coordination Unit. Project Number: 17L0447001, 2019.

References

Agliassa, C, Narayana R, Bertea CM, Rodgers CT, Maffei ME (2018). Reduction of the geomagnetic field delays arabidopsis thaliana flowering time through downregulation of flowering-related genes. Bioelectromagnetics 39: 361-374. doi: 10.1002/bem.22123

Alisoltani A, Shiran B, Ebrahimi E, Fallahi H, Mousavi S et al. (2015). Expression of genes related to macromolecule metabolic process under cold stress in almond (*Prunus dulcis* Mill) using RNA-seq analysis. Modern Genetics Journal 12 (1): 21-32.

Barros PM, Cherian S, Costa M, Sapeta H, Saibo NJM et al. (2017). The identification of almond GIGANTEA gene and its expression under cold stress, variable photoperiod, and seasonal dormancy. Biologia Plantarum 61 (4): 631-640. doi:10.1007/s10535-017-0711-1

Barros PM, Gonçalves N, Nelson JM, Saibo NJM, Oliveira MM (2012a). Functional characterization of two almond C-repeat-binding factors involved in cold response. Tree Physiology 32: 1113-1128. doi: 10.1093/treephys/tps067

Barros PM, Gonçalves N, Nelson JM, Saibo NJM, Oliveira MM (2012b). Cold acclimation and floral development in almond bud break:insights into the regulatory pathways. Journal of Experimental Botany 63 (12): 4585-4596. doi: 10.1093/jxb/ers144

Baurle I, Dean C (2006). The timing of developmental transitions in plants. Cell 125: 655-664. doi: 10.1016/j.cell.2006.05.005

Bayazit S, Çalışkan O, Irmak B (2011). Comparison of pollen production and quality characteristics of cultivated and wild almond species. Chilean Journal of Agricultural Research 71 (4): 536-541. doi: 10.4067/S0718-58392011000400006

Benedict C, Skinner JS, Meng R, Chang Y, Bhalerao R et al. (2006). The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in Populus spp. Plant, Cell & Environment 29: 1259-1272. doi: 10.1111/j.1365-3040.2006.01505.x

Blazquez MA, Ahn JH, Weigel D (2003). A thermosensory pathway controlling flowering time in Arabidopsis thaliana. Nature Genetics 33: 168-171. doi: 10.1038/ng1085

Boss PK, Bastow RM, Mylne JS, Dean C (2004). Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell 16 (Suppl.): S18-S31. doi: 10.1105/tpc.015958

Chew YH, Halliday KJ (2010). A stress-free walk from Arabidopsis to crops. Current Opinion in Biotechnology 22 (2): 281-286. doi: 10.1016/j.copbio.2010.11.011

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X et al. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes & Development 17: 1043-1054. doi: 10.1101/gad.1077503

Chinnusamy V, Zhu J, Zhu JK (2007). Cold stress regulation of gene expression in plants. Trends in Plant Science 12: 444-451. doi: 10.1016/j.tplants.2007.07.002

Erez A (2000). Bud dormancy; phenomenon, problems and solutions in the tropics and subtropics. In: Erez A (editor). Temperate Fruit Crops in Warm Climates. Dordrecht, The Netherlands: Springer, pp 17-48.

Gilmour SJ, Fowler SG, Thomashow MF (2004). Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Molecular Biology 54: 767-781. doi: 10.1023/B:plan.0000040902.06881.d4

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000). Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology 124 (4): 1854-1865. doi: 10.1104/pp.124.4.1854

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM et al. (1998). Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant Journal 16 (4): 433-442. doi: 10.1046/j.1365-313x.1998.00310.x

Hoagland DR, Arnon DI (1950). The ater culture method for growing plants without soil. California Agricultural Experimental Station Circular 347:1-32.

Imani A, Mahamadkhani Y (2011). Characteristics of almond selections in relation to late frost spring. International Journal of Nuts and Related Sciences 2 (2): 77-80.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M et al. (2006). Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant & Cell Physiology 47: 141-145. doi: 10.1093/pcp/pci230

İbrahime M, Kibar U, Kazan K, Yüksel Özmen C, Mutaf F et al. (2019). Genome-wide identification of the LEA protein gene family in grapevine (*Vitis vinifera* L.). Tree Genetics and Genomes 15 (4):55. doi: 10.1007/s11295-019-1364-3

Kitamura Y, Takeuchia T, Yamanea H, Taoa R (2016). Simultaneous down-regulation of dormancy-associated MADS-box6 and SOC1 during dormancy release in Japanese apricot (Prunus mume) flower buds. Journal of Horticultural Science and Biotechnology 91 (5): 476-482. doi: 10.1080/14620316.2016.1173524

