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The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus* spp.

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ABSTRACT

The meristematic tissues of temperate woody perennials must acclimate to freezing temperatures to survive the winter and resume growth the following year. To determine whether the C-repeat binding factor (CBF) family of transcription factors contributing to this process in annual herbaceous species also functions in woody perennials, we investigated the changes in phenotype and transcript profile of transgenic *Populus* constitutively expressing *CBF1* from *Arabidopsis* (*AtCBF1*). Ectopic expression of *AtCBF1* was sufficient to significantly increase the freezing tolerance of non-acclimated leaves and stems relative to wild-type plants. cDNA microarray experiments identified genes up-regulated by ectopic *AtCBF1* expression in *Populus*, demonstrated a strong conservation of the CBF regulon between *Populus* and *Arabidopsis* and identified differences between leaf and stem regulons. We studied the induction kinetics and tissue specificity of four *CBF* paralogues identified from the *Populus balsamifera* subsp. *trichocarpa* genome sequence (*PtCBF*s). All four *PtCBF*s are cold-inducible in leaves, but only *PtCBF1* and *PtCBF3* show significant induction in stems. Our results suggest that the central role played by the CBF family of transcriptional activators in cold acclimation of *Arabidopsis* has been maintained in *Populus*. However, the differential expression of the *PtCBF*s and differing clusters of CBF-responsive genes in annual (leaf) and perennial (stem) tissues suggest that the perennial-driven evolution of winter dormancy may have given rise to specific roles for these 'master-switches' in the different annual and perennial tissues of woody species.

Key-words: cold tolerance; microarray.

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INTRODUCTION

Adaptation to low temperatures is one of the most important components in the evolutionary process in temperate and boreal tree species (Saxe *et al*. 2001). Even when non-lethal, the accumulated lifetime costs of recurring freeze damage to tree fitness are significant; spring frosts damage flushing buds and flowers, decreasing tree growth and seed production (Selas *et al*. 2002), and leaves prematurely lost to autumn frosts reduce the ability of the tree to cold harden (Howell & Stockhause 1973). Under field conditions, woody perennials develop deep winter hardiness in response to two environmental cues (Weiser 1970). Shortening day length first initiates the transition from active growth to winter dormancy, which may take many weeks to complete (Weiser 1970; Fuchigami, Weiser & Evert 1971; Junttila 1976). The onset of winter dormancy results in a moderate increase in freezing tolerance (FT), but subsequent exposure to low temperatures is required to promote the deep winter hardiness that is unique to woody perennials (Weiser 1970; Fuchigami *et al*. 1971). Woody perennials, like herbaceous species, can also acquire cold tolerance when exposed to low temperatures during long days, but full winter hardiness only develops under the combined stimuli of short photoperiods and low temperatures (Christersson 1978; Li *et al*. 2002, 2003a; Puhakainen *et al*. 2004).

The molecular mechanisms governing the acquisition of FT are largely unknown in woody plants. The accumulation of dehydrins has been associated with the development of FT in a number of woody species (Arora & Wisniewski 1994; Arora, Wisniewski & Rowland 1996; Campalans, Pages & Messeguer 2000; Richard *et al*. 2000) and have been shown to correlate with seasonal variations in FT (Arora *et al*. 1996; Wisniewski *et al*. 1996; Welling, Kaikuranta & Rinne 1997; Rinne, Welling & Kaikuranta 1998; Lim, Krebs & Arora 1999). However, although significant efforts have been made to assign roles to different dehydrins (Wisniewski *et al*. 1999; Bravo *et al*. 2003), their biochemical

functions remain largely unknown (Svensson *et al*. 2002). Cold acclimation is better understood in herbaceous annuals such as *Arabidopsis thaliana*, where members of the C-repeat binding factor (CBF or DREB1) family of transcriptional activators, which bind the *cis*-element known as the C-repeat (CRT)/dehydration-responsive element (DRE) (Stockinger, Gilmour & Thomashow 1997), have been shown to control the transcription of a suite of genes that play important roles in cold acclimation and the development of FT. The *Arabidopsis* CBF/DREB1 family consists of six paralogues, but only three [*CBF1* (*DREB1b*), *CBF2* (*DREB1c*) and *CBF3* (*DREB1a*)] are cold-inducible (Sakuma *et al*. 2002). These three low-temperature-responsive *CBF*s colocalize to an 8.7 kb region of chromosome 4 and share a conserved AP2 DNA binding region flanked by the characteristic amino acid motifs PKKPAGRxKFxETRHP and DSAWR (Jaglo *et al*. 2001). Based on a number of experiments using transgenic plants, at least 12% of the cold-induced transcriptional changes observed in *Arabidopsis* are accounted for by the members of the *CBF* transcription factor family (*CBF1-3*) (Fowler & Thomashow 2002; Vogel *et al*. 2005; van Buskirk & Thomashow 2006), and ectopic expression of *Arabidopsis* CBF1-4 has been shown to improve FT of non-acclimated plants (Jaglo-Ottosen *et al*. 1998; Gilmour *et al*. 2000, Haake *et al*. 2002; Gilmour, Fowler & Thomashow 2004).

The accumulation of CBF transcripts and the activity of the CRT regulatory motif in *Arabidopsis* is also modulated by the presence and quality of light during cold stress (Kim *et al*. 2002; Fowler, Cook & Thomashow 2005). A short period of exposure to red light has been shown to be sufficient to induce CRT-driven GUS reporter expression in the cold, and this induction is photo-reversible with far-red light. GUS reporter expression is also altered in phytochrome mutant backgrounds, suggesting that day length mediates expression of the CBF regulon through phytochrome B in *Arabidopsis* (Kim *et al*. 2002). Furthermore, the kinetics of endogenous CBF transcript accumulation is reduced in complete darkness (Kim *et al*. 2002), suggesting that light regulation occurs at a point upstream of CBF expression. These data raise the question of whether CBF or related transcriptional regulators may be involved in the day length-mediated growth cessation in woody species. It has been suggested that growth cessation in woody perennials, mediated by short days and phytochrome, operates independently of the low-temperature-driven acquisition of deep FT (Welling *et al*. 2002). However, the observation that pre-treatment with short days enhances the expression of low temperature response genes (Puhakainen *et al*. 2004) and the finding that a CBF-like transcription factor is induced by short days in the cambial meristem of poplar (Schrader *et al*. 2004) indicates that there may be cross-talk between these two acclimation mechanisms.

Following their original discovery in *Arabidopsis*, the list of plants with cold-inducible *CBF* orthologues has expanded to include cold-sensitive and cold-tolerant annual species (Jaglo *et al*. 2001) and woody perennials (Kitashiba *et al*. 2002; Owens *et al*. 2002). Puhakainen *et al*. (2004) have also demonstrated that AtCBF3 can activate gene transcription via the birch *Bplti36* promoter sequence in transgenic *Arabidopsis*, indicating that the structure and function of the active domain in the promoters of CBF-mediated cold-responsive genes have been conserved in herbaceous and woody perennials. However, detailed studies of the role of CBF-related proteins in cold acclimation in tree species have been lacking. Based on the demonstrated effects of both low temperature and light on CBF transcript accumulation in *Arabidopsis*, we have examined how CBF expression affected the FT of non-dormant poplar leaf and meristematic stem tissue under long-day growth conditions. This experimental design enabled us to focus on the role the CBF-mediated signalling pathway plays in the temperature response of different tissues in a woody perennial, without complicating interactions with day length and dormancy. Our data demonstrate: (1) that CBFs are involved in the FT mechanism of temperate trees species such as poplar; (2) that distinct regulons control stem (meristematic) versus leaf (non-meristematic) FT; and (3) that the *Populus* genome contains multiple CBFs that are likely to be differentially employed in perennial (stem) and annual (leaf) tissues.

MATERIALS AND METHODS

Construction of transformation vector

A CaMV $35S_{Promoter}$::*Arabidopsis* CBF1 cDNA::$35S_{Terminator}$ cassette (Jaglo-Ottosen *et al*. 1998) was ligated as a *Hind*III fragment into the binary vector pGAH (Onouchi *et al*. 1991) that had been linearised with *Hind*III.

