Journal of Experimental Botany, Vol. 57, No. 10, pp. 2455–2469, 2006
doi:10.1093/jxb/erl019 Advance Access publication 30 June, 2006
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany

RESEARCH PAPER

Expression profile of *CBF*-like transcriptional factor genes from *Eucalyptus* in response to cold

Walid El Kayal*, Marie Navarro, Gilles Marque, Guylaine Keller, Christiane Marque and Chantal Teulieres†

Université Paul Sabatier: UMR 5546, 'Surfaces Cellulaires et Signalisation chez les Végétaux' Pôle de Biotechnologie Végétale, 24 Chemin de Borde Rouge, BP 42617 Auzeville, F-31326 Castanet-Tolosan, France

Received 4 November 2005; Accepted 10 April 2006

Abstract

Two *CBF* (CRT/DRE-binding factor) homologues isolated from *Eucalyptus gunnii* were designated Egu*CBF*1a and Egu*CBF*1b and belong to a gene family which includes at least five members. Both promoter and coding sequences were found to exhibit the main characteristics of a *CBF* transcription activator gene and, as expected, the corresponding protein targeted the nucleus. Gene expression was quantitatively analysed using real-time reverse transcription–polymerase chain reaction (RT–PCR) after a short exposure to different environmental conditions or along a two-step cold acclimation programme with either short or long daylengths. A very strong and fast response to cold was observed, with dark conditions and cold intensity (down to 0 °C) having a positive effect on the magnitude of induction. The two genes under study exhibited several similar features such as light response. However, interestingly, their regulation by cold proved differential and complementary as Egu*CBF*1a was more transiently induced by a direct and intense exposure while Egu*CBF*1b responded to milder treatments and exhibited a longer (i.e. which started earlier and finished later) time course. During acclimation, the short daylength positively affected the freezing tolerance in the same way as it positively affected the *CBF* transcript accumulation, suggesting a potential involvement of these genes in the adaptive response. Although very quick after the first signal, the up-regulation of the two Egu*CBF*1 genes unexpectedly lasted throughout the chilling culture, and new inductions were seen during the thermoperiod transitions. Using a quantitative and highly sensitive measurement of gene expression combined with the application of a cold treatment consistent with natural environmental conditions, this study provides new information on the regulation of *CBF*-like genes by cold *in planta.*

Key words: CBF/DREB1 transcription factor, cold acclimation, *Eucalyptus*, freezing tolerance, gene expression, real-time RT–PCR.

Introduction

Cold, drought, and high salinity, which all cause dehydration damage to the plant cell, are the most common environmental stresses that influence plant growth and development, limiting productivity in cultivated areas worldwide. In the last decade, numerous investigations have focused on the identification of stress-regulated genes, with the aim of better understanding their functions in stress tolerance mechanisms (Thomashow, 2001; Zhu, 2002; Seki *et al*., 2003; Shinozaki *et al*., 2003) and to provide the basis for efficient strategies in plant breeding programmes.

Due to its fast growth and fibre quality, *Eucalyptus* is the most commonly planted hardwood in the world, in particular for paper making. However, although widely distributed, its extension is mostly restricted to southern areas because of freezing sensitivity. In the absence of any physiological or morphological adaptive strategy to avoid frost, overwintering of this perennial species essentially depends on the capacity of cells to tolerate apoplastic freezing.

* Present address: Biotechnology Research Center, Forest Resources and Environmental Science, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931-1295, USA
† To whom correspondence should be addressed. E-mail: teulieres@scsv.ups-tlse.fr

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; AFOCEL, Association Forêt Cellulose; CaMV, cauliflower mosaic virus; CRT/DRE, C-repeat/dehydration-responsive element; EST, expressed sequence tag; GFP, green fluorescent protein; LD, long day; ORF, open reading frame; RT–PCR, reverse transcription–polymerase chain reaction; SD, short day.

Eucalyptus, as well as many other plant species, develops increased freezing tolerance in response to low but non-freezing temperatures. This adaptive response, known as cold acclimation, takes place on the time scale of days or weeks as a result of a combination of physiological and metabolic changes depending on transcriptome modifications (Thomashow, 1999).

At present, the best understood genetic system with a role in cold acclimation is the *Arabidopsis* CBF (CRT/DRE-binding factor) cold response pathway. The CBF/DREB1 proteins belong to the AP2/ERF DNA-binding protein family (Riechmann and Meyerowitz, 1998), which includes >140 members in *Arabidopsis thaliana. CBF* gene expression is induced within 1 h by low temperature, and the encoded transcriptional factors in turn activate the expression of many cold-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997). The CBF regulon comprising several dozen genes (Seki *et al*., 2001; Fowler and Thomashow, 2002; Cook *et al*., 2004; Gilmour *et al*., 2004; Maruyama *et al*., 2004) represents up to 12% of the cold-regulated genes (Fowler *et al.*, 2002; Cook *et al.*, 2004). Moreover, when constitutively overexpressed in *Arabidopsis*, *CBF* genes induce the expression of the downstream genes (CBF regulon) under non-stress conditions and confer freezing, drought, and salt tolerance to the corresponding transgenic plants (Gilmour *et al*., 1998; Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999). In addition, the CBF cold response pathway was found to play a prominent role in the configuration of the low temperature metabolome of *Arabidopsis* (Cook *et al*., 2004). Therefore, this class of genes represents a critical component in signal transduction of cold acclimation.

