Tree Physiology 33, 866–877
doi:10.1093/treephys/tpt056

Research paper

CBF gene expression in peach leaf and bark tissues is gated by a circadian clock

Timothy S. Artlip, Michael E. Wisniewski[1], Carole L. Bassett and John L. Norelli

USDA-ARS, Appalachian Fruit Research Station, Kearneysville, WV 25430, USA; [1]Corresponding author (michael.wisniewski@ars.usda.gov)

Received Feburary 25, 2013; accepted July 4, 2013; published online August 15, 2013; handling Editor Chung-Jui Tsai

CBF (C-repeat Binding Factor) transcription factors are part of the AP2/ERF (Apetala2-ethylene responsive factor) domain family of DNA-binding proteins that recognize a C-repeat response *cis*-acting element that regulates a number of cold-responsive genes (*CBF* regulon). Induction of *CBF* gene expression by low temperature in *Arabidopsis* has been shown to be gated by a circadian clock. In peach (*Prunus persica* L.), five *CBF* genes are arranged in tandem on scaffold (linkage group) 5 of the peach genome. Since *CBF* gene regulation has been shown to be more complex in woody plants than herbaceous plants, the present study was conducted to determine if temperature-modulated *CBF* gene expression in peach leaf and bark tissues was also influenced by a circadian clock. One-year-old 'Loring' peach trees grafted on 'Bailey' rootstocks were entrained to a 12-h day/12-h night photoperiod at 25 °C. After 2 weeks, trees were exposed to 4 °C under continuous light for up to 48 h beginning at either subjective dawn + 4 h (ZT4; where ZT is Zeitgeber time) or subjective dawn + 16 h (ZT16) with leaf and bark tissues harvested at various time points. Gene expression of the five peach *CBF* genes and a *DREB2* gene was assessed by real-time quantitative polymerase chain reaction. Results revealed a distinct gating of *CBF* gene expression by a circadian clock for four *CBF* genes in both leaf and bark tissues. *CBF* genes were highly induced by 4 °C in ZT4 leaf samples with expression peaking at 6–24 h depending on the specific *CBF* gene. In contrast, *CBF* gene expression was highly attenuated in leaf, and to a lesser extent in bark, samples exposed to 4 °C at ZT16. These results are similar to reports for *Arabidopsis*. Further experiments were conducted to verify environmental influence on the induction of *CBF* and *DREB2* genes. In contrast to *DREB2* genes from other dicots, the peach *DREB2* ortholog was induced by both low temperature and dehydration. Induction of the peach *CBFs* and *DREB2* by either low temperature or dehydration corresponded with regulatory motifs present in their promoter sequences. Low temperature and dehydration induction data for three peach dehydrin genes indicated that the regulation of these genes in peach is complex, with individual dehydrin gene expression being correlated with the expression of one or more *CBF* genes.

Keywords: CBF, circadian rhythm, dehydrin, DREB, low temperature, peach, *Prunus persica*, Zeitgeber time.

Introduction

CBF (C-repeat Binding Factor) proteins belong to the CBF/DRE binding (DREB1) sub-family of the Apetala2-ethylene responsive factor (AP2/ERF) superfamily of transcription factors (Sakuma et al. 2002, Nakano et al. 2006). They bind to a *cis*-element (*DRE*/*CRT*/*LTRE*) containing the conserved CCGA core sequence (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994).

Low temperature (LT)-inducible *CBF* genes regulate a large number of cold-regulated (*COR*) genes (the *CBF* regulon) whose products are thought to contribute to freezing tolerance. For example, it is well known that LT-inducible dehydrins display expression patterns correlated with *CBF* expression and are part of the *CBF* regulon (e.g., Novillo et al. 2007). Overexpression of *Arabidopsis CBF* genes has been shown to increase LT tolerance

in several plant systems via the increased expression of target genes (Novillo et al. 2007, Mizoi et al. 2012).

Closely related DREB2 transcription factors also bind to the same CCGA core sequence as CBF/DREB1 and can induce the expression of many of the same downstream target genes (Sakuma et al. 2002, Nakano et al. 2006). DREB2 proteins in dicots, however, are not cold responsive and overexpression does not result in increased cold tolerance (Mizoi et al. 2012). Instead, DREB2 members from dicots are responsive to, and components of, heat-shock, salinity and dehydration signaling pathways (Qin et al. 2011, Mizoi et al. 2012). Slight differences in the *DRE/CRT/LTRE cis*-element impact the binding preferences of CBF/DREB1 and DREB2 members and thus may provide a measure of specificity for their response to different abiotic stresses (Sakuma et al. 2002, Qin et al. 2011, Mizoi et al. 2012).

Low temperature regulates *CBF* expression through at least two signaling pathways. One, dependent on changes in Ca^{2+} concentration, is modulated via a complex of calmodulin and a CAMTA (Calmodulin binding Transcription Activator) transcription factor which together binds to a *cis*-element in the *CBF* promoter and positively regulates *CBF* transcription. A second pathway involves the activation of an ICE (Inducer of CBF Expression) transcription factor that acts as a positive regulator of *CBF* expression and a negative regulator of *Myb15*, which in the absence of LT represses *CBF* gene expression. These pathways impact *CBF* genes differentially (Mizoi et al. 2012, Wisniewski et al. 2013).

In addition to LT, expression of *CBF*s in *Arabidopsis* has been shown to be impacted by a circadian rhythm (Harmer et al. 2000, Fowler et al. 2005). During the day, CCA1 (Circadian Clock Associated1) and LHY (Late elongated HYpocotyl) levels are high and they positively regulate *CBF* expression. During evening, levels of TOC1 (Timing Of Cab Expression1), PIF7 (Phytochrome Interacting Factor7) and Phytochrome B are high; TOC1, PIF7 and PhyB interact to form a complex that represses *CBF* expression. The CCA1/LHY complex also represses TOC1 expression, but this effect ceases as their levels fall at subjective dusk. As TOC1 levels rise, it positively regulates CCA1 and LHY expression, thus turning itself off (Dong et al. 2011, Mizoi et al. 2012).

In their studies of *CBF* regulation in *Arabidopsis*, Harmer et al. (2000) and Fowler et al. (2005) shifted plants, entrained to a 12 h light/12 h dark photoperiod at warm temperatures, to LT at various times after subjective dawn. In this protocol, ZT0 (Zeitgeber time (ZT); Aschoff 1965) represents subjective dawn, while ZT4 is 4 h after subjective dawn and ZT16 is 16 h after subjective dawn, i.e., 4 h after subjective dusk. Harmer et al. (2000) demonstrated that *AtCBF3* exhibits circadian-regulated cycling at warm temperatures. Fowler et al. (2005) examined the response of *AtCBFs1–3* to LT at various ZTs and demonstrated that *AtCBFs1–3* transcript accumulation was much greater when LT was imposed at ZT4 than at ZT16. The results further indicated that members of the *CBF* regulon also exhibit an attenuated response to LT due to the circadian gating of CBF. Fowler et al. (2005) demonstrated that constitutive expression of CCA1 abolishes the circadian gating of *AtCBFs1–3* in response to LT.