Poplar transformation

Plants of poplar clone 717-1B4 (*Populus tremula* × *alba*) were grown *in vitro* in a culture room at 24 °C under cool-white fluorescent lights (40–60 μmol m^{-2} s^{-1}, 16 h photoperiod). Stem internodes (7–10 mm in length) and leaf discs (5 mm in diameter) from fully expanded young leaves were used as the explants for the transformation (DeBlock 1990).

Agrobacterium tumefaciens strain EHA105 carrying the plasmid pGAH35SAtCBF1 was grown overnight at 28 °C in liquid YEP medium supplemented with 50 mg L^{-1} hygromycin and 50 mg L^{-1} kanamycin. The cells were collected by centrifugation at 1500 g for 20 min and resuspended to an OD_{600} = 0.4–0.6 in Murashige and Skoog (MS) medium with 20 μg L^{-1} acetosyringone (AS). Explants (stem internodes or leaf discs) were soaked for 10–20 min in the bacterial suspension, incubated on a shaker (300 g) for 1 h at room temperature, followed by cultivation on callus induction medium (CIM, pH 5.2) supplemented with 20 μg L^{-1} AS at 25 °C in the dark for 2–3 d. After 2–3 d of cocultivation, explants were washed four times with double-distilled water and once with wash solution (WS, pH 5.8) supplemented with 500 mg L^{-1} cefotaxime. The washed explants were blotted dry with paper towels, allowed to stand in a

laminar flow hood for drying for about 30 s and then transferred to CIM supplemented with 250 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin for 2 weeks at 25 °C in darkness. Explants were transferred to shoot induction medium (SIM, pH 5.8) supplemented with 50 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime to induce shoot formation for 2–3 weeks. Explants were then transferred to shoot development and elongation medium (SEM, pH 5.8) supplemented with 50 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime until shoots were well developed (> 1 cm in length). During this period, the explants were transferred to fresh SEM supplemented with 50 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime every two weeks. Regenerated shoots (> 1.0 cm) were excised from the explants and further screened for kanamycin resistance on rooting medium (RM, pH 5.8) supplemented with 50 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime. Leaves from the rooted plants were collected for DNA extraction for PCR to verify the presence of CaMV 35S promoter::AtCBF1 transgene in the poplar genome, and for RNA extraction for Northern blot analysis to verify lines expressing detectable levels of the transgene.

Leaf and stem FT assays

Plants of wild-type (WT) and transgenic lines 1 and 2 were propagated by cuttings *in vitro*, and rooted plants were transplanted into 1 gallon pots containing Sunshine SB40 Professional Growing Mix (Sun Gro Horticulture Inc., Bellevue, WA) supplemented with 7% pumice (weight/volume) and 0.14% perlite, and fertilized with 0.04% osmocote (The Scotts Company, Marysville, OH). Plants were grown in a greenhouse maintained at a 16 h day (25 ± 3 °C) and 8 h night (20 ± 3 °C) with supplemental lighting (mercury halide lamps providing 400–500 μmol/m^2 s^{-1}). After 6 weeks of growth, plants of both transgenic lines and WT were divided into two groups. One group of plants was transferred to a cold room (2 °C, 16 h photoperiod) for 1 week, while the other group was maintained in the greenhouse under the greenhouse conditions mentioned earlier. For freezing tests tissue samples, 1-cm-diameter leaf discs were excised from each side of the two to three uppermost fully expanded leaves, and 2 cm stem segments were harvested. Three leaf discs or stem segments were used per assay, and FT was determined by ion leakage (Sukumaran & Weiser 1972).

RNA extraction for microarray analysis

Leaf and stem samples were collected from three WT plants and three individuals from each transgenic line (1 and 2) grown under either the warm greenhouse conditions discussed earlier or after 1 week of cold acclimation (2 °C with 16 h photoperiod). Total RNA was extracted from frozen stem and leaf samples (Hughes & Galau 1988), the RNA samples were reconstituted in TE buffer and the total RNA was further purified using QIAGEN RNeasy columns (Qiagen, Valencia, CA, USA). Aliquots of these total RNA preparations from each clonally propagated genetic background were then pooled before being used for fluorescent probe synthesis. This experimental protocol was repeated on clonally propagated material grown at a later date, resulting in two independent biologically replicated experiments.

Preparation of fluorescent targets

Targets were labelled indirectly by incorporating aminoallyl-dUTPs (Sigma, St Louis, MO, USA; Pharmacia, Fairfield, CT, USA) and subsequently coupling with either Cy3 or Cy5 dye. The reverse transcription reaction mixture (30 μL) contained 20 μg total RNA with 5 μg of oligo(dT) 20 mer, 10 mM DTT, 500 μM each of dATP, dCTP, and dGTP, 300 μM dTTP, 200 μM aa-dUTP, 30–40 units RNase inhibitor, and 200 units of SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY) in 1X Superscript first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 20 mM DTT) (Life Technologies). After incubation at 42 °C for 2 h, the reaction products were treated with 10 μL 1 M NaOH, 10 μL 0.5 M EDTA, incubated at 65 °C for 15 min, and neutralized with 50 μL 1 M HEPES (pH 7.0). Samples were purified on QIAGEN MinElute Clean-up columns (Qiagen) as directed, except that the final elution was performed with 15 μL of $NaHCO_3$, pH 9.0 into an amber eppendorf containing the appropriate vacuum-dried Cy dye pellet [1/7 of the commercial aliquot (Amersham Pharmacia Biotech; 1 mg protein-labelling aliquots)]. The eppendorf contents were mixed until the Cy dye pellet was fully resuspended, spun down and incubated in darkness at room temperature for 2 h. At the end of the incubation period, the labelling mixture was diluted to 100 μL using sterile water and QIAGEN PCR purification columns (Qiagen) were used as directed, except that the final elution was performed using 30 μL sterile water. Respective Cy3 and Cy5 samples for comparison were combined and reduced under vacuum to a volume of 13 μL at 40 °C. This probe mixture was combined with 0.5 μL of 25 μg μL^{-1} yeast tRNA, 2.0 μL of 10 μg μL^{-1} oligo(dA), 6.0 μL of 20X SSC, 7.5 μL of deonized formamide and 1.0 μL 10% sodium dodecyl sulphate (SDS) and denatured at 95 °C for 1 min. After 2 min cooling on ice, the prepared probe mixture was used for hybridization.

Microarray hybridization and scanning

Populus 13K duplicate array slides (Andersson *et al*. 2004) were prehybridized and hybridized using the solutions and protocols reported previously (Hertzberg *et al*. 2001). The slides were scanned with a ScanArray Lite scanner (Perkin Elmer, Wellesley, MA, USA) and ScanArray express software. Separate images were acquired for each fluor at a resolution of 10 μm per pixel. Scanning photomultiplier and laser power settings were optimized by the software to minimize saturated spots and maximize the signal-to-noise ratio. Microarray spots were flagged and quantified using Genepix 5.0 (Axon Instruments, Foster City, CA, USA)

software. The resulting mean signal and median background data for each channel and spot were imported and normalized using the Bioconductor package (www.bioconductor.org), applying Edwards background correction and loess normalizations within individual arrays and Aquartile between-array normalization before import into Genespring 6.0 (Silicon Genetics, Redwood City, CA, USA) for visualization. Gene lists for each comparison (Supplemental Tables) were constructed from the data collected from four hybridizations of each line (including two independent biological replicates and dye-swapping). Duplicate printing of probes on the hybridized UPSC–KTH 13K *Populus* slides also increased the total replication twofold for each probe. Therefore, the final gene lists (incorporating expression data from both transgenic lines) represent the mean expression change and statistical data from 16 replicates. We set statistical significance at $P < 0.05$ plus an arbitrary fold-change (FC) cut-off of FC > 1.75X to construct the gene lists. The raw and normalized microarray data are publicly available at http://www.upscbase.db.umu.se/.