Highly conserved in plants, *CBF* orthologues exhibiting the same characteristic elements as *Arabidopsis thaliana* have been found in several plant species, including herbaceous species such as *Brassica napus* (Jaglo *et al*., 2001), *Lycopersicon esculentum* (Hsieh *et al*., 2002), *Oryza sativa* (Dubouzet *et al*., 2003), and *Capsicum annuum* (Kim *et al*., 2004; Hong and Kim, 2005), as well as the woody plant *Prunus avium* (Kitashiba *et al*., 2004). Moreover, as in *Arabidopsis*, overexpression of two *Brassica CBF*-like genes in *Brassica napus* allowed freezing tolerance to increase (Savitch *et al*., 2005). However, despite this apparent high conservation, some structural and regulatory differences were observed in the CBF cold response pathway among the plant species. For example, the CBF regulon of *L. esculentum* (a chilling-sensitive species) was found to be smaller and less diverse in function than the regulon of frost-tolerant plants such as *A. thaliana*, which partly explains the differences in plant behaviour in low temperatures (Zhang *et al*., 2004). Another recent work on wheat showed that several *CBF* genes have dramatically different levels of induction after cold exposure linked to the frost tolerance of the corresponding recombinant lines (Vagujfalvi *et al*., 2005). It could therefore be hypothesized that the CBF pathway is more or less complete, depending on the complexity of the cold-tolerance mechanisms for the considered plant species.

Although recent breakthroughs have increased the knowledge of the molecular basis of frost hardiness and the CBF pathway in herbaceous species, very little is known concerning woody plants. To date, the only published information on a *CBF* gene from woody plants is a functional analysis of Pa*CBF* (Kitashiba *et al*., 2004) from wild cherry by heterologous expression in *Arabidopsis*. The present study reports the molecular characterization and the transcriptional patterns of two *CBF*-like genes, Egu*CBF*1a and Egu*CBF*1b, isolated from *Eucalyptus gunnii*. The real-time reverse transcription–polymerase chain reaction (RT–PCR) method was used to quantify sensitively the influence of various conditions of cold culture (light, photoperiod, intensity, or duration of cold treatment) on the expression of both Egu*CBF*1a and b. The experimental system was completed by the design of two chilling programmes imitating natural conditions and leading to a differential level of freezing tolerance measured by ion leakage. Among the numerous data, the detailed kinetics of *CBF* transcript abundance during a cold culture programme, aligned with the level of freezing tolerance, suggest that the two Egu*CBF*1 genes are involved in the development of *Eucalyptus* cold acclimation.

Materials and methods

Plant material

Two *Eucalyptus* lines from a plant breeding programme managed by AFOCEL were studied. The *E. gunnii* cell suspension culture from line 634, maintained as previously described (Teulières *et al*., 1989), was used for gene isolation, Southern analysis, and protoplast isolation. Plantlets from a hybrid *Eucalyptus gunnii*×*Eucalyptus dalrympleana* ('E. gundal 208') were provided by TEMBEC R&D KRAFT as material for gene expression studies. The 1-year-old plantlets were grown in controlled-environment chambers at 25 °C day/22 °C night, with a long-day (LD) photoperiod (16 h/light=115 μmol m^{-2} s^{-1} supplied by Lumilux Daylight 58 W Osram).

Gene isolation and sequence analysis of EguCBF1

Based on the sequence similarity between the published plant *CBF*/DREB genes, two degenerate primers (Table 1) were designated from the conserved regions to amplify the *CBF* orthologues from *E. gunnii*, using the High-Fidelity PCR system (Roche). The resulting product, cloned in the pGEM-T Easy vector (Promega, France), was identified as *CBF*-like using BLAST analysis and designated Egu*CBF*1. The isolation of the promoter sequence and complete ORF (open reading frame) was carried out using a Universal Genome Walker Kit (Clontech, USA) on 10 μg of *E. gunnii* genomic DNA digested by four blunt-end-generating restriction enzymes (*Eco*RV, *Dra*I, *Pvu*II, and *Stu*I). After purification, the restriction fragments were ligated with Genome Walker adaptors. The first PCR was performed on each restriction fragment set using an Egu*CBF*1 sequence-specific primer (Egu*CBF*1a-ext.5′, Table 1) and the adaptor primer 1 (5′-GTAATACGACTCACTACTAGGGC-3′). Using another Egu*CBF* sequence-specific primer (EguCBF1a-int-5′, Table 1) and the adaptor primer 2 (5′-ACTATAGGGCACGCGTGGT-3′),

Table 1. *List of oligonucleotide PCR primers sequences used for isolation of Egu*CBF*1*