The role of *CBF* in cold response has been documented in both herbaceous (Thomashow et al. 2001, Qin et al. 2011, Mizoi et al. 2012) and woody plants (Welling and Palva 2008, Wisniewski et al. 2013). Regulation of *CBF*s in woody plants is complex and exhibits gene, tissue and age-related specificity, as well as temporal differences in the timing of induction, not observed in herbaceous plants (Wisniewski et al. 2013). Benedict et al. (2006) reported that the expression pattern of poplar *CBF* genes was different in annual vs. perennial tissues. Xiao et al. (2006, 2008) reported a similar phenomenon in grape, where *Vitis CBFs1–3* were expressed only in young tissue in response to LT, while *Vitis CBF4* was expressed in both young and old tissue in response to LT. *Eucalyptus gunnii* (Hook f.) *CBFs1a–d* exhibit a differential response to a number of variables including temperature, the rate of induction and photoperiod (El Kayal et al. 2006, Navarro et al. 2009). Under long days, *CBF* induction in birch occurs within 15 min after exposure to LT (Welling and Palva 2008). Under short days (SD), however, *CBF* induction is delayed and upregulated for a longer period of time. Welling and Palva (2008) further demonstrated that the exposure of dormant birch trees to freezing temperatures (–10 °C) only induced *CBF* expression and *COR* genes after trees had thawed.

Wisniewski et al. (2011) constitutively expressed *PpCBF1* from peach (*Prunus persica* [L.] Batsch cv. 'Loring') in apple (*Malus* × *domestica* Borkh.) 'M.26' rootstock. Freezing tolerance was significantly greater in both non-acclimated and cold-acclimated transgenic trees compared with untransformed trees. Unexpectedly, dormancy and leaf senescence were triggered by SD in the transgenic trees, a response that is atypical for apple (Heide and Prestrud 2005). In order to increase our understanding of *CBF* regulation in fruit trees, the present study was conducted to (i) determine if LT induction of *CBF* gene expression in peach leaf and bark tissues is gated by a circadian clock as in *Arabidopsis*, and (ii) characterize the natural expression of *PpCBF1* in its native context, along with other peach *CBF* genes, to better understand the impact of *PpCBF1* overexpression in apple. Such information may be critical for adapting fruit trees to predicted changes in climate resulting from global warming and increased levels of atmospheric carbon dioxide.

Materials and methods

Plants

One-year-old 'Loring' peach trees grafted on 'Bailey' rootstocks (0.95 cm caliper; Adams County Nursery, Aspers, PA, USA) in

11.3-L pots with MetroMix 360 (Sun Gro Horticulture, Bellevue, WA, USA) were allowed to break dormancy and leaf out in a greenhouse in mid-spring (ambient light and daylength; temperatures ca. 15–30 °C; fertilized twice with MiracleGro; Scott's Miracle-Gro Products, Marysville, OH, USA). Actively growing trees were then moved to a PGV36 growth chamber (Conviron, Winnipeg, MN, Canada) for 2 weeks with 12 h day/12 h night photoperiod at a constant 25 °C. The light level during the day period was ~300 μmoles photons m^{-2} s^{-1}.

Low-temperature treatment

Low-temperature exposure (4 °C) was initiated at a ZT of ZT4 (subjective dawn + 4 h), with 12 trees moved to a separate chamber with a lower light level to reduce the possibility of photoinhibition (continuous light; 100 μmoles photons m^{-2} s^{-1}). Random leaves from each of three trees were harvested at 0, 1, 4, 6, 24 and 48 h. Bark tissue (phloem, cambium and epidermis) was destructively sampled (i.e., trees completely destroyed) from three trees at 0, 4, 24 and 48 h. Leaf and bark tissues were frozen in liquid N_2, and stored at −80 °C until use. An additional LT exposure was initiated at ZT16 (subjective dawn + 16 h; i.e., dark + 4 h) with continuous light (100 μmoles photons m^{-2} s^{-1}). Leaves from three trees were harvested at 0, 1, 4, 6 and 48 h. Bark tissues were destructively sampled at 0 and 48 h. The leaf and bark tissues were flash frozen in liquid N_2, and stored at −80 °C until use. The tissue from each tree was collected and stored separately as biological replicates (three biological replicates per time point per ZT).

Bioinformatic analyses

Putative *CBF* genes were subjected to BLAST (Thompson et al. (1994) within the Genome Database for Rosaceae (GDR; http://www.rosaceae.org, 30 July 2013 date last accessed). The 5′-UTRs (up to 1000 bp upstream of the putative translational start site) were analyzed by PLACE (http://www.dna.affrc.go.jp/PLACE/, 30 July 2013 date last accessed; Higo et al. 1999), PAN (http://plantpan.mbc.nctu.edu.tw/gene_group/index.php, 30 July 2013 date last accessed; Chang et al. 2008) and PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, 30 July 2013 date last accessed; Lescot et al. 2002).

Real-time quantitative polymerase chain reaction

Total RNA was isolated from leaf and bark tissues using Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase (Turbo DNA-free Kit; Ambion, Austin, TX, USA) and then diluted to 25 ng μl^{-1}. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed using 50 ng of total RNA as a template, SuperScript III Platinum SYBR Green One-Step RT-qPCR Kit with ROX (Invitrogen) and 2.0 pmol of each primer per reaction; no-RT control reactions were included to ensure no residual DNA contamination. The ABI 7900 (Applied Biosystems, Foster City, CA, USA) was set to cycle as follows: cDNA synthesis at 48.0 °C for 30 min; 95.0 °C denaturation for 5 min; 40 cycles of 95.0 °C for 15 s followed by 52.0–57.0 °C (depending on primers used; Table 1) for 1 min; followed by ABI-specified hold and melt curve stages. Primers were verified for specificity by using genomic DNA template and assessing the resulting amplicon by agarose gel electrophoresis and qPCR with genomic DNA on the ABI 7900; all primers had a single band and single peak. Primer efficiency was also verified for all primer sets by qPCR analysis of a standard curve, constructed by serially diluting RNAs from the sample set starting at some concentration above what was used in unknown samples and ending at a concentration well below it. Three technical replicates were used for each biological replicate (tree). The standard curve method was used to calculate transcript abundance relative to *β-tubulin* as a reference gene (user bulletin no. 2; Applied Biosystems http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf, 30 July 2013 date last accessed; Tong et al. 2009). The *β-tubulin* gene along with other endogenous reference genes (*actin*, *translation elongation factor 2* and *26S rRNA*) were assessed as to their stability within a tissue and across time points (see Table 1 for primer sequences), since Nicot et al. (2005) and Oakley et al. (2007) demonstrated potential problems with the use of *β-tubulin* as an endogenous reference gene. *β-tubulin* was deemed the best overall reference gene according to the NormFinder software (Anderson et al. 2004; Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online). To weight the importance of biological variation over technical variation, technical replicates were nested within biological replicates in calculating the mean square error term. Normalized data were then re-normalized to the respective values at time 0, and the means taken from the biological replicates. Standard errors (SEs) were derived by dividing the standard deviations by the square root of n, where $n = 3$. Significance of differences between ZT4 and ZT16 time points was calculated by a Log2 transformation (to satisfy the statistical normality assumption) of the T0 re-normalized biological replicate values and performing an independent two-sample Student's *t*-test; the null hypothesis was that the two means were equal.