Multiplex and real-time RT-PCR transcript quantification

Total RNA was isolated from 50 to 100 mg of powder from leaf or stem tissue from 6-week-old hybrid aspen (*Populus tremula* × *tremuloides* clone T89) trees using Trizol reagent (Gibco BRL, Paisley, UK) according to the manufacturer's instructions except for an extra 10 min centrifugation step at 4 °C after the addition of Trizol, the use of 0.5 volumes of bromochloropropane in place of 1 volume of chloroform during phase separation, and use of 1:1 isopropanol:high salt solution (0.8 M sodium citrate, 1.2 M sodium chloride) in place of isopropanol during RNA precipitation. QIAGEN RNeasy columns (Qiagen) were used as directed to purify samples. Three micrograms total RNA was used in reverse transcription with the First Strand cDNA kit (Amersham) according to the manufacturers instructions. Semi-quantitative multiplex PCR was used to determine relative amounts of AtCBF1 transcript in the transgenic *Populus* lines using the Cbf-f (ACGAATCCCGGAGT CAACATGC) and Cbf-r (ccttcgctctgttccggtgtataaat) gene-specific primers and Universal 18S (Quantum RNA 18S standards, Ambion, Austin, TX, USA) primers with competimer (2:8 ratio) as the endogenous standard. Real-time PCR quantification was performed using iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA) as instructed with gene-specific primers [PtCBF1F, ACACAGGATGCCT TGTTTCC; PtCBF1R, GGAGTTCAACCAGGTG CAAT; PtCBF2F, GGGAGGTGAGTTGATGAGGA; PtCBF2R, TATTAGCCAACAACCCTGGC; PtCBF3F, TTTCAAATGAGGCCAAGGAC; PtCBF3R, CCTC CCTCCTGAAATCTTCC; PtCBF4F, GGCAGCAAAT GAGGCAGCAG; PtCBF4R, CTTGAGCAATCCTCTA GCACTGCAT; Universal 18S rRNA primers (Ambion), and ~2 ng cDNA template. Relative *PtCBF* abundance was quantified and then normalized using the $\Delta\Delta$ct method of reference gene: 18S; calibrator sample: 2 ng, 3 h cold-treated leaf cDNA].

RESULTS

Ectopic expression of *AtCBF1* in *Populus*

Arabidopsis CBF paralogues have been used to manipulate FT in a number of herbaceous plant species (Jaglo *et al*. 2001; Hsieh *et al*. 2002; Lee *et al*. 2003, 2004). To test whether a CBF regulon exists and plays a role in the FT of overwintering woody perennials, we generated transgenic *Populus tremula* × *alba* (clone 717-1B4) ectopically expressing the well-characterized *AtCBF1* gene from *Arabidopsis* (Fig. 1a). We generated 19 independent transgenic lines expressing *AtCBF1* and characterized two of these lines expressing *AtCBF1* at rates equivalent to endogenous *AtCBF1* transcript levels in *Arabidopsis* after 3 h cold treatment, as assessed by RT-PCR (Fig. 1b & c). The selected lines, with high *AtCBF1* transcript abundance (AtCBF1-Poplar), had fewer roots and slower growth rates when cultivated *in vitro*(Fig. 2a), but fully recovered after 3–6 weeks growth in soil (Fig. 2b–d).

AtCBF1-Poplar lines have improved leaf and stem FT

The effect of ectopic expression of *AtCBF1* in poplar on FT of leaves and stems was tested using an electrolyte leakage test. This assay provides an estimate of cell damage (Dexter, Tottingham & Garber 1932; Sukumaran & Weiser 1972) and is quantified by determining the temperature leading to leakage of 50% of cellular electrolytes (T_{EL50}). Non-acclimated leaves from both AtCBF1-Poplar lines showed a significant ($P < 0.001$) gain in FT, from –3.9 °C for non-acclimated WT to an average of –6.9 °C for AtCBF1-Poplar (Table 1). Ectopic expression of *AtCBF1* also significantly improved stem FT in non-acclimated plants (T_{EL50} –5.4 °C for the AtCBF1 lines versus –4.1 °C for WT, $P < 0.001$) (Table 1). These FT data demonstrate

Table 1. T_{EL50} measurements for leaf and stem tissues of non-acclimated (NA) and 1 week cold-acclimated (CA) hybrid aspen trees. Values represent the mean of six independent experiments ± standard error. Statistical significance was tested first by one-way ANOVA with Dunnett's post-test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA)

	Leaves		Stems	
	NA	CA	NA	CA
T_{EL50} (°C)				
WT	–3.9 ± 0.2	–17.3 ± 1.2	–4.1 ± 0.3	–9.9 ± 0.2
Line 1	–6.7 ± 1.0***	–18.3 ± 1.1	–5.2 ± 0.4***	–10.7 ± 1.0
Line 2	–7.1 ± 0.7***	–18.7 ± 2.5	–5.7 ± 0.6***	–11.3 ± 1.7

WT, wild type.
***$P < 0.001$

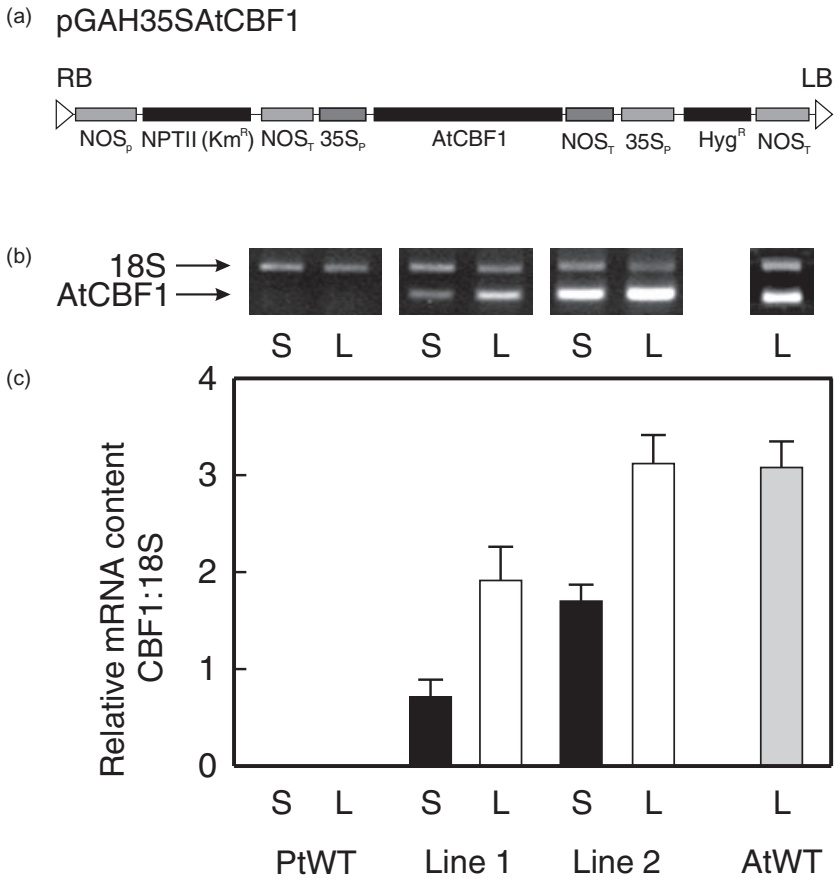

Figure 1. Expression of the *AtCBF1* transgene. (a) pGAH35SAtCBF1 expression construct. (b) Relative expression of the *AtCBF1* gene in the stem (S) and leaf (L) tissue of wild-type *Populus* (PtWT), AtCBF1-Poplar line 1, AtCBF1-Poplar line 2 grown for 6 weeks in the greenhouse at 23 °C, and in warm (23 °C) grown wild-type *Arabidopsis*, Col 0 (AtWT) after 3 h exposure to 5 °C, respectively. (c) Semi-quantitative multiplex RT-PCR analysis was performed on first-strand cDNA generated from total RNA extracted from wild-type and transgenic plants 6 weeks after transfer to soil in the greenhouse. Differences in template starting quantity in the wild-type and transgenic lines were corrected by normalizing to the expression of the 18S rRNA (18S) in the respective samples. Error bars indicate standard errors of six independent experiments.

that the cryoprotective benefit of ectopic *AtCBF1* expression shown in *Arabidopsis* leaves (Jaglo-Ottosen *et al.* 1998; Gilmour *et al.* 2000) extends to leaves and stems of poplar, and suggests that *Populus* spp. utilize a CBF-mediated signalling pathway to control at least part of the cold acclimation response.