Name	Oligonucleotide sequence	Purpose of the reaction
Egu*CBF*-FW	5′-GAG ACT CGG CAC CCG MTD TAC-3′	Isolation of Eg*CBF*1 (AP2+signatures)
Egu*CBF*-Rev	5′-CCT CCA GGC GGA GTC RGM GAA GTT-3′	
Egu*CBF*1A-ext.3′	5′-GAGACTCGGCACCCGATGTACC-3′	Extension of 3'TR and nTR
Egu*CBF*1A-int.3′	5′-GAGATGGCGGCGCGCCCACG-3′	
Egu*CBF*1A-ext.5′	5′-TCTTCCGAACGCAACTTCTTCTC-3′	Extension of 5'TR and nTR
Egu*CBF*1A-int.5′	5′-GCTGCCATCTCCACAGTAGGGA-3′	
Egu*CBF*1B-ext.5′	5′-GGGAGGACGTCTGCACAGGGATACTGCG-3′	Extension of 5'TR and nTR
Egu*CBF*1B-int.5′	5′-CAAATGATTAGACTCATTTCATTCG-3′	

3′TR, 3′ translated sequence; 3′nTR, 3′-non-translated sequence; 5′TR, 5′ translated sequence; 5′nTR, 5′ non-translated sequence.

the second PCR amplification was performed on the products of the first PCR. Finally, the PCR products were cloned into pGEM-T Easy vector and mobilized into *Escherichia coli* (DH5α).

In the second round, the Egu*CBF*1 sequence was used for BLAST analysis against the *Eucalyptus* cold cDNA library available in the laboratory (unpublished data). The result allowed the identification of a matching clone which was presumed to be a part of another *CBF*1-like gene. As previously described for the first Egu*CBF*1 expressed sequence tag (EST), the extension of the upstream sequence of this EST was carried out. The comparison between the two sequences led to the conclusion that two different *CBF*1-like genes had been isolated, which were designated as Egu*CBF*1a and b, respectively.

Promoter sequence analysis was performed on the Egu*CBF*1a sequence using the PLACE Signal Scan Search program (Prestridge, 1991; Higo *et al*., 1999), (http://www.dna.affrc.go.jp/PLACE/signalscan.html). The alignment of the CBF/DREB1 proteins was performed using ClustalW, and shading was done using Esprit 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The phylogenetic tree was constructed using TreeTop-Phylogenetic Tree Prediction, (http://www.genebee.msu.su/services/phtree_reduced.html).

Southern blotting analysis

A 10 μg aliquot of genomic DNA was isolated from *E. gunnii* using the DNeasy Plant Maxi kit (Qiagen) and digested with *Bam*HI, *Eco*RI, *Eco*RV, or *Hin*dIII enzymes. The restriction fragments were separated by electrophoresis in a 0.8% agarose gel, then blotted onto a nylon membrane filter (Amersham). Under high-stringency conditions, the membrane was successively hybridized to two different ^{32}P-labelled probes: the first one was the *CBF* full-length ORF sequence and the second was a partial Egu*CBF*1 sequence corresponding to the 3′-terminal region (DSAWR to stop codon). The second hybridization on the stripped filter was performed in order to improve hybridization specificity and avoid putative cross-hybridization between the *CBF* coding sequence probe and the numerous genomic sequences which contain the highly conserved ERF/AP2 DNA-binding domain present in the ORF.

Protoplast isolation and transient expression of EguCBF1::GFP fusion proteins

The coding sequences of Egu*CBF*1s were cloned in-frame with the green fluorescent protein (GFP) into the pGreen vector (Hellens *et al*., 2000) as a C-terminal fusion expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The ORFs were amplified by PCR (Expand High Fidelity, Roche) using the following primers: Egu*CBF*1a-*Xma*I (5′-TAACCCGGGATGAACCCTTTCTCTTCTCATTCCCAT-3′) and Egu*CBF*1a-*Nco*I (5′-TAACCATGGAGATGGAATAGCTCCATAATGACGTGTG-3′) or Egu*CBF*1b-*Bam*HI (5′-AATAGGATCCATGAACTCTTCCTCTTATATCTCCCAT-3′) and Egu*CBF*1b-*Nco*I (5′-AATTCCATGGTTGGAATAGCTCCATAATGACGTGTACGC-3′). The corresponding ORFs of Egu*CBF*1a and b were cloned using the *Xma*I–*Nco*I and *Bam*HI–*Nco*I restriction sites of the pGreen vector, respectively.

Protoplasts were obtained from *E. gunnii* cell suspension culture and transfected using a polyethylene glycol method as previously described (Teulières *et al*., 1989). A 0.3 ml aliquot of protoplast suspension (0.5×10^6) was transfected using 50 μg of sheared salmon sperm DNA as carrier and 30 μg of Egu*CBF*1a::GFP or Egu*CBF*1b::GFP or GFP (control) plasmid DNA. After transfection, the protoplasts were incubated for 16 h at 25 °C and then analysed for GFP fluorescence (500–520 nm) using confocal microscopy. All transient expression assays were performed in three independent experiments.

Cold treatments

All the results were obtained from at least two independent experiments on two or three plantlets of E. gundal, depending on the treatment. For the corresponding gene expression studies, total RNA was extracted from a pool of leaves randomly harvested on the plantlets.