Results

Peach CBF and DREB2 genes

An in silico analysis of the peach genome revealed five peach *CBF* genes (*PpCBFs1–5*) in a tandem array on Linkage Group (LG) 5 with high amino acid homology to each other (Figure 1a and b; see also Wisniewski et al. 2013). An additional *CBF* gene located on LG 2, *PpCBF6*, also exists (Wisniewski et al. 2013) but was not investigated. Another gene, termed *PpDREB2C*, located on LG 2, was investigated as part of this study. *PpDREB2C* is a member of the *DREB2* sub-family of *AP2/ERF*

Table 1. Primers tested or used for RT-qPCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)
PpCBF1 (ppa014628m)	**GCACATTGTGGATATGGGAAAAAG**	**GGGTTGGGGTGGAGAAAGAAG**
	GGAAGAGAAGAAGAAGAAG	GGGTGGAGAAAGAAGAAG
	GGTGGAAGAGAAGAAGAAGAAG	GGGGTGGAGAAAGAAGAAG
PpCBF2 (ppa010909m)	**CTTCTTCTTTCTCCACCT**C	**GCAACTCACACATGAACAA**
	AACTGAAGCTGATGCCAA	GCAACTCACACATGAACAAA
PpCBF3 (ppa010800m)	**TCTTTCTCCACCGCAACC**	**AACAATAATCGCTCGCACAA**
	TTCTTTCTCCACCGCAAC	AACAATAATCGCTCGCAC
PpCBF4 (ppa017761m)	**AGAAGGAGAGTAAGGGGG**	**AGCACTGAGGTGGACAAA**
	GAGAAGGAGAGTAAGGGG	AGGTGGACAAAGCATAAG
PpCBF5 (ppa021197m)	TTGCCTGCCTCAACTTCC	CCTTCTTCTTCTTCTTCTCTTCC
	CTTGCCTGCCTCAACTTC	TCCTTCTTCTTCTTCTTCTCTTCC
	CTTCTCTCATTTTTCTGACTCC	CTCATTTCACACACCCAC
	AAGCGGAGTCAGGGAAGT	GTGGGTGTGTGAAATGAGAG
	AGGTGGAAGAGAAGAAGAAG	GAGGTGGAGTAAGAAGAAGG
	TGGGATGAGGAAGAAGTG	AGGTGGAGTAAGAAGAAGG
	GGAAGAGAAGAAGAAGAAGAAG	AGGTGGAGTAAGAAGAAGG
	GAAGAGAAGAAGAAGAAGAAGG	AGGTGGAGTAAGAAGAAGG
	GTGGAGTTTGGTGGAGTG	TGAGGTGGAGTAAGAAGAAGG
PpDhn1 (ppa005514m)	**TGACACCCAGACAACCAC**	**TCATCCTTTTGCCCACCT**
	TGACACCCAGACAACCAC	CTTCTTCTCCTGGTGCTCTCCT
	GAGCAGAGGACCACGAGAAGAA	TGGGTGGGTGTCATGAGAG
PpDhn2 (ppa011637m)	**GGAGGGAGGAGGAAGAAGAA**	**GAGTCTGAGATGGGTAGGG**
	AGGGAGGAGGAAGAAGAAGG	GTCTGAGATGGGTAGGGGT
	AGGAGGGAGGAGGAAGAAGAA	GTCTGAGATGGGTAGGGGT
PpDhn3 (ppa010326m)	**AGAAAAGAAGGGATTGAAGG**	**TCTTCTGCTGCCCTGGTA**
	TGATCAGAAGGTGGAGGAC	CCCTGGTAGCTTTTCCTTT
	GGAGAAGATCTGTGGTGAT	TTCTTCTGCTGCCCTGGT
PpDREB2C (ppa007606m)	**AGGCGAATCGGCAATTCATGCT**	**TTGACGGCCGGTTGATCATTGT**
TUB (ppa005644m)	**CCGAGAATTGTGACTGCCTTCAAG**	**AGCATCATCCTGTCTGGGTATTCC**
26S rRNA	**GCAGCCAAGCCTTCATAGCG**	**GTGCGAATCAACGGTTCCTC**
TEF2 (ppa001368m)	**GGTGTGACGATGAAGAGTGATG**	**TGAAGGAGAGGGAAGGTGAAAG**
actin (ppa007242m)	**CACCGAAAGAGGGTACATGTTCA**	**TGCGAGCTTCTCCTTCATATCA**

Gene names include Genome Database for Rosaceae predicted transcript accession numbers. Bold face denotes primer pairs used to generate RT-qPCR results, while regular face denotes primer pairs that were deemed unacceptable, including reference genes. *TUB*, *β-tubulin*; *TEF2*, *translation elongation factor 2*.

genes, and was named due to its similarity to *AtDREB2C* (GenBank Accessions NM129594 and NP565929; data not shown). The AP2 domain which defines the AP2/ERF family was evident in the conceptual translation of all five peach CBFs, as were domains which define the CBF/DREB1 sub-family, as per Nakano et al. (2006) and Wisniewski et al. (2013). Conceptual translation of the *PpDREB2C* gene includes an AP2 domain and domains consistent with the DREB2 sub-family as per Nakano et al. (2006) (Figure 1c).

An examination of the 5′ 1000 bases upstream of the translation start sites of *PpCBFs1–5* and *PpDREB2C* indicated the presence of abiotic stress regulatory motifs and many *cis*-elements related to light or photoperiod regulation (Table 2). The examination was done due to the relative paucity of such information for woody plants, particularly fruit trees, and may lead to a better understanding of how circadian rhythm, cold acclimation and dormancy interact in such trees. A variable number of the LT conserved motifs (CMs) described by Doherty et al. (2009) were found in the promoters of *PpCBFs1–5*. Partial matches for the ICEr1 and ICEr2 binding sites (Zarka et al. 2003) were found in the promoter for *PpCBF2*, along with exact or partial matches to all seven CMs, including CM2, which represents a CAMTA-binding element (Doherty et al. 2009). In addition, canonical C-repeats involved in LT responses (Baker et al. 1994, Wisniewski et al. 2013) were found in the promoters of *PpCBFs1–5* and in *PpDREB2C*, indicating the potential for self- or cross-regulation. Numerous putative ABREs (ABscisic acid Response Elements) were also found, implying regulation by the abscisic acid-dependent abiotic stress signal transduction pathway in *PpCBFs1–5*, and in *PpDREB2C*. CCA, PIF, evening element, GATA box and G-box elements are all regulatory elements recognized as binding sites for transcription factors involved in circadian regulation, and were found to varying extents in *PpCBFs1–5* and *PpDREB2C* (Table 2).