The *AtCBF1* regulon(s) in *Populus*

To determine whether *AtCBF1*-driven transcriptional changes mirrored the endogenous transcriptional response to low temperature, the transcriptomes of WT warm-grown leaf and stem tissue were compared with the transcriptomes of leaf and stem tissues of warm-grown poplar ectopically expressing *AtCBF1*, and with the transcriptomes of leaf and stem tissues from WT trees after 7 d of cold acclimation at 2 °C. The cDNA microarray experiments utilized mRNA from WT plants, and the two independent AtCBF1-Poplar lines and gene lists were constructed using the average result of two independent growth experiments. These experiments enabled us to determine: (1) which genes were susceptible to AtCBF1 regulation; (2) whether the genes that respond to AtCBF1 control showed differential expression in the meristematic stem tissues versus leaf tissue; and (3) how much overlap there was between the CBF regulon and the endogenous (untransformed) response of poplar leaves and stems to cold.

Using our cut-offs (FC >1.8, $P < 0.05$), we identified 63 genes up-regulated in leaves from warm (23 °C) grown AtCBF1-Poplar relative to leaves from warm-grown WT plants (Table S1a). Almost half of the total list (29/63) of AtCBF1-Poplar leaf regulon members was composed of novel 'expressed proteins' and other proteins with no known function. Of the 34 AtCBF1-Poplar leaf regulon genes annotated to have a known function, more than one-third (35%) encoded metabolic enzymes. Together, the classes 'Cell rescue' (15%) and 'Transcription' (21%) made up another third, with the remaining classes represented being: 'Development' (9%), 'Cellular communication/signal transduction' (6%), 'Storage' (6%), 'Cellular biogenesis' (6%) and 'Protein fate' (3%) (Fig. 3). This functional distribution of genes in the AtCBF1-Poplar leaf regulon mirrored that of the genes endogenously up-regulated in WT leaves by 7 d at low temperature (Fig. 3, Table S2). In contrast, the functional class 'Transcription' made up the dominant fraction (41%) of the AtCBF1-Poplar regulon in warm-grown perennial stem tissue, followed by 'Cellular communication/signal transduction' (18%), 'Cell rescue' (12%) and 'Metabolism' (12%) (Fig. 3).

The functional analysis of the genes induced by 7 d of cold (2 °C) treatment in WT *Populus* leaves and stems showed that these tissue-specific regulons were not as innately different as the AtCBF1-Poplar leaf and stem reg-

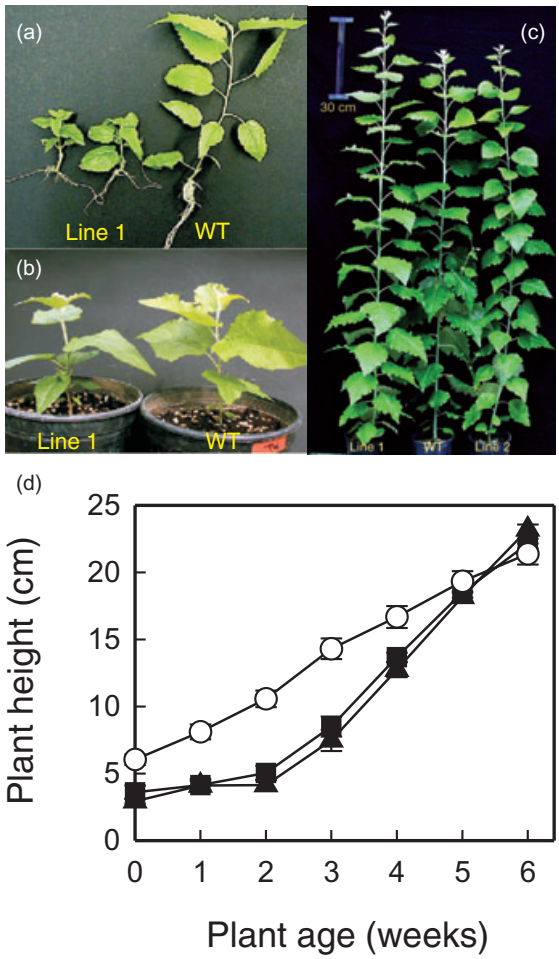

Figure 2. Morphology of the AtCBF1-Poplar trees. (a) AtCBF1-Poplar were propagated on agar under long-day conditions. (b) AtCBF1-Poplar 3 weeks after transfer from propagation on agar to soil. (c) AtCBF1-Poplar 10 weeks after transfer from propagation on agar to soil. (d) Average plant height during 6 weeks growth on soil in wild-type and AtCBF1-Poplar lines. Error bars represent standard error in three independent experiments. Open circles, wild type; closed squares, AtCBF1-Poplar line 1; closed triangles, AtCBF1-Poplar line 2.

ulon data (Fig. 3). This comparison held true even at the level of individual genes: more than half (57%) of the up-regulated genes with known function from WT cold-treated stem tissue were also significantly up-regulated in WT cold-treated leaf tissue. In fact, in terms of functional composition, the AtCBF1-Poplar leaf, WT cold leaf and WT cold stem regulons showed high similarity, while the AtCBF1-Poplar stem regulon differed largely on the basis of a substantial depletion of metabolic genes (12% versus 35% in AtCBF1-Poplar leaf tissue and 38% in WT cold stem) and an enrichment of genes regulating transcription (41% versus 21% in AtCBF1-Poplar leaf tissue and 22% for WT cold stem) (Fig. 3).

As indicated by the functional analysis, the normalized expression data for all genes significantly up-regulated by *AtCBF1* in at least one tissue showed that the leaf (Table S1a) and stem (Table S1b) AtCBF1-Poplar regulons contained clusters specific for that tissue (Fig. 4 gene subsets B and C, respectively), suggesting different functional roles for the AtCBF1-Poplar regulons in the annual (leaf) and perennial (stem) tissues. However, the leaf and stem AtCBF1-Poplar regulons also shared clusters of up-regulated genes with each other and with the WT leaf and stem cold regulons (Fig. 4 gene subset A and Table 2). Twenty-two of the 63 AtCBF1-Poplar leaf regulon genes identified were also up-regulated in the WT 7 d cold-acclimated leaves (Table 2). Five of the 26 AtCBF1-Poplar stem regulon genes were also up-regulated in WT stems by 7 d cold treatment (Table 2). Although the total AtCBF1-Poplar gene number in stems was lower than that in leaves, approximately the same fraction of the total 7 d cold induced list could be linked to *AtCBF1* expression in the stem (5/152 or 3%, see Table 2 and Table S3) as in the leaf (22/462 or 5%, Table 2 and Table S2). Twenty-nine genes were also found to be down-regulated by *AtCBF1* expression in leaves, and 24 genes were down-regulated in stems (Table S1c).

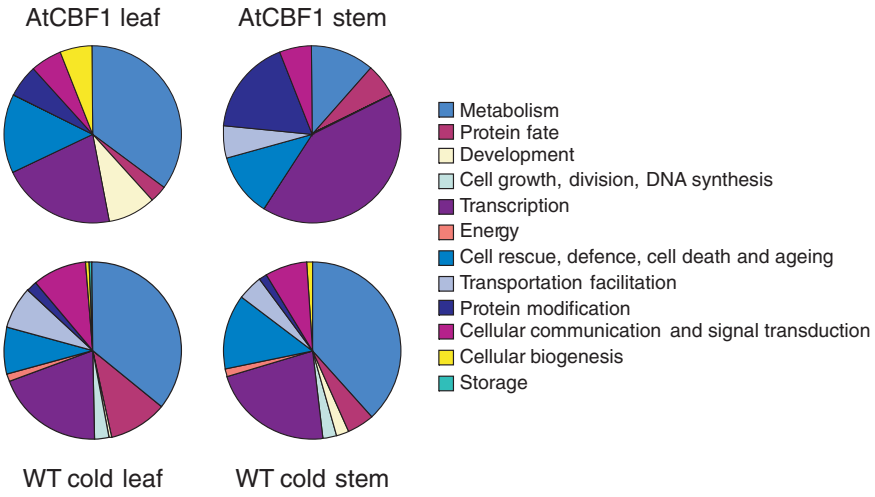

Figure 3. Functional distribution of the genes annotated with known functions significantly up-regulated in warm-grown AtCBF1-Poplar (51 genes in total) and 7 d cold-treated wild-type *Populus* (387 genes in total) relative to warm-grown wild-type *Populus*.