First, the time-course of Egu*CBF*1 transcript production was studied at 15 min, 30 min, 2 h, 5 h, and 24 h, on two plantlets grown at 4 °C in the dark or in continuous light (45 μmol m^{-2} s^{-1} supplied by Lumilux Daylight 58 W Osram). Then, the effect of the cold shock intensity on the Egu*CBF*1 transcription rate was evaluated on the plantlets after transferring them directly from 22 °C/night to lower temperatures (12, 8, 4, 0, or −4 °C) for 2 h in the dark.

For cold acclimation, the chilling programme consisted of two steps: 4 d at 12 °C/day and 8 °C/night followed by 6 d at 4 °C day and night (Fig. 1). In two different experiments, this chilling programme was coupled with a photoperiod corresponding either to a long daylength (LD=16 h light) or a short daylength (SD=8 h light). Before the acclimation programme, the plantlets were cultured with the appropriate photoperiod for 3 d, and the thermoperiod transition was started at the same time as the light was switched on (from 22 °C/night to 12 °C/day and then 8 °C/night to 4 °C/day). In addition, in order to avoid photoinhibition stress on plants, the light intensity was reduced in the culture chamber during cold treatment (45 μmol m^{-2} s^{-1} supplied by Lumilux Daylight 58 W Osram). Frost tolerance was evaluated on day 4, day 7, and day 10 by measuring the electrolyte leakage from leaf discs after freezing at −2 °C h^{-1}, using previously described methods (Leborgne *et al*., 1995). The measurement was expressed as the percentage of cell viability after freezing at −6 and −8 °C compared with the viability of unfrozen leaf discs. It was given as the mean of at least three plantlet measurements.

For expression studies during the acclimation programme, RNA was extracted from a pool of leaves randomly harvested on the three plantlets at the indicated times (Fig. 1): (i) 30 min, 2 h, and 5 h after the temperature changes from 22 °C to 12 °C (day 1) or from 8 °C to 4 °C (day 5); (ii) 15 min before and 2 h after the daily thermoperiod transition (12 °C day/8 °C night) for SD culture conditions; and (iii) 24 h for each day except days 8 and 9.

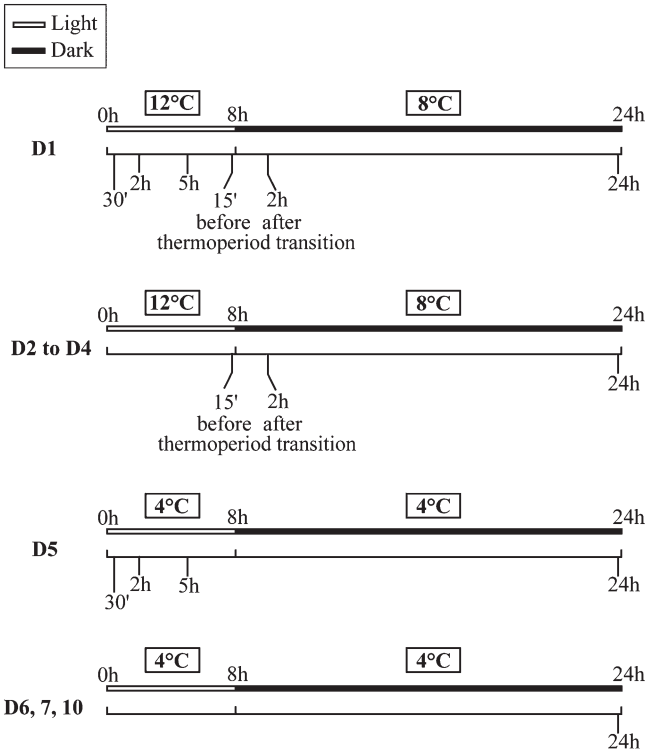


Fig. 1. RNA sampling during the acclimation programme: the leaves were randomly harvested 30 min, 2 h, and 5 h after the transfer of plantlets from 22 °C to 12 °C (D1) or from 8 °C to 4 °C (D5), 15 min before and 2 h after the daily (D1 to D4) thermoperiod change (12 °C day/8 °C night) and finally at 24 h for each day except D8 and D9. The applied cold culture conditions are associated with a short photoperiod represented on the graph as white and black lines for day and night periods, respectively.

Abiotic stress treatments and ABA application on leaf discs

For cold, salt, or abscisic (ABA) treatments, 10 leaf discs from fully expanded leaves of the E. gundal line were incubated for 30 min, 2 h, or 24 h at 4 °C, or in 200 mM NaCl or 100 μM ABA. Leaf discs were incubated for similar periods in distilled water as a control for cold and salt treatments, or in dimethylsulphoxide (DMSO) as a control for ABA application. For gene expression studies, total RNA was extracted from all the tested discs.