Expression analysis in leaves in response to LT

PpCBFs1–4 were all responsive to LT (Figure 2a–d). Expression levels, however, were significantly higher ($P \leq 0.05$) at ZT4

Figure 1. AP2-domain genes used in this research. (a) Alignment deduced amino acid sequences of *PpCBF* genes on LG 5. CBF-specific motifs are indicated in gray; the AP2 domain is outlined. Stars indicate identical residues, while colons and periods indicate synonymous or near-synonymous residues. Alignment was performed with Clustal W (Thompson et al. 1994). (b) Location of *PpCBF* genes on LG 5. *CBF* genes are circled. (c) Amino acid sequence of PpDREB2. DREB2-specific motifs are indicated in various shades of gray; the AP2 domain is outlined. CBF- and DREB2-specific motifs are thought to confer binding specificity either to DNA or other transcriptional machinery components.

than at ZT16 at 4 and 8 h post-LT treatment, indicating that *PpCBFs1–4* are gated by a circadian rhythm (Table S1 available as Supplementary Data at *Tree Physiology* Online). Expression of *PpCBF5* could not be detected by RT-qPCR despite numerous attempts and utilization of different primer combinations. Therefore, it was considered to be not expressed, and no data on this gene are presented. Unexpectedly, *PpDREB2C*, which was initially used as a marker gene for response to dehydration, was also highly responsive to LT (Figure 2e). Expression levels were significantly higher ($P \leq 0.05$) for ZT4 compared with ZT16 after 6 h exposure to LT, indicating that *PpDREB2C* is also gated by a circadian rhythm.

The level of *PpDHN1* expression was examined since it is cold-inducible and its promoter has two C-repeat motifs capable of binding by CBF (Wisniewski et al. 2006). *PpDHN1*

Table 2. Selected circadian rhythm, abiotic stress or light responsive promoter elements of *PpCBFs1–5* and *PpDREB2C*.

Gene	Motif	Position	Sequence in promoter	Published consensus sequence
PpCBF1	LTRE	18 (+)	GCCGAC	A/GCCGAC
	ABRE/G-box	880 (+)	CACGTGTC	YACGTGGC
	CCA	464 (–)	AGATTTTT	AAMAATCT
	Evening Element	610 (+)	AAAATATCC	AAAATATCT
	GATA	318 (+)	GATA	GATA
	PIF	713 (+)	aaagatCACGTgtaccaa	GKRGGMCACGTGRMSWCK
	PIF	860 (+)	cgtgttCACGTgtccact	GKRGGMCACGTGRMSWCK
PpCBF2	ICEr1-like	350 (+)	GGACACCATGACATGA	GGACACATGTCAGA
	ICRr2-like	850 (–)	GGAGGC	TGAGGC
	CM1-like	890 (+)	GGCCCCA	GACCCCA
	CM2 (CAMTA)	840 (+)	TGGCGCC (CCGCGGT)	VCGCGB
	CM3	645 (–)	AGAGAC	AGAGAC
	CM4 (ICEr4)-like	700 (–)	TCCACGT	TCCACGT
	CM5-like	270 (+)	GTGCTTC (CTTCGGTG)	CTTA/CGCTG
	CM6-like	230 (–)	ATTCTCA	AGATTCTCA
	CM7-like	350 (+)	GGGTAAGG	GGGTCAAAG
	LTRE	730 (–)	CAGCCA (ACCGAC)	A/GCCGAC
	ABRE/G-box	649 (–)	GCCACGTG	YACGTGGC
	CCA	920 (+)	CAATCTA	AAMAATCT
	Evening Element	971 (+)	AAAATATCT	AAAATATCT
	PIF	635 (+)	gggagcCACGTggacgta	GKRGGMCACGTGRMSWCK
	PIF	693 (+)	aacgatCACGTgtggcaa	GKRGGMCACGTGRMSWCK
	GATA	111 (+)	GATA	GATA
	GATA	311 (+)	GATA	GATA
PpCBF3	CM5-like	700 (+)	CTTAGTTC	CTTA/CGCTG
	LTRE	837 (+)	TCCGAC	A/GCCGAC
	ABRE/G-box	231 (+)	ACGT	YACGTGGC
	CCA-like	150 (–)	AAATCT	AAMAATCT
	Evening Element-like	220 (+)	AAAATATCA	AAMAATCT
	GATA	148 (+)	GATA	GATA
	GATA	661 (+)	GATA	GATA
	PIF	221 (+)	aaatatCACGTttgaaaa	GKRGGMCACGTGRMSWCK
PpCBF4	ICEr2-like	180 (–)	GGAGGC	TGAGGC
	CM1-like	265 (+)	GACCTCA	GACCCCA
	CM2 (CAMTA)	845 (+)	GCGCGT	VCGCGB
	CM3-like	910 (–)	AGAGAG	AGAGAC
	CM5-like	860 (+)	CTTCGCAT	
	CM6	10 (+)	GAGGTCTCA	AGATTCTCA
	LTRE	612 (–)	GTCGG (CCGAC)	CCGAC
	ABRE	320 (+)	ACGTG	YACGTGGC
	ABRE	152 (+)	AACGCGC	MACGYGB
	ABRE	319 (+)	CACGTGT	MACGYGB
	CCA	400 (+)	AAAATCT	AAMAATCT
	CCA	880 (+)	AAAATCT	AAMAATCT
	Evening Element-like	585 (+)	TAAATATCT	AAAATATCT
	GATA	232 (+)	GATA	GATA
	GATA	423 (+)	GATA	GATA
	GATA	434 (+)	GATA	GATA
	PIF	308 (+)	atttagCACGTgtgattt	GKRGGMCACGTGRMSWCK
PpCBF5	ICEr2-like	520 (–)	GGAGGC	TGAGGC
	CM2 (CAMTA)	760 (+)	TGGCGCA (ACGCGGT)	VCGCGB
	CM2 (CAMTA)	790 (+)	TGGCGCA (ACGCGGT)	VCGCGB
	CM2 (CAMTA)	875 (+)	CCGCGT	VCGCGB
	CM3	815 (–)	AGAGAC	AGAGAC
	CM3	615 (–)	AGAGAC	AGAGAC

(Continued)