Lee J, Lee I (2010). Regulation and function of SOC1, a flowering pathway integrator. Journal of Experimental Botany 61(9):2247-2254. doi:10.1093/jxb/erq098

Lee S, Kim J, Han JJ, Han MJ, An.G (2004). Functional analyses of the flowering time gene OsMADS50, the putative SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 (SOC1/AGL20) ortholog in rice. Plant Journal 8 (5): 754-64. doi: 10.1111/j.1365-313X.2004.02082.x

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408. doi: 10.1006/meth.2001.1262

Medina J, Bargues M, Terol J, Perez-Alonso M, Salinas J (1999). The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiology 119: 463-470. doi: 10.104/pp.119.2.463

Medina J, Catala R, Salinas J (2011). The CBFs: three Arabidopsis transcription factors to cold acclimate. Plant Science 180: 3-11. doi: 10.1016/j.plantsci.2010.06.019

Moon J, Lee H, Kim M, Lee I (2005). Analysis of flowering pathway integrators in Arabidopsis. Plant & Cell Physiology 46: 292-299. doi: 10.1093/pcp/pci024

Mouradov A, Cremer F, Coupland G (2002). Control of flowering time: interacting pathways as a basis for diversity. Plant Cell 14 (suppl.) S111-S130. doi: 10.1105/tpc.001362

Novillo F, Medina J, Salinas J (2007). Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. PNAS 104 (52): 21002-21007. doi: 10.1073/pnas.0705639105

Onouchi H, Igeno MI, Perilleux C, Graves K, Coupland G (2000). Mutagenesis of plants overexpressing CONSTANS demonstrates novel interactions among Arabidopsis flowering-time genes. Plant Cell 12: 885-900. doi: 10.1105/tpc.12.6.885

Parcy F (2005). Flowering: a time for integration. International Journal of Developmental Biology 49: 585-593. doi: 10.1387/ijdb.041930fp

Park S, Lee CM, Doherty CJ, Gilmour SJ, Kim Y et al. (2015). Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. Plant Journal 82 (2): 193-207. doi: 10.1111/tpj.12796

Seo E, Lee H, Jeon J, Park H, Kim J et al. (2009). Crosstalk between cold response and flowering in arabidopsis ıs mediated through the flowering-time gene soc1 and ıts upstream negative regulator flc. Plant Cell 21: 3185-3197. doi: 10.1105/tpc.108.063883

Shi H, Qian Y, Tan DX, Reiter RJ, He C (2015). Melatonin induces the transcripts of CBF/DREB1s and their involvement in both abiotic and biotic stresses in Arabidopsis. Journal of Pineal Research 59: 334-342. doi: 10.1111/jpi.12262

Silva C, Garcia-Mas J, Sanchez MA, Arus P, Oliveira MM (2005). Looking into flowering time in almond (Prunus dulcis (Mill) D. A. Webb): the candidate gene approach. Theoretical and Applied Genetics 110: 959-968. doi: 10.1007/s00122-004-1918-z

Simpson GG, Dean C (2002). Arabidopsis, the rosetta stone of flowering time? Science 296: 285-289. doi: 10.1126/science.296.5566.285

Socias i Company R, Felipe AJ, Gómez Aparisi J (1999). A major gene for flowering time in almond. Plant Breeding 118: 443-448. doi: 10.1046/j.1439-0523.1999.00400.x

Sorkeh K, Shiran B, Gradziel TM, Epperson BK Martı´nez-Go´mez P et al. (2007). Amplified fragment length polymorphism as a tool for molecular characterization of almond germplasm: genetic diversity among cultivated genotypes and related wild species of almond, and its relationships with agronomic traits. Euphytica 156: 327-344. doi: 10.1007/s10681-007-9382-x

Tan FC, Swain SM (2007). Functional characterization of AP3, SOC1 and WUS homologues from citrus (Citrus sinensis). Physiologia Plantarum 131 (3): 481-495. doi: 10.1111/j.1399-3054.2007.00971.x

Trainin T, Bar-Ya'akov I, Holland D (2013). ParSOC1, a MADS-box gene closely related to Arabidopsis AGL20/SOC1, is expressed in apricot leaves in a diurnal manner and is linked with chilling requirements for dormancy break. Tree Genetics and Genomes 9: 753-766. doi: 10.1007/s11295-012-0590-8

Turck F, Fornara F, Coupland G (2008). Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annual Review of Plant Biology 59: 573-594. doi: 10.1146/annurev.arplant.59.032607.092755

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF (2005). Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. Plant Journal 41: 195-211. doi: 10.1111/j.1365-313X.2004.02288.x

Yılmaz A (2017). Gaziantep ili Araban ve Yavuzeli ilçelerinde doğal olarak yetişen bademlerin (*Prunus amygdalus* Batsch) seleksiyonu. PhD, Ankara University, Ankara, Turkey (in Turkish).