Real time RT–PCR

The total RNA was extracted from E. gundal leaves using the SV Total RNA Isolation System (Promega, France). Using SuperScript II and random primers (Invitrogen, France), cDNAs were produced according to the manufacturer's instructions. The Egu*CBF*1-specific primers were designated (Table 2) using the Primer Express software (version 2.0, Applied Biosystems, France). The PCRs were performed in 20 μl of 2× SYBR Green Master mix (Applied Biosystems), with 10 ng of cDNA and 300 nM of each primer. Three replicates of each PCR were run in an ABI PRISM 7900HT Sequence Detection System (Applied Biosciences, France) using a programme including a first step (50 °C/2 min and 95 °C/10 min) followed by 40 cycles (95 °C/15 s and 60 °C/1 min). The non-specific products could be detected after the end of the amplification programme, when the PCR assays were submitted to a temperature ramp in order to create the dissociation curve, measured in terms of the changes in fluorescence as a function of temperature. The dissociation programme was 95 °C /15 s, 60 °C /15 s followed by 20 min of slow ramp from 60 to 95 °C. Specific primers for 18S RNA were used as the internal control for the normalization of the RNA steady-state level, and the relative changes in gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The results of Egu*CBF*1 relative transcript abundance are presented as a mean value of the three assay replicates compared with the mean of the three control values (leaves from control plants).

Table 2. *List of oligonucleotide sequence used in real time RT–PCR*

Name	Oligonucleotide sequence
Eg*CBF*1A-FW	5′-GGTACGAAGCCATTTTTTTGGT-3′
Eg*CBF*1A-Rev	5′-GCCCAACATCATCATCGATATG-3′
Eg*CBF*1B-FW	5′-AAAGCTGTGTTCCCCATGACA-3′
Eg*CBF*1B-Rev	5′-CACAATGACTCGGGCAGTTTC-3′
Eg18S-FW	5′-CGCGCTACACTGATGTATTC-3′
Eg18S-Rev	5′-GTACAAAGGGCAGGGACGTA-3′

Results

*Isolation and structural characterization of Egu*CBF*1a and Egu*CBF*1b*

Two *CBF*-like genes were isolated from *E. gunnii*, one through a PCR-based approach and the other from a cDNA library (unpublished data). The full-length ORF sequences were designated Egu*CBF*1a and Egu*CBF*1b (DQ241820 and DQ241821) due to their strong similarity (BLAST analysis) to other known *CBF*1 genes. The two genes do not exhibit any introns and encode two putative ORFs (of 220 and 223 amino acids, respectively) which bear a close resemblance to each other (82% identical).

The predicted molecular masses are 24.78 kDa for Egu*CBF*1a and 25.13 kDa for Egu*CBF*1b, and the estimated isoelectric points are 7.24 and 5.72, respectively. A database search revealed that the deduced amino acid sequences of Egu*CBF*1A and B proteins contain the conserved DNA-binding domain of 60 amino acids which is characteristic of the AP2 family of plant DNA-binding proteins (underlined in Fig. 2). In addition, the two genes exhibit the CBF signature sequences ETRHP and DSAWR found upstream and downstream of the AP2 motif, and the basic residue region 'PKKP\RAGRKKFR' located on the N-terminal domain which might function as a nuclear localization signal (NLS).

The amino acid sequence alignments used for comparison with other CBF proteins showed that Egu*CBF*1A shares 67% similarity with Le*CBF*1 (AY034473) from tomato and 60% similarity with At*CBF*1 (AY667247) from *Arabidopsis*; Egu*CBF*1B shares 66% similarity with Ca*CBF*1 (AY368483) from *Capsicum* and 53% similarity with At*CBF*1. Based on the sequence divergence in the conserved domains of these *CBF*/DREB sequences, the phylogenetic tree analysis defines three main groups (Fig. 3). As expected, *Eucalyptus* genes belong to the group consisting of *Capsicum*, *Lycopersicon*, *Gossypium*,