Table 2. Continued

Gene	Motif	Position	Sequence in promoter	Published consensus sequence
	CM4 (ICEr4)-like	400 (+)	ACCACGT	TCCACGT
	LTRE	461 (+)	CCGAC	CCGAC
	ABRE/G-box	221 (–)	TACACGTG	YACGTGGC
	ABRE/G-box	347 (–)	GCCACGTA	YACGTGGC
	ABRE/G-box	497 (+)	CACGTGGC	YACGTGGC
	ABRE/G-box	882 (+)	TACGTGTC	YACGTGGC
	ABRE/G-box	126 (+)	CACGTGT	YACGTGGC
	ABRE/G-box	380 (+)	AACGTGT	YACGTGGC
	ABRE/G-box	497 (+)	CACGTGG	YACGTGGC
	GATA	287 (+)	GATA	GATA
	PIF	118 (+)	cttgtgCACGTgttataa	GKRGGMCACGTGRMSWCK
	PIF	214 (+)	attataCACGTgactgta	GKRGGMCACGTGRMSWCK
	PIF	338 (+)	tggagcCACGTaacgcac	GKRGGMCACGTGRMSWCK
	PIF	394 (+)	taaaacCACGTgtgatta	GKRGGMCACGTGRMSWCK
	PIF	483 (+)	tactgcCACGTggcagag	GKRGGMCACGTGRMSWCK
PpDREB2C	LTRE	616 (–)	GTCGG (CCGAC)	CCGAC
	ABRE/G-Box	86 (+)	CACGTGGC	YACGTGGC
	ABRE/G-Box	139 (+)	CACGTGTC	YACGTGGC
	ABRE/G-Box	570 (–)	CACGT	YACGTGGC
	ABRE/G-Box	984 (–)	ACGTGGC	YACGTGGC
	CCA1	882 (+)	AAAAATCT	YACGTGGC
	CCA1	882 (+)	AAAAATCT	AAMAATCT
	Evening Element	630 (–)	AAAATATCT	AAAATATCT
	GATA	122 (+)	GATA	GATA
	GATA	240 (–)	TATC	TATC
	GATA	314 (–)	TATC	TATC
	GATA	330 (–)	TATC	TATC
	GATA	38 (+)	GATA	GATA
	GATA	545 (–)	TATC	TATC
	GATA	59 (+)	GATA	GATA
	GATA	591 (+)	GATA	GATA
	GATA	643 (+)	GATA	GATA
	GATA	68 (+)	GATA	GATA
	GATA	751 (–)	TATC	TATC
	GATA	776 (+)	GATA	GATA
	GATA	802 (+)	GATA	GATA
	PIF	131 (+)	ttccagCACGTgtaccc	GKRGGMCACGTGRMSWCK
	PIF	553 (+)	ttaaatCACGTctcacat	GKRGGMCACGTGRMSWCK
	PIF	959 (+)	cagtttCACGTtaggggg	GKRGGMCACGTGRMSWCK

Promoter elements found in *PpCBFs1–5* and *PpDREB2C*. The 5′ 1000 bp upstream of the putative translational start site were analyzed by PLACE (http://www.dna.affrc.go.jp/PLACE/, 30 July 2013 date last accessed; Higo et al. 1999), PAN (http://plantpan.mbc.nctu.edu.tw/gene_group/index.php, 30 July 2013 date last accessed; Chang et al. 2008) and PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, 30 July 2013 date last accessed; Lescot et al. 2002). Position is starting 1000 bp from translational start site; (+) or (–) indicates strand. Sequence in the promoter is as indicated, while published consensus sequence data are from the PLACE, PAN and PLANTCARE databases. The promoter elements for each gene are arranged in the following order: circadian rhythm elements (ICE, CM), low-temperature response (LTRE), abscisic acid or G-Box (ABRE/G-Box) and light-responsive elements (CCA, evening element, GATA and PIF). Standard genetic code; R = A/G, Y = C/T, M = A/C, K = G/T, S = C/G, W = A/T, B = C/G/T, V = A/C/G.

was cold-inducible and its expression was much higher in ZT4 samples than in ZT16 samples (Figure 2f). The induction kinetics of *PpDHN1* is consistent with regulation by CBF(s) as expected, since *PpDHN1* expression increased after *CBF* genes had been upregulated. In contrast, *PpDHN2* and *PpDHN3* had minimal responses to LT with some evidence of circadian gating observed for *PpDHN3* (Figure 2f–h).

Expression analysis in bark tissues in response to LT

PpCBFs1–4 were all observed to be responsive to LT in bark tissue (Figure 3a–d). Unfortunately, insufficient material was available for taking ZT16 samples at 48 h. In addition, based on the reported kinetics of *CBF* expression in comparable systems such as poplar (Benedict et al. 2006), a 24-h sampling was thought to be adequate. Plant material was also limited because

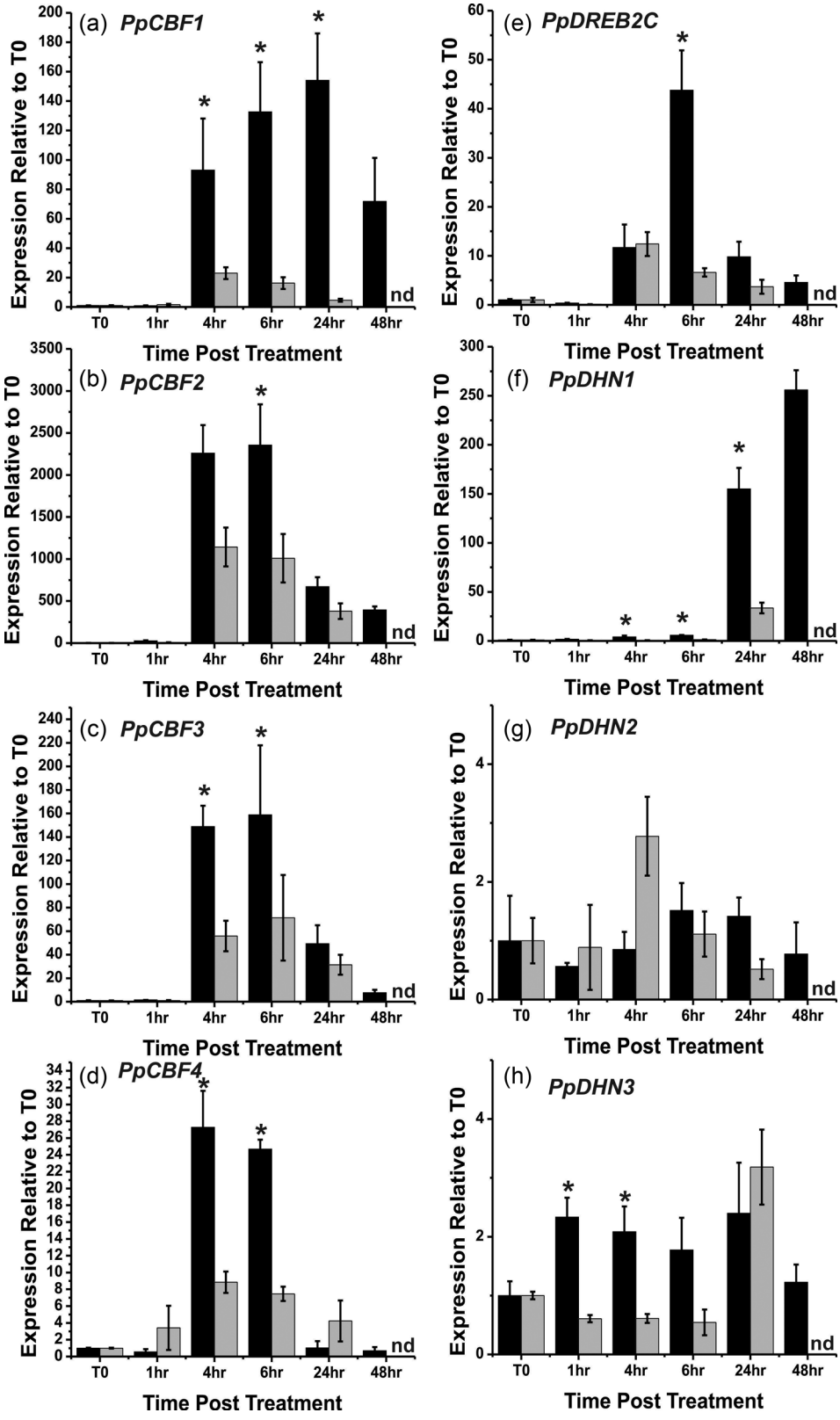

Figure 2. Real-time quantitative polymerase chain reaction time course expression data for *PpCBFs1–4*, *PpDREB2C* and *PpDHNs1–3* in leaves of plants shifted to 4 °C at ZT4 (black bars) vs. ZT16 (gray bars). Values are expression relative to the T0 time points for each gene at ZT4 or ZT16. Mean of three biological replicates ± SE. (a) *PpCBF1*. (b) *PpCBF2*. (c) *PpCBF3*. (d) *PpCBF4*. (e) *PpDREB2C*. (f) *PpDHN1*. (g) *PpDHN2*. (h) *PpDHN3*. A star above the error bars at a particular time point indicates a significant difference between ZT4 and ZT16 at the $P \leq 0.05$ level by Student's *t*-test. Note that scales may differ between panels. Significant differences between ZT4 and ZT16 suggest gating by the circadian rhythm.