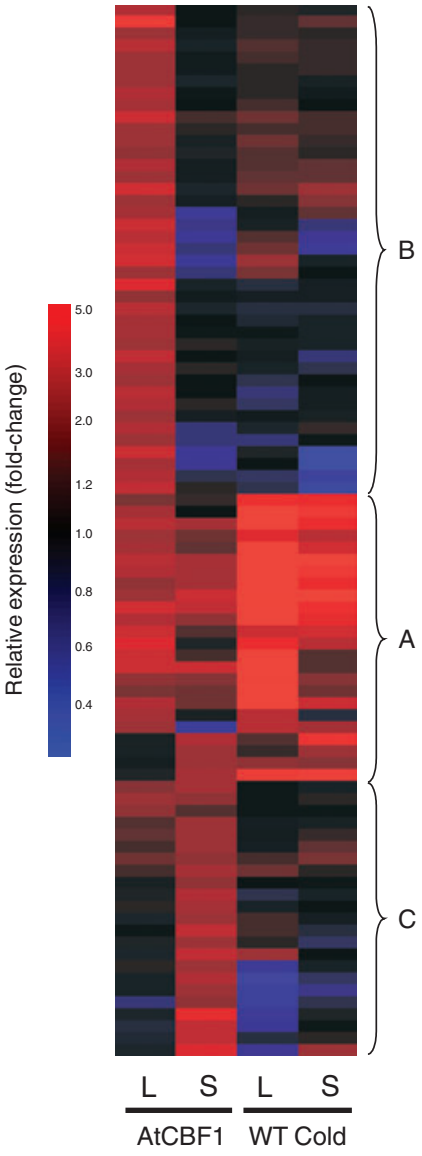

Figure 4. Hierarchical tree of genes belonging to the CBF regulon based on similarities in expression profiles of the leaf and stem tissues of AtCBF1-Poplar and 7 d cold-treated wild-type *Populus*. Gene subsets up-regulated in the leaf and stem tissue of AtCBF1-Poplar are indicated to the right with brackets, where (A) indicates the gene subset similarly up-regulated in both AtCBF1-Poplar and wild-type cold-treated *Populus* tissues, (B) indicates the AtCBF1-Poplar leaf-specific regulon, and (C) indicates the AtCBF1-Poplar stem-specific regulon.

Comparison of *Populus* and *Arabidopsis* CBF regulons

Comprehensive transcriptome changes due to ectopic *AtCBF1* expression have previously only been published for *Arabidopsis* as a combined AtCBF1/CBF2/CBF3 regulon (Fowler & Thomashow 2002). However, paralogue-specific studies have identified gene members of the AtCBF2 (Vogel *et al*. 2005) and AtCBF3 regulons in *Arabidopsis* (Maruyama *et al*. 2004). We next examined the similarity between our identified annual (leaf) and perennial (stem) tissue AtCBF1 regulons in *Populus* and the reported *Arabidopsis* CBF regulons. We surveyed our *Populus* cDNA microarray DB (Sterky *et al*. 2004) to find all the closest protein homologues to previously reported CBF-responsive genes from the combined AtCBF1-3 regulon and the AtCBF2- and AtCBF3-paralogue regulons. The *Arabidopsis* CBF3 regulon shared the most similarity with the CBF regulon in *Populus*. Twelve of the 38 reported AtCBF3-up-regulated genes that had representative orthologues on our microarray, and a gene comparison showed that more than half (7/12 having FC > 1.3) of the AtCBF3-up-regulated gene orthologues present on the POP1 13K cDNA array were similarly up-regulated in one or both AtCBF1-Poplar tissues and possessed DREs in their 1500 bp promoters (Table 3). Examination of the induction of AtCBF2- and AtCBF1/2/3 regulon orthologues in *Populus* (Table S4) showed greater disagreement. Whether this reduced response of the CBF2 and CBF1/2/3 regulon orthologues in *Populus* to ectopic *AtCBF1* expression was indicative of differences in species and/or paralogue-specific regulon composition remains a question that will require additional experiments to answer.

AtCBF1 regulon promoter analysis in *Populus* reveals conserved linkage of the DRE and abscisic acid-responsive element (ABRE)

Given the broad similarity between the *Populus* and *Arabidopsis* CBF regulons, we determined whether there was also a conserved enrichment of the DRE in the promoters of the AtCBF1-Poplar regulon genes. Sixteen of the 63 (25%) AtCBF1-up-regulated leaf regulon gene promoters (defined as the 1500 bp of genomic sequence found upstream of the ATG site) contained the basic DRE nucleic acid sequence 'RCCGAC'. Eight of 26 (31%) AtCBF1-up-regulated stem regulon promoters contained the DRE. Positional analysis of the RCCGAC revealed no significant enrichment along the 1500 bp promoter. However, it is worth noting that 7 of 33 DREs appeared between 200 and 400 bp upstream of ATG (Table S5), leaving the possibility that a larger sample set, using currently unavailable transcriptional start sites instead of the ATG, would verify the –450/–51 positional enrichment of the DRE reported for *Arabidopsis* (Maruyama *et al*. 2004) in *Populus*. Recent studies have also noted an enrichment of the ABRE (Marcotte, Russell & Quatrano 1989) 'ACGTGTC' in the CBF3 regulon of *Arabidopsis* Maruyama *et al*. 2004). In *Populus*, an ABRE was found in 12 of 87 AtCBF1 regulon 1500 bp promoters, representing a significant enrichment (χ^2, $P < 0.05$). There was also a significant overlap between ABRE- and DRE-containing gene promoters in *Populus* (4/12 ABRE-containing gene promoters also contained the RCCGAC consensus: χ^2, $P < 0.001$) and positional enrichment of the ABRE at positions 100–199 bp and 300–399 bp upstream of the start codon (χ^2, $P < 0.001$).

Table 2. A selection of genes up-regulated in AtCBF1-Poplar (*Poplar tremula* × *alba*) and wild-type (WT) *Populus tremula* × *alba* cold acclimated for 1 week at 2 °C (versus WT *Populus tremula* × *alba* grown at 23 °C). Normalized mean fold-change (FC) values are shown (bold face indicates $P < 0.05$). The poplar gene probe (PU) number is matched to its closest *Arabidopsis* gene (AGI) based on homology to the *Arabidopsis* genome sequence. This table corresponds to gene subset 'A' in Fig. 4.

CBF1 Leaf FC	CBF1 Stem FC	WT Leaf FC	WT Stem FC	Clone ID	AGI	Description	Category
2.1	1.1	**3.7**	**2.8**	PU03647	At1g54410	SRC2	Cell rescue
2.0	1.0	**5.0**	**4.3**	PU03776	At5g66780	Expressed protein	Unknown role
2.2	2.0	**3.5**	3.4	PU04037	At2g21120	P0491F11.21 protein. *Oryza sativa*	Metabolism
1.8	1.5	**3.1**	**2.2**	PU03803	At2g19370	Expressed protein	Unknown role
2.0	1.4	**5.0**	**2.7**	PU03505	At5g09390	Expressed protein	Unknown role
2.2	2.0	**12.3**	**5.2**	PU03995	At3g06660	Expressed protein	Unknown role
2.0	1.9	**7.9**	**3.7**	PU01895	At2g15970	WCOR413-like protein	Cell rescue
1.8	2.0	**7.8**	3.4	PU03971	At4g39450	Expressed protein	Unknown role
1.9	**1.9**	**10.0**	**5.8**	PU03418	At3g05880	LTI6A	Cell rescue
2.6	2.4	**14.5**	**4.1**	PU03755	At5g38760	Pollen coat protein	Development
2.1	1.7	**9.4**	3.2	PU03503	None	Arabinogalactan protein AGP21	Development
2.5	1.3	**2.5**	2.8	PU03276	At5g61660	Glycine-rich protein	Unknown role
2.8	1.1	**3.5**	2.2	PU03151	At1g71691	GDSL-motif lipase/hydrolase	Metabolism
2.5	1.3	**9.4**	1.3	PU03398	At4g29680	Nucleotide pyrophosphatase	Metabolism
2.6	2.5	**14.0**	1.3	PU03390	At2g28680	Legumin-like protein	Storage
1.8	1.6	**8.5**	1.6	PU03329	At5g67080	Expressed protein	Unknown role
2.2	1.8	**7.4**	1.5	PU00815	At4g24220	Dehydrin	Cell rescue
2.1	1.5	**4.7**	2.5	PU03208	At5g54470	CONSTANS B-box zinc finger protein	Transcription
1.9	1.1	**2.3**	–1.3	PU10341	At5g55430	Expressed protein	Unknown role
1.8	–1.7	**2.3**	2.0	PU00410	None	Expressed protein	Unknown role
1.0	**2.1**	1.3	**4.0**	PU10603	At2g41380	Embryo abundant protein	Unknown role
1.0	**1.8**	1.2	**1.8**	PU10452	At5g39670	Calcium binding protein	Signal transduction
1.0	**1.8**	**1.7**	1.6	PU03954	At5g62165	MADS-box transcription factor FBP22	Transcription
1.1	**2.0**	**4.6**	**4.8**	PU03748	At1g06330	Copper-binding protein	Unknown role