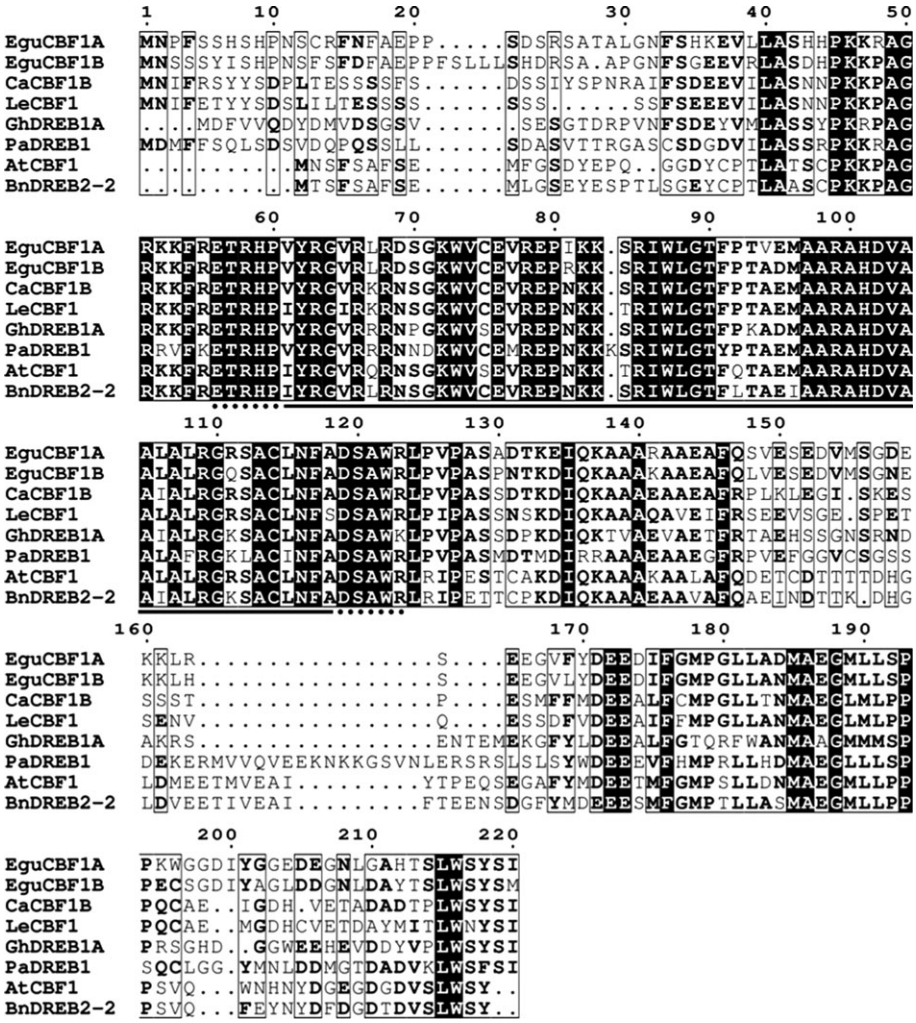


Fig. 2. Multiple alignment of the amino acid sequences of *CBF*/DREB proteins. Identical amino acid sequences are highlighted on a black background while white boxes indicate at least three identical amino acids. The CBF signatures (ETRHP/DSAWR) and the ERF/AP2 DNA-binding domain are underlined with dots and black lines, respectively. The GenBank accession numbers are reported as follows: Egu*CBF*1A (DQ241820), Egu*CBF*1B (DQ241821), Ca*CBF*1B (AY368483), Le*CBF*1 (AY034473), GhDREB1A (AY321150), PaDREB1 (AB080966), At*CBF*1 (AY667247), and BnDREB2-2 (AY444874).

and *Prunus*, which represents one of the two dicotyledon groups, the second of which contains *Arabidopsis*.

Promoter sequence analysis: The promoter sequence from the Egu*CBF*1a DNA fragment contained several predictive *cis*-acting elements presumed to be involved in transcriptional regulation. The typical TATA box sequence was located about −30 bp from the transcription initiation point (Fig. 4). In addition, motifs related to MYB (five sites) and MYC (eight sites) recognition sites, and three ABRE *cis*-elements (ABA-responsive element) were detected on the Egu*CBF*1a promoter sequence.

DNA gel blotting: To determine the Egu*CBF* copy number in the *Eucalyptus* genome, DNA was digested by *Eco*RI, *Bam*HI, or *Hin*dIII which did not cut, or by *Eco*RV cutting once within the Egu*CBF*1a and b sequences. After blotting, the filter was successively hybridized to two labelled probes containing the *CBF* well-conserved coding region or the Egu*CBF*1-specific sequence from the 3′-terminal region. The profiles obtained with the two probes were found to be strictly identical, indicating that their hybridization is equally efficient in these experimental conditions, and providing confirmation of Southern data. The resulting complex hybridization patterns (shown only for the full-length ORF, Fig. 5) suggest that at least five Egu*CBF*-related genes are present in the *Eucalyptus* genome. The *Eco*RI and *Bam*HI profiles presented some similarities, in particular for the shortest band (1 kb), which was unexpected given the Egu*CBF*1a and b restriction maps. This may result from the digestion of another Egu*CBF* family member.

*The Egu*CBF*1a- and b-encoded proteins are targeted to the nucleus*

The subcellular localization of Egu*CBF*1 proteins was studied using a gene fusion with the coding sequence of