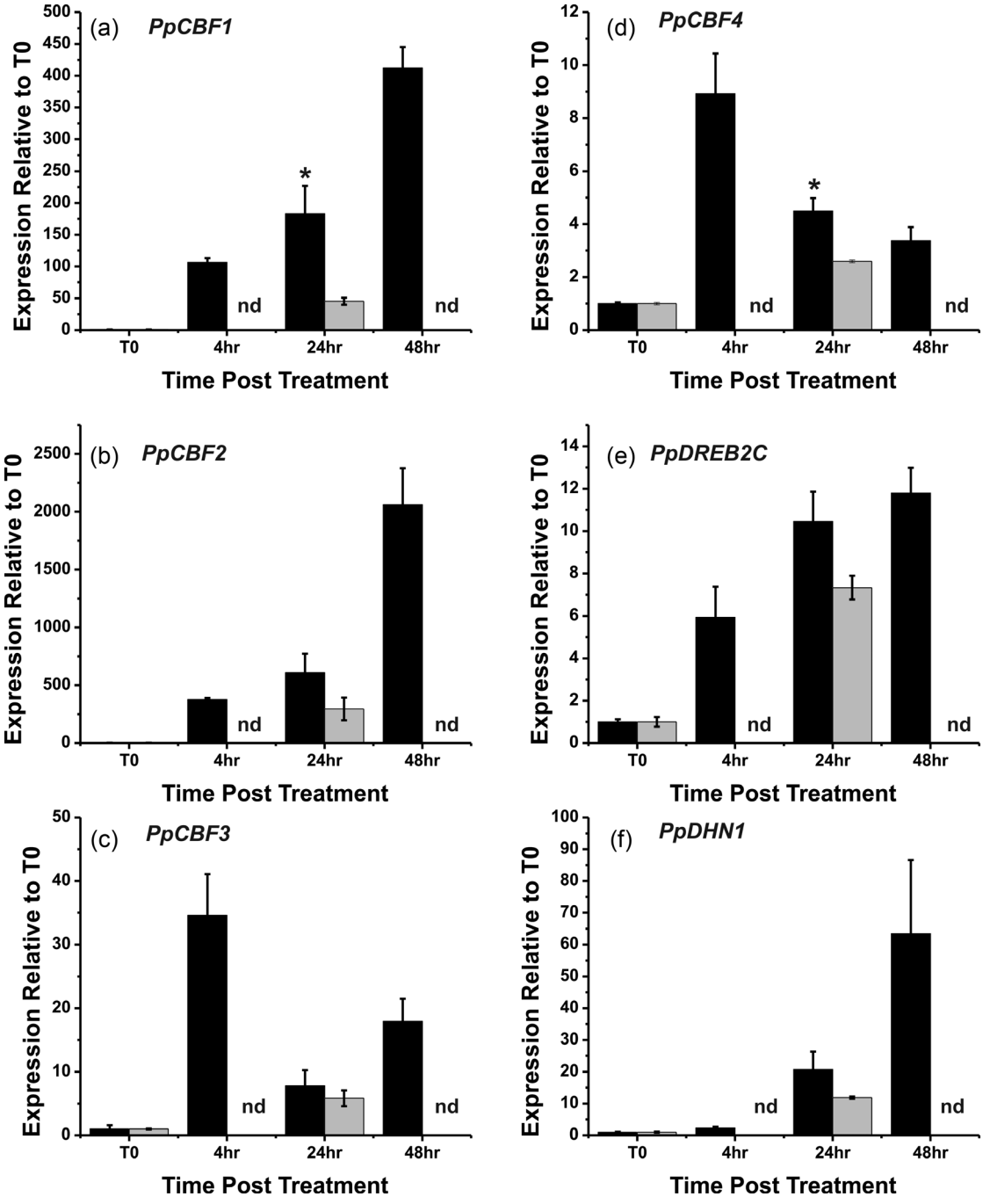

Figure 3. Real-time quantitative polymerase chain reaction time course expression data for *PpCBFs1–4*, *PpDREB2C* and *PpDHN1* in bark of plants shifted to 4 °C at ZT4 (black bars) vs. ZT16 (gray bars). Values are expression relative to the T0 time points for each gene at ZT4 or ZT16. Mean of three biological replicates ± SE. (a) *PpCBF1*. (b) *PpCBF2*. (c) *PpCBF3*. (d) *PpCBF4*. (e) *PpDREB2C*. (f) *PpDHN1*. A star above the error bars at a particular time point indicates a significant difference between ZT4 and ZT16 at the $P \leq 0.05$ level by Student's *t*-test. Note that scales may differ between panels. Significant differences between ZT4 and ZT16 suggest gating by the circadian rhythm.

the collection of bark tissues required destructive sampling of the entire tree, which meant that it could not be sampled again. However, despite limited comparative time points (T0 and 24 h), it was evident that *PpCBF1* and 4 were more highly expressed in ZT4 than ZT16 bark samples at 24 h. No significant differences ($P \leq 0.05$) were observed in the expression of *PpCBF2* and *3* in ZT4 and ZT16 bark tissue at 24 h. *PpCBF5* was again found to be undetectable in bark tissues.

PpDREB2C was responsive to LT in bark tissues (Figure 3e). *PpDHN1* was responsive to LT in bark tissue, and expression at 24 h was slightly higher in ZT4 than in ZT16 samples (Figure 3e). Since less than twofold increases were observed

for *PpDHN2* and *3*, it was concluded that these genes did not respond to LT in bark (data not shown).

Discussion

Peach CBF gene structure and homology

The five peach *CBF* genes identified in this study are highly homologous to each other with many identical amino acid residues or conserved amino acid substitutions (Figure 1a). The AP2 domain exhibited the greatest degree of identity between the sequences due to its high level of conservation (see also Sakuma et al. 2002). Several CBF-specific domains, as defined by Nakano et al. (2006) and Wisniewski et al. (2013), are also evident. The in silico analysis of the *Prunus* reference genome (http://www.rosaceae.org, 30 July 2013 date last accessed) indicated that *PpCBFs1–5* are located in tandem on LG 5 (Figure 1b). This arrangement is similar to the grouping of *AtCBFs1–3/DREBs1B*, *C*, *A* genes in *Arabidopsis thaliana* [L.] Heynh. (Shinwari et al. 1998). A general level of microsynteny between *P. persica* and *A. thaliana* has been noted before by Georgi et al. (2003) and specifically for dehydrin genes by Wisniewski et al. (2006).