The *Populus balsamifera* ssp. *trichocarpa* genome encodes six *CBF*-like transcription factors

The AtCBF1-Poplar leaf and stem regulons contained many genes previously linked to the process of cold acclimation in herbaceous annuals such as *Arabidopsis* and, when combined with the observed improvement in FT in AtCBF1-Poplar, implied that cold-responsive CBF transcription factors were encoded by the *Populus* genome and involved in the cold acclimation process. Iterative

Table 3. Normalized microarray expression values for *Populus* genes matched to reported *Arabidopsis* CBF3 regulon members in AtCBF1-Poplar and trees acclimated for 1 week at 2 °C. The poplar gene probe (PU) number is matched to its closest *Arabidopsis* gene (AGI) based on homology to the *Arabidopsis* genome sequence. Gene names presented in bold have a dehydration-responsive element (DRE) *cis*-element in within 1500 bp of the start codon.

			AtCBF1 trees		WT cold trees	
			Leaf FC	Stem FC	Leaf FC	Stem FC
CBF3 regulon						
Name	CloneID	AGI				
Pdc1	PU00414	At4g33070	1.1	1.2	1.4	1.0
ATFP6	**PU00465**	**At4g38580**	**1.3**	**1.2**	**7.5**	**2.9**
expressed protein	PU07867	At4g35300	1.1	1.0	2.0	1.3
expressed protein	**PU04708**	**At5g62530**	**1.5**	**1.1**	**2.5**	**1.7**
expressed protein	**PU10962**	**At4g14000**	**1.1**	**1.0**	**1.2**	**1.1**
Lti6	**PU10681**	**At4g30650**	**1.1**	**0.9**	**5.2**	**1.3**
erd7	**PU03488**	**At2g17840**	**1.3**	**1.5**	**2.5**	**2.8**
expressed protein	**PU11420**	**At1g27730**	**1.2**	**1.2**	**1.5**	**1.4**
expressed protein	PU07579	At1g51090	1.2	1.4	1.0	1.3
rap2.1	**PU10438**	**At1g46768**	**1.4**	**1.7**	**1.4**	**1.8**
expressed protein	PU05694	At4g15910	1.5	0.9	1.5	0.8
src2	**PU10619**	**At1g54410**	**1.5**	**0.6**	**1.2**	**1.3**

WT, wild type; FC, fold-change.

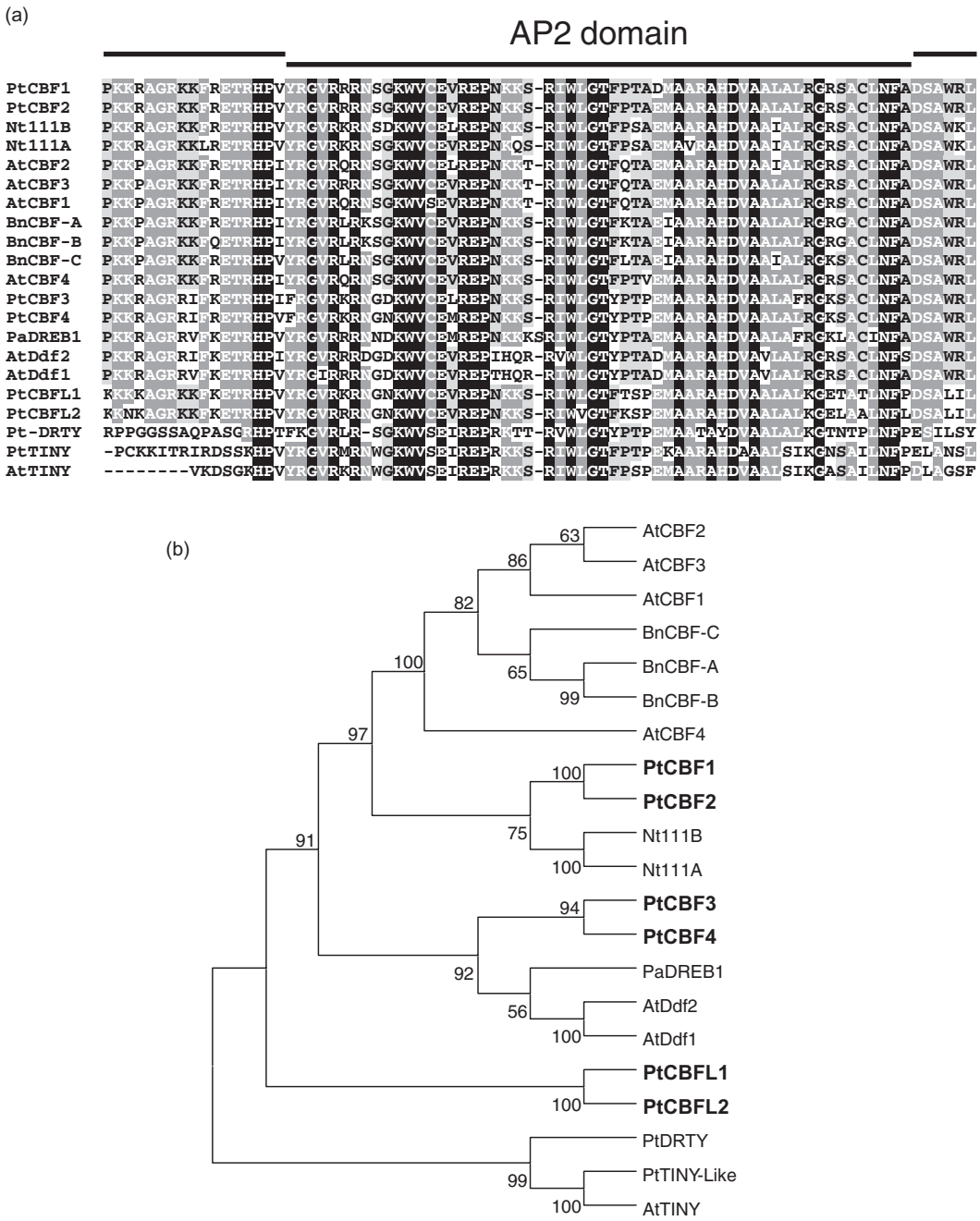

Figure 5. Comparison of representative C-repeat binding factor (CBF) protein family members in dicots. (a) Alignment of AP2 and flanking region of CBF factors. (b) Phylogenetic analysis, based on minimum evolution, was performed with the full-length protein sequences (Fig. S1). Polypeptides used for alignments were the six CBFs of *Arabidopsis*: AtCBF1 (U77378), AtCBF2 (AF074601), AtCBF3 (AF074602), AtCBF4 (NM_124578), AtDDF1/At1g12610 (NM_101131), AtDDF2/At1g63030 and (NM_104981); three *Brassica napus* CBFs: BnCBF-A (AAL38242), BnCBF-B (AAL38243), BnCBF-C (AAD45623); two tobacco EREBPs: Nt111A (AAG43548), Nt111B (AAG43549), *Prunus avium* PaDREB1 (AB121674); and the six poplar CBFs identified in this study: PtCBF1 (JGI666968), PtCBF2 (JGI346104), PtCBF3 (JGI548519), PtCBF4 (JGI63608), PtCBFL1 (JGI594822), and PtCBFL2 (JGI649103). PtDRTY (JGI253714) was also used and is a related AP2 domain protein with extended homology to CBFs in the C-terminal domain. *Arabidopsis* TINY (AtTINY) (AAC29139) was utilised as an outlier and represents the closest AP2 domain-containing protein group that falls outside the CBF/DREB1 family clade. A likely poplar TINY orthologue, PtTINY-Like (JGI653993), was also utilised as an outlier and assembled from the draft poplar genome sequence data. CBF proteins were initially aligned using CLUSTAL-W, the output alignments hand refined using GeneDoc Version 2.6 software (http://www.psc.edu/biomed/genedoc), and used to conduct phylogenetic analysis using MEGA Version 2.1 software (http://www.megasoftware.net/). Phylogenetic trees were generated using MEGA Version 2.1 Neighbor Joining and Minimum Evolution methodologies on 1000 bootstrap replications. Bootstrap numbers are shown on the dendrogram branch points.