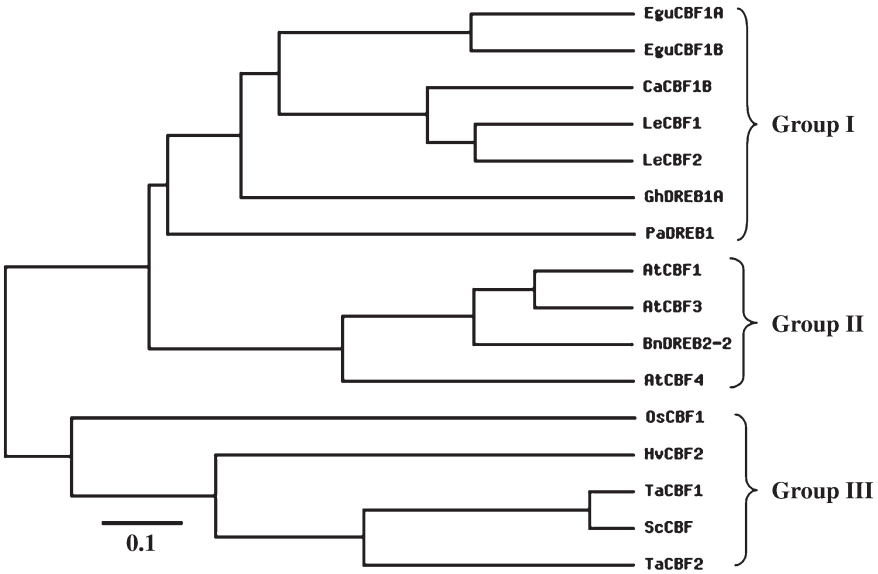


Fig. 3. Phylogenetic relationship among *CBF*/DREB proteins. The dendrogram is based on the amino acid sequence alignment of the following proteins: *Eucalyptus gunnii* (Egu*CBF*1A, DQ241820; Egu*CBF*1B, DQ241821), *Capsicum annuum* (Ca*CBF*1B, AY368483), *Lycopersicon esculentum* (Le*CBF*1, AY034473; Le*CBF*2, AY497899), *Gossypium hirsutum* (GhDREB1A, AY321150), *Prunus avium* (PaDREB1, AB080966), *Arabidopsis thaliana* (At*CBF*1, AY667247; At*CBF*3, AY667247), *Brassica napus* (BnDREB2-2, AY444874), *Arabidopsis thaliana* (At*CBF*4, AB015478), *Oryza sativa* (Os*CBF*1, AP001168), *Hordeum vulgare* (Hv*CBF*2, AF442489), *Triticum aestivum* (Ta*CBF*1, AF376136), *Secale cereale* (Sc*CBF*, AF370728), and *Triticum aestivum* (Ta*CBF*2, AY572831).

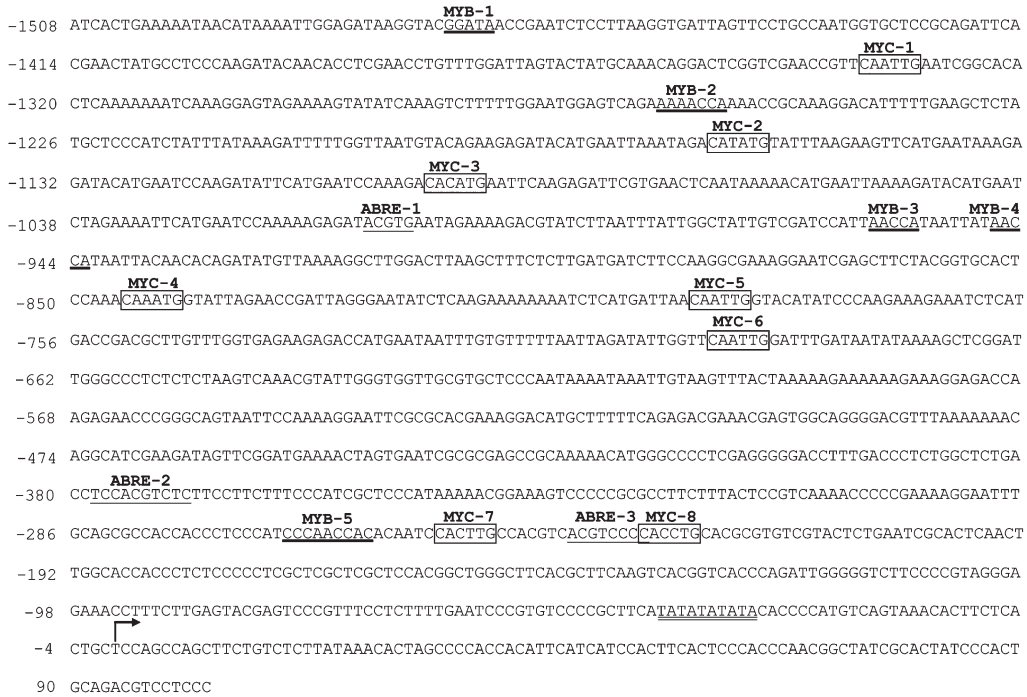


Fig. 4. Nucleotide sequence of the promoter region of the Egu*CBF*1a gene including some predicted known *cis*-elements. The sequences homologous to MYB, MYC recognition sites, and ABRE-related sequences are highlighted. A putative TATA box is double underlined. The arrow shows the initiation point of transcription.

the GFP associated with the Egu*CBF*1a and b coding sequences under the control of the CaMV 35S promoter. Plasmids bearing either the GFP gene alone, or the Egu*CBF*1a::GFP and Egu*CBF*1b::gene fusions were transiently expressed in *Eucalyptus* protoplasts. Fluorescence microscopy analysis associated with image overlay techniques demonstrated that control cells transformed with GFP alone displayed fluorescence spread throughout the