Promoter analyses of *PpCBFs1–5* and *PpDREB2C* revealed the presence of *cis*-elements associated with circadian rhythm (Table 2). There is a marked degree of difference, however, between the number and type of regulatory elements present. *PpCBF2* has numerous perfect and imperfect versions of the ICEr1 and ICEr2 and CM1-7 regulatory motifs reported by Zarka et al. (2003) and Doherty et al. (2009) in *Arabidopsis*. An uncharacterized peach version (ppa005038m) of *ICE1* is present on LG 5 (Wisniewski et al. 2013).

In contrast to *PpCBF2*, the complement of regulatory elements associated with LT induction and circadian rhythm is not as extensive in the other peach *CBF* genes. Several have ICEr2 and CM2 (CAMTA) sites, but only *PpCBF2* harbors an ICEr1-like site. Lack of ICEr1 motifs is not unusual, as *AtCBF1* and *3* do not exhibit one, but are cold-inducible (Doherty et al. 2009), and *AtCBFs1–3* are all subject to regulatory influence by circadian rhythm (Fowler et al. 2005).

The *A. thaliana CBFs1–3* gene family does not contain C-repeat/DRE motifs in their promoters (Gilmour et al. 1998). In contrast, *PpCBFs1–5* do contain C-repeat/DREs, indicating that these peach *CBF* genes may be subject to self- or cross-regulation. Wisniewski et al. (2011) also reported the presence of the core CCGAC portion of the C-repeat in *PpCBF1* and in *MdCBF1* and *MdCBF2* of apple.

Peach CBF gene expression and circadian gating

Our data indicate that *PpCBFs1–4* are LT-inducible and gated by a circadian rhythm, particularly in leaves (Figure 2), and less so in bark (Figure 3). The differences in the timing and pattern of expression between the peach *CBF*s may be a reflection of their underlying regulatory complexity. Barros et al. (2012), in a study of *PdCBF2* (homologue of *PpCBF3*) in almond (*Prunus dulcis* [L.] D.A. Webb) reported a similar circadian response using in vitro shoot cultures although with different expression kinetics. Marked differences in the induction kinetics and tissue specificity of LT-inducible *CBF* genes have been also reported in poplar (*Populus tremula* × *alba*) by Benedict et al. (2006). Some of the *CBF*s were more inducible in leaves compared with stem tissue, while others appeared to be equally inducible in either tissue. Welling and Palva (2008) also reported differences in induction kinetics and relative expression levels of *CBF* genes in leaves of birch (*Betula pendula* Roth). Relevant to the present study, when the same LT treatment occurred under SD photoperiod conditions, three of the four *BpCBF* genes responded strongly while one *BpCBF* gene had no response.

The inability to detect the induction of *PpCBF5* by either LT or dehydration in the present study is problematic since an ICEr2-like element, a C-repeat, three CAMTA elements, numerous motifs for circadian rhythm transcription factors and ABREs are present in its promoter. Therefore, additional research will be needed to clarify this issue.

The LT induction and circadian gating of *PpDREB2C* in peach leaves were unexpected since the *DREB2* family in *A. thaliana* and other dicot species has been shown to be heat, salt and/or dehydration responsive rather than LT-inducible (Mizoi et al. 2012). *DREB2* genes from grass species, however, have been reported to be LT responsive (as reviewed by Mizoi et al. 2012). Regulation of *PpDREB2C* by LT may be due to the presence of a C-repeat element in its promoter, which would allow for transcriptional activation by other peach *CBF* genes.

Bark tissues have frequently been used to examine responses to LT in perennial plants (e.g., Artlip et al., 1997, Bassett et al. 2006, Wisniewski et al. 2006). In the present study, a shift to LT induced the expression of *PpCBFs1–4*, with *PpCBF1* and *4* being more highly expressed at ZT4 than at ZT16 in bark (Figure 3). This implies some level of circadian gating, but it is unknown if photoperception occurred in the bark itself or via a signal transduction pathway from leaves. No comparable differences were observed for *PpCBFs2* and *3* in bark tissues.

Low-temperature-inducible dehydrin gene expression and circadian gating in leaf and bark tissues of peach

Induction of *CBF* genes by LT is accompanied by the up-regulation of downstream targets, and many dehydrin (*DHN*) genes have been reported to be a part of the *CBF*-regulon (Qin et al. 2011, Mizoi et al. 2012). The requisite for *DHN* induction by CBF protein is the presence of a C-repeat/DRE element in the promoter region of the dehydrin gene. *PpDHN1* exhibits seasonal- and LT-induced expression (Artlip et al. 1997) and its promoter contains two C-repeats (Bassett et al. 2006, 2009, Wisniewski et al. 2006). Therefore, expression levels of *PpDHN1* should

correlate with peach *CBF* expression. *PpDHN2* has been shown to be dehydration rather than cold-inducible and has no C-repeat in its promoter (Wisniewski et al. 2006, Bassett et al. 2009). *PpDHN3* is moderately cold-inducible and does contain a C-repeat in its promoter (Bassett et al. 2006, 2009).

The timing and level of the response of *PpDHN1* expression mimicked the circadian response of cold-inducible *PpCBFs1–4* expression. Expression of *PpDHN1* increased after the induction of *PpCBFs1–4* and was much higher at ZT4 than at ZT16. *PpDHN3* was only minimally induced in leaves by LT, but did exhibit a differential response in ZT4 and ZT16 samples starting at 1 h after the temperature shift.

PpDHN2 was not induced by LT in leaf tissues, which is consistent with previous reports (Wisniewski et al. 2006, Bassett et al. 2009) where *PpDHN2* was shown to be dehydration- but not cold-inducible. The data also indicate that the induction of *PpDHN2* is not gated by a circadian rhythm (Figure 2).

The response of *PpDHNs1–3* was also examined in bark tissues. As in earlier studies, *PpDHN1* responded strongly to LT (Artlip and Wisniewski, 1997, Artlip et al. 1997, Wisniewski et al. 2006). Although no significant difference (at $P \leq 0.05$) between ZT4 and ZT16 samples at 24 h were observed, the highest level of expression in ZT4 samples was observed at 48 h, a time period for which data for ZT16 were not available. Similar to earlier reports (Bassett et al. 2006, 2009, Wisniewski et al. 2006), *PpDHN2* and *3* had no or minimal response to LT in bark tissues.

Since dehydrins can also be induced by desiccation, the response of *PpDHNs1–3* to dehydration (Figures S3–S5 available as Supplementary Data at *Tree Physiology* Online) was evaluated and found to be consistent with previous observations (Artlip et al. 1997, Artlip and Wisniewski, 1997, Wisniewski et al. 2006, Bassett et al. 2009).