tBLASTn searches of the *Populus balsamifera* ssp. *trichocarpa* (Torr. & A. Gray) Brayshaw genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) using the conserved AP2 and flanking CBF ID sequences from *Arabidopsis* (Jaglo *et al*. 2001) identified six potential CBF-encoding genes. An amino acid sequence alignment of the highly conserved AP2 domain and flanking CBF ID regions of the candidate poplar CBFs with other dicotyledonous CBFs (Fig. 5a) shows that two of the six candidate genes (*PtCBF1* and *PtCBF2*) have 100% conservation of the previously reported *Arabidopsis* CBF consensus sequences (PKKR/PRAGRxKFxETRHP and DSAWR), two other candidate genes (*PtCBF3* and *PtCBF4*) possess a single amino acid substitution in the N-terminal consensus (I instead of K in position 10) with the remaining two sequences (*PtCBFL1* and *PtCBFL2*) deviating from the consensus sequences by four and five amino acids, respectively. Interestingly, none of the poplar CBFs utilizes the commonly occurring proline at the fourth position, but PtCBF1-4 use the common alternate arginine residue while PtCBFL1-2 have lysine at this position, in contrast to all the other dicot CBFs. A phylogenetic analysis of the six poplar CBF candidates with known full-length sequences from dicotyledonous plants, using the *TINY* AP2 transcription factor as the out-group, confirms that *PtCBFL1* and *PtCBFL2* share the least amino acid similarity to the characterized CBFs from *Arabidopsis* (Fig. 5b). Also of some note, a *Populus* gene previously reported to be 'CBF1-like' and up-regulated in dormant cambium (*PtDRTY*) (Schrader *et al*. 2004) groups with *TINY*, not the CBFs. However, unlike *TINY*, *PtDRTY* shares some extended blocks of homology in the C-terminal domain with the CBFs. Investigation of the 1500 bp promoters of the *PtCBFs* also demonstrated that they possess numerous potential ICE-binding sites (CANNTG) and a *tBLASTn* search of the *Populus balsamifera* ssp. *trichocarpa* genome identified *Populus*-encoded ICE proteins with high similarity to *Arabidopsis* ICE1 (data not shown), indicating that ICE-mediation of the CBF signalling pathway may also be conserved in this woody perennial.

PtCBFs are cold-inducible in leaf and stem tissues

Based on the phylogenetic analysis, we selected *PtCBF1-4* for further study, and real-time PCR demonstrated that all were cold-inducible, although the kinetics of induction and tissue specificity differed between the orthologues (Fig. 6). *PtCBF1* was cold-inducible in leaf and stem tissue. *PtCBF1* transcript levels in both tissue types peaked 6 h after transfer to cold and decreased again overnight (Fig. 6a). Similarly, *PtCBF3* was inducible in both leaf and stem tissue (Fig. 6c). *PtCBF3* transcript accumulated rapidly after cold-shifting, peaking at 3 h, and returned to near starting levels within 6 h. In contrast, both *PtCBF2* and *PtCBF4* were cold-inducible in leaf tissue but were only weakly cold-induced in the stem (Fig. 6b & d). Leaf *PtCBF2* expression peaked 9 h after transfer, with stem transcripts peaking between 3 and 6 h. *PtCBF4* expression peaked at 3 h (Fig. 6d). These data strongly support the conclusion that poplar utilizes a cold-inducible CBF-based response to cold.

DISCUSSION

In this study, we have characterised the role of the CBF transcription factors in leaves and stems of a woody perennial, *Populus*. As shown previously for herbaceous plants (Jaglo *et al*. 2001; Hsieh *et al*. 2002; Lee *et al*. 2003, 2004), ectopic expression of *AtCBF1* from *Arabidopsis* increased the constitutive FT of non-acclimated meristematic stem and leaf tissues in both independent AtCBF1-Poplar lines,

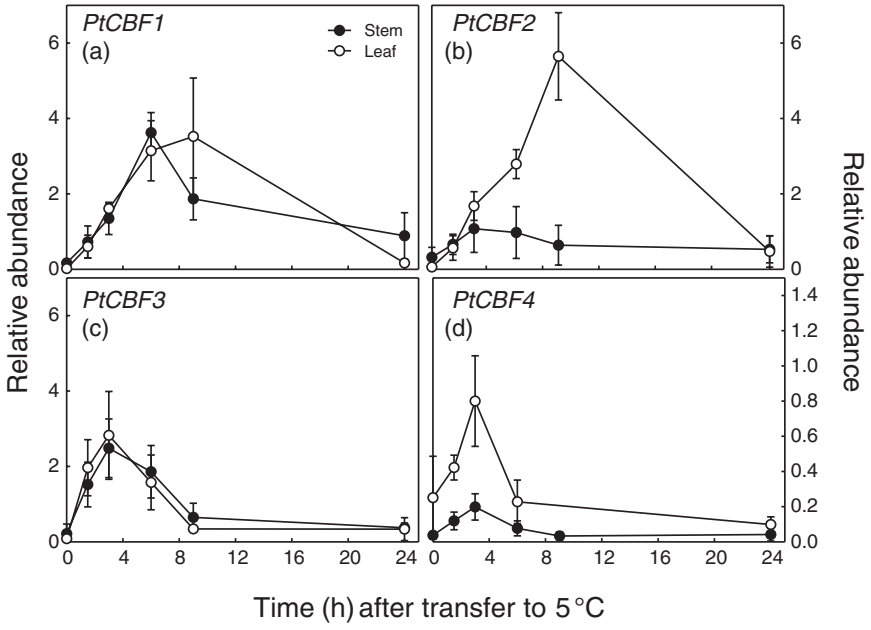

Figure 6. Expression response of *Populus CBF* paralogues to low temperature in wild-type trees: (a) *PtCBF1*; (b) *PtCBF2*; (c) *PtCBF3*; (d) *PtCBF4*. Expression was determined by RT-PCR. Each reaction used 30 ng first-strand cDNA and normalized to 18S rRNA quantity. Open circle, leaf; closed circle, stem. Error bars represent the standard error on three independent experiments.

demonstrating that the CBF-mediated signalling pathway and regulon are conserved in annual and perennial tissues of woody perennials. This conclusion is supported by earlier reports that the promoters of cold-responsive genes such as *Bplti36* from birch are activated by *Arabidopsis* CBFs (Puhakainen *et al*. 2004) and that CBF orthologues from woody species are functional when transferred to *Arabidopsis* (Kitashiba *et al*. 2004). Our subsequent microarray analysis, showing broad agreement between the transcriptomes of AtCBF1-Poplar and that reported for the AtCBF1-3 regulon orthologues in *Arabidopsis* (Fowler & Thomashow 2002; Vogel *et al*. 2005), demonstrates that AtCBF1 acts in a similar manner in *Populus* as AtCBF1-3 act in *Arabidopsis*.