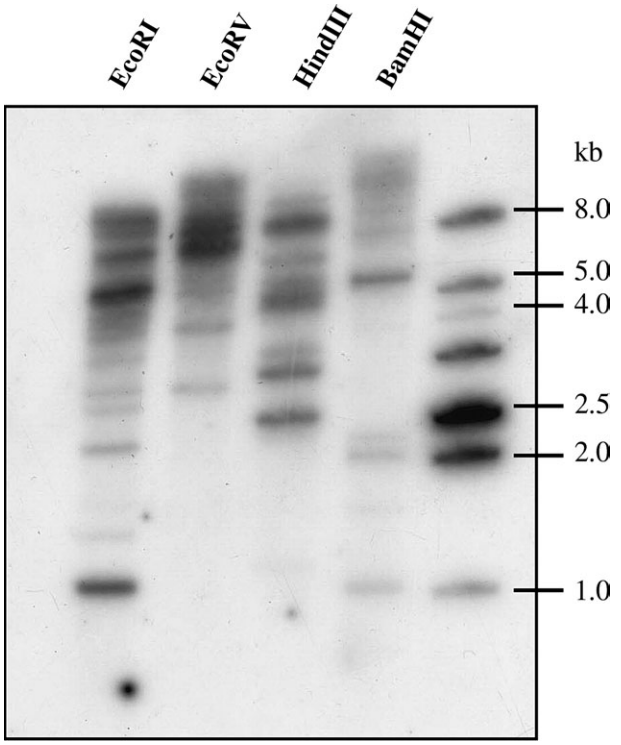


Fig. 5. Southern blot analysis of the Egu*CBF*1 gene. *Eucalyptus gunnii* genomic DNA was digested with *Eco*RI, *Eco*RV, *Hin*dIII, or *Bam*HI. After separation of the restriction fragments and blotting to a nylon filter, the hybridization was performed using the full-length Egu*CBF*1a ORF as probe. Molecular markers are shown on the right lane with the corresponding molecular weight scale.

cytoplasm, in accordance with the expected cytosolic localization of the GFP proteins (Fig. 6a). In contrast, both the Egu*CBF*1a::GFP (Fig. 6b) and Egu*CBF*1b::GFP (Fig. 6c) fusion proteins were localized exclusively in the nucleus, indicating that both Egu*CBF*1a and b were fully able to redirect the GFP from the cytosol to the nucleus.

*Comparative gene expression analysis of Egu*CBF*1a and Egu*CBF*1b in response to various environmental conditions*

For the two aligned genes, the expression was quantified by real-time RT–PCR after cold shocks to plants, or leaf disc treatments representing various abiotic conditions.

First, the cold response was evaluated on whole leaves from plants grown in controlled cold conditions. The results provided another highly significant similarity to the known *CBF*1: Egu*CBF*1a and b were quickly (15 min) and strongly (up to 500 times) induced by low temperature (Figs 7, 9, 10). After exposure at 4 °C in the dark, the transcript accumulation of Egu*CBF*1a and b reached a maximal level after 2–5 h (Fig. 7). Whereas at 24 h Egu*CBF*1a induction declined, it was still strong for Egu*CBF*1b. The two genes very clearly showed different levels of cold induction, Egu*CBF*1a mRNA accumulating up to twice as much as Egu*CBF*1b at the maximum level of induction. Interestingly, this differential pattern is the opposite for the earliest time-courses (15 and 30 min) as well as for the latest point (24 h).

Surprisingly, light did not amplify the Egu*CBF*1 response to cold. On the contrary, it exhibited a negative effect on induction (Fig. 7A, B). Although detectable at 4 °C in the light, Egu*CBF*1a and b induction was 3–9 times lower compared with dark conditions, with a similar time-course of up-regulation for both conditions. As a control, the light influence on Egu*CBF*1a and b expression in the absence of cold was also evaluated by comparison between plants cultured for 2 h at 22 °C in light or dark conditions. The slight fold change in the RNA level (2.85) observed in the dark compared with the light condition for the Egu*CBF*1b transcript, and not shown for Egu*CBF*1a, cannot be considered as significant compared with the ratios (up to 500) obtained after cold exposure. In addition, the importance of the time of day at which the plants were exposed to cold was checked, since this accumulation was reported in *Arabidopsis* to be regulated by the circadian clock (Fowler *et al*., 2005). Less than 11% variation (454/509-fold change) was detected over 6 h on Egu*CBF*1a and b induction, indicating that on whole plants under cold conditions, this parameter does not significantly interfere with the other factors under study, thus allowing any induction change above 11% to be taken into account.

The effect of the magnitude of the cold shock on gene expression was then determined on the same plant material after a 2 h exposure in dark conditions (Fig. 8). For the two genes, a decreasing positive temperature resulted in higher accumulation of Egu*CBF*1 transcripts with a dramatic increase (×25) below 8 °C and a maximum at 0 °C. At 4 °C, the same result as in the previous experiment (Fig. 7) was obtained for the two genes, showing the reproducibility of the measurements. For a freezing temperature (−4 °C), the Egu*CBF*1 induction dropped to a very low, although still significant ratio. This decrease could be partly explained by the stress symptoms which the plants exhibited after this treatment.

In a particularly striking result, when the two genes were compared, they were found to respond similarly to 8 °C, but above this temperature, Egu*CBF*1b was the more highly induced, whereas Egu*CBF*1a transcripts accumulated more below 8 °C.

In order to investigate the specificity of the Egu*CBF*1 gene response, their expression was then measured on leaf discs exposed to cold, salt, or ABA treatment. Using this simplified system which allows the rapid and controlled application of different treatments, the level of induction was found to be ~25 times lower than in the integrated system (cf. Figs 7, 8) as shown by the control exposed for 2 h at 4 °C (Fig. 9). However, the high sensitivity of the measurements meant that changes in gene expression could be reliably detected and the fold change values were considered significant when >2-fold.