Conclusions

Low-temperature induction of peach *PpCBFs1–4* genes in leaf and bark tissues is gated by a circadian clock. The promoters of *PpCBFs1–4* contain C-repeat elements indicative of self- or cross-regulation. Such elements have not been reported for *Arabidopsis*, suggesting that while some aspects of *CBF* regulation appear common between the species, there are potential differences as well. A *DREB2* gene family member, *PpDREB2C*, is also cold responsive and gated by a circadian rhythm. This is the first report of this pattern of gene expression in a woody plant (and dicots in general) for this type of transcription factor. A potential downstream target of these transcription factors (*PpDHN1*) increased after the expression maxima of *PpCBFs1–4* and *PpDREB2C*. Owing to a longer time frame for the kinetics of *CBF* and *DHN* gene induction in bark tissues, the bark data are informative but not definitive. Finally, additional studies are needed to compare the overall patterns of *CBF* gene expression in other fruit tree species in order to develop a more complete understanding of the role(s) and evolution of *CBF* genes in LT stress response of woody plants.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Acknowledgments

The authors thank Mr Erik Burchard for his dedication, hard work and technical expertise. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Conflict of interest

None declared.

Literature cited

Anderson CL, Jensen JL, Ømtoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250.

Artlip TS, Wisniewski ME (1997) Tissue-specific expression of a dehydrin gene in one-year-old Rio Oso Gem peach trees. J Am Soc Hortic Sci 122:784–787.

Artlip TS, Callahan AS, Bassett CL, Wisniewski ME (1997) Seasonal expression of a dehydrin gene in sibling deciduous and evergreen genotypes of peach (*Prunus persica* [L.] Batsch). Plant Mol Biol 33:61–70.

Aschoff J (1965) The phase-angle difference in circadian periodicity. In: Aschoff J (eds) Circadian clocks. North Holland Press, Amsterdam, pp 262–278.

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′-region of *Arabidopsis thaliana cor15a* has cis-acting elements that confer cold, drought, and ABA-regulated gene expression. Plant Mol Biol 24:701–713.

Barros PM, Goncalves N, Saibo NJM, Oliveira MM (2012) Functional characterization of two almond C-repeat-binding factors involved in cold response. Tree Physiol 32:1113–1128.

Bassett CL, Wisniewski ME, Artlip TS, Norelli JL, Renaut J, Farrell RE Jr (2006) Global analysis of genes regulated by low temperature and photoperiod in peach bark. J Am Soc Hortic Sci 131:551–563.

Bassett CL, Wisniewski ME, Artlip TS, Richart G, Norelli JL, Farrell RE Jr (2009) Comparative expression profiling and transcript initiation of three peach dehydrin genes. Planta 230:107–118.

Benedict C, Skinner JS, Meng R, Chang Y, Bhalerao R, Huner NPA, Finn CE, Chen THH, Hurry V (2006) The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp. Plant Cell Environ 29:1259–1272.

Chang WC, Lee TY, Huang HD, Pan RL (2008) PlantPAN: plant promoter analysis navigator for identifying combinatorial cis-regulatory elements with distance constrain in plant gene group. BMC Genomics 9:561.

Doherty CJ, Van Buskerk HA, Myers SJ, Thomashow MF (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. Plant Cell 21:972–984.

Dong MA, Farre EM, Thomashow MF (2011) CIRCADIAN CLOCK-ASSOCIATED1 and LATE ELONGATED HYPOCOTYL regulated expression of the C-REPEAT BINDING FACTOR (CBF) pathway in *Arabidopsis*. Proc Natl Acad Sci USA 108:7241–7246.

El Kayal W, Navarro M, Marque G, Keller G, Marque C, Teulières C (2006) Expression profile of *CBF*-like transcriptional factor genes from *Eucalyptus* in response to cold. J Exp Bot 57:2455–2469.

Fowler SG, Cook D, Thomashow MF (2005) Low temperature induction of *Arabidopsis* CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol 137:961–968.

Georgi LL, Wang Y, Reighard GL, Mao L, Wing RA, Abbott AG (2003) Comparison of peach and *Arabidopsis* genomic sequences: fragmentary conservation of gene neighborhoods. Genome 46:268–276.

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J 14:433–442.

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290:2110–2113.

Heide OM, Prestrud AK (2005) Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. Tree Physiol 25:109–114.

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27:297–300.

Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327.

Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF transcription factors in plant abiotic stress responses. Biochim Biophys Acta 1819:86–96.

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. Plant Physiol 140:411–432.

Navarro M, Marque G, Ayax C, Keller G, Borges JP, Marque C, Teulières C (2009) Complementary regulation of four *Eucalyptus CBF* genes under various cold conditions. J Exp Bot 60:2713–2724.

Nicot N, Hausman J-F, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907–2914.

Novillo F, Alonso JM, Ecker JR, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis*. Proc Natl Acad Sci USA 101:3985–3990.

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. Proc Natl Acad Sci USA 104:21002–21007.

Oakley RV, Want Y-S, Ramakrishna W, Harding SA, Tsai C-J (2007) Differential expansion and expression of α- and β-tubulin gene familes in *Populus*. Plant Physiol 145:961–973.

Qin F, Shinozaki K, Yamaguchi-Shinozaki K (2011) Achievements and challenges in understanding plant abiotic stress responses. Plant Cell Physiol 52:1569–1582.

Sakuma Y, Liu Q, Dobouzer JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Commun 290:998–1009.

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun 250:161–170.

Thomashow MF, Gilmour SJ, Stockinger EJ, Jaglo-Ottosen K, Zarka DG (2001) Role of the *Arabidopsis* CBF transcriptional activators in cold acclimation. Physiol Plant 112:171–175.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Tong Z, Gao Z, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:71 doi:10.1186/1471-2199-10-71.

Welling A, Palva ET (2008) Involvement of CBF transcription factors in winter hardiness in birch. Plant Physiol 147:1199–1211.

Wisniewski ME, Bassett CL, Renaut J, Farrell R Jr, Tworkoski T, Artlip TS (2006) Effect of photoperiod and temperature on expression of two dehydrin genes from peach (*Prunus persica* [L.] Batsch). Tree Physiol 26:575–584.

Wisniewski M, Norelli J, Bassett C, Artlip T, Macarisin D (2011) Ectopic expression of a novel peach (*Prunus persica*) *CBF* transcription factor in apple (*Malus* × *domestica*) results in short-day induced dormancy and increased cold hardiness. Planta 233:971–983.

Wisniewski M, Nassuth A, Teulieres C, Marque C, Rowland J, Cao P-B, Brown A (2013) Genomics of cold hardiness in woody plants. Crit Rev Plant Sci. In press.

Xiao H, Siddiqua M, Braybrook S, Nassuth A (2006) Three grape CBF/DREB1 genes respond to low temperature, drought, and abscisic acid. Plant Cell Environ 29:1410–1421.

Xiao H, Tattersal E, Siddiqua M, Cramer G, Nassuth A (2008) CBF4 is a unique member of the CBF transcription factor family of *Vitis vinifera* and *Vitis riparia*. Plant Cell Environ 31:1–10.

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251–264.

Zarka DG, Vogel JT, Cook D, Thomashow MF (2003) Cold induction of *Arabidopsis* CBF genes involved multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. Plant Physiol 133:910–918.