Interestingly, the functional distribution of the *Populus* AtCBF1 regulon genes differed between the annual (leaf) and perennial (stem) tissues of this woody species. In stem tissues, 59% of the AtCBF1-Poplar regulon was composed of genes involved in the processes of 'Transcription' and 'Cellular Communication/Signal Transduction', while these two processes composed only 26% of the AtCBF1-Poplar leaf regulon. It has been shown previously that the CBF regulon contains a number of transcription factors, which themselves target additional gene sets (Fowler & Thomashow 2002; Gilmour *et al*. 2004; Novillo *et al*. 2004; Vogel *et al*. 2005; Nakashima & Yamaguchi-Shinozaki 2006). However, the difference in composition of the poplar leaf and stem regulons was unexpected and could not be attributed to a general difference in the low temperature transcriptomes of the annual and perennial tissues because the same comparison made between 7 d cold-acclimated WT leaf and stem tissues demonstrated general agreement in the distribution of up-regulated functional categories (Fig. 3). Examination of the identities of the leaf and stem cold regulon genes showed that their functional similarities were not superficial: the WT cold-responsive regulons of the leaf and stem were compositionally similar. It is possible that the AtCBF1-Poplar leaf/stem regulon functional difference is due to the small size of the stem regulon; however, examination of the largest functional group ('Metabolism') in the leaf and stem regulons contained significantly (χ^2, $P < 0.05$) fewer genes (2) than expected (6) in the stem regulon. The composition of the AtCBF1-Poplar stem regulon was also not representative of the functional distribution of the genes printed on our array, indicating that this compositional difference has a biological basis.

Associated with the difference in regulon composition between annual leaf tissue and the perennial stem meristem tissue, another finding was that a *FRY2* orthologue was down-regulated in AtCBF1-Poplar leaves. FRY2 is a double stranded RNA-binding transcriptional repressor that is known to repress expression of CBF regulon members by working at a point upstream of their transcription (Xiong *et al*. 2002). Like the majority of genes down-regulated in the AtCBF1-Poplar, the FRY2 1500 bp promoter lacks the DRE consensus, indicating that a CBF-responsive transcription factor or post-transcriptional process, and not CBF itself, leads to its differential expression. Nevertheless, because the *fry2* mutation is known to alter ABA sensitivity in *Arabidopsis* seeds and seedlings (Xiong *et al*. 2002) and the CBF regulon in *Populus* was significantly enriched for the ABRE, differential expression of FRY2 in leaves (versus stems) of AtCBF1-Poplar may also have contributed to the CBF leaf/stem regulon differences. The up-regulation of *PtCBF3* in AtCBF1-Poplar stem tissues also suggests that individual *Populus* CBF factors are, as in *Arabidopsis* (Novillo *et al*. 2004), regulated by their own paralogues. *PtCBF* promoters contain numerous ICE1 binding sites, and the genome encodes ICE1-like proteins (data not shown).

Despite the broad similarity between the *Populus* and *Arabidopsis* CBF leaf regulons and the substantial overlap of the AtCBF1-Poplar leaf regulon with the endogenous transcriptional response to low temperature, we found no significant enrichment of the DRE (RCCGAC) in the 1500 bp promoters of our identified *Populus* CBF regulon. The lack of DRE enrichment may have been due to the small number of genes identified in the regulon, but the current lack of annotated transcriptional start sites in the *Populus* genome assembly, the larger genome and intergenic size of *Populus* or an altered AtCBF1-binding consensus sequence may all have contributed to this. In contrast, the ABRE, previously shown to be enriched in CBF3 regulon promoters and to impart ABA-responsiveness (Hattori *et al*. 2002), showed significant positional enrichment (between 100 and 400 bp upstream of the ATG) and co-occurrence with the DRE in our *Populus* CBF regulon, indicating that this *cis*-element relationship is important.

As noted earlier, the accumulation of CBF transcripts and the activity of the CRT regulatory motif in *Arabidopsis* are also modulated by the presence and quality of light during cold stress (Kim *et al*. 2002; Fowler *et al*. 2005) and that CRT-driven GUS reporter activity is altered in phytochrome mutant backgrounds, suggesting that day length mediates expression of the CBF regulon through phytochrome B in *Arabidopsis* (Kim *et al*. 2002). Pre-treatment with short days has also been shown to enhance the expression of low temperature-responsive, CRT-containing genes in birch (Puhakainen *et al*. 2004) and a CBF-like transcription factor is induced by short days in the cambial meristem of poplar (Schrader *et al*. 2004). These data suggest that CBF or related transcriptional regulators may be involved in the day-length-mediated growth cessation in woody species. ABA is known to mediate the response of many abiotic stress-response genes (Chen, Li & Brenner 1983; Mäntyla, Lång & Palva 1995) via activation of the ABRE (Marcotte *et al*. 1989; Lam & Chua 1991), and initiation and maintenance of bud dormancy in woody species has been associated with high endogenous ABA levels (Wright 1975; Rinne, Saarelainen & Junttila 1994; Rinne, Tuominen & Junttila 1994; Rohde *et al*. 2002; Li *et al*. 2003b). The strong concurrence of the DRE and ABRE in the promoters of our *Populus* CBF regulon supports the conclusion that there is direct interaction between the day-length-

dependent ABA and low-temperature-dependent CBF signalling pathways. Although unravelling the role(s) that the different PtCBFs play in this interaction will require additional studies, our data nevertheless indicate that in poplar the initial perception of low temperature (biologically indicated by the accumulation of *CBF* transcripts in WT trees and artificially mimicked by ectopic expression of *AtCBF1* transcript in AtCBF1-Poplar) results in up-regulation of a functionally different CBF regulon in annual leaf and perennial stem tissues. This conclusion is supported by the differential inducibility of the endogenous PtCBFs in *Populus*. Although all four *PtCBF*s are cold-inducible in leaf tissue, only *PtCBF1* and *PtCBF3* are significantly induced in the stem. PtCBF1/2 and PtCBF3/4 form distinct phylogenetic clusters, and one gene from each cluster is tissue-specific, suggesting that the different CBF gene clusters may be controlling slightly different CBF regulons in the different tissues. In accordance with their predicted role in cold acclimation in trees (protection of temperature-sensitive tissues such as buds and flowers during spring flushing), the poplar expressed sequence tag (EST) sequencing project (Sterky *et al*. 2004) has also detected *PtCBF3* expression in flower buds (as well as roots), and *PtCBF1* in dormant buds.

Overall, our studies of the CBF regulon in *Populus* have demonstrated that the central role played by the CBF family of transcriptional activators in cold acclimation of herbaceous annuals such as *Arabidopsis* has been maintained in temperate woody perennials, despite the evolutionary pressure imposed by the development of a winter dormant phenotype. However, these results also point to several key differences when we compare results from *Populus* with *Arabidopsis*: (1) unlike data from all herbaceous species, two of the four poplar CBF paralogues show differential expression in leaf (annual) and stem (perennial) tissues; (2) the functional composition of the stem and leaf AtCBF1-Poplar regulons are different, perhaps reflecting in part the different transcriptomes of these divergent tissues and also possibly indicating that different functional requirements in these tissues, have driven the evolution of specific leaf and stem cold-responsive CBF paralogues; and (3) this divergence in functional composition is reinforced by the finding of leaf- and stem-specific AtCBF1-Poplar regulon members. Taken together, these data suggest that the perennial-driven evolution of winter dormancy has led to the development of specific roles for abiotic stress response regulators, such as the CBFs, in annual and perennial tissues. Future studies exploring the FT and transcriptomic changes in poplar constitutively/inducibly expressing native PtCBFs should help elucidate these specific roles in greater detail.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this paper online:

Table S1. (a) Genes up-regulated by ectopic AtCBF1 expression in Poplar leaves. The assigned gene identity (AGI) is based on homology to the *Arabidopsis* genome sequence. (b) Genes up-regulated by ectopic AtCBF1 expression in Poplar stems. The assigned gene identity (AGI) is based on homology to the *Arabidopsis* genome sequence. (c) Genes down-regulated by ectopic AtCBF1 expression in Poplar leaves and stems. The assigned gene identity (AGI) is based on homology to the *Arabidopsis* genome sequence.

Table S2. Genes up-regulated by 7 d cold treatment in Poplar (leaf). The assigned function is based on homology to the *Arabidopsis* genome sequence.

Table S3. Genes up-regulated by 7 d cold treatment in Poplar (stem). The assigned function is based on homology to the *Arabidopsis* genome sequence.

Table S4. Normalized expression levels for orthologues to the *Arabidopsis* CBF2 and CBF1/2/3 regulons. The assigned gene identity (AGI) is based on homology to the *Arabidopsis* genome sequence.

Table S5. Positional analysis of DRE and ABRE distances from ATG in Populus balsamifera subsp trichocarpa. The assigned gene identity (AGI) is based on homology to the *Arabidopsis* genome sequence.

Figure S1. Sequence alignments used to produce phylogenetic tree in Fig. 5.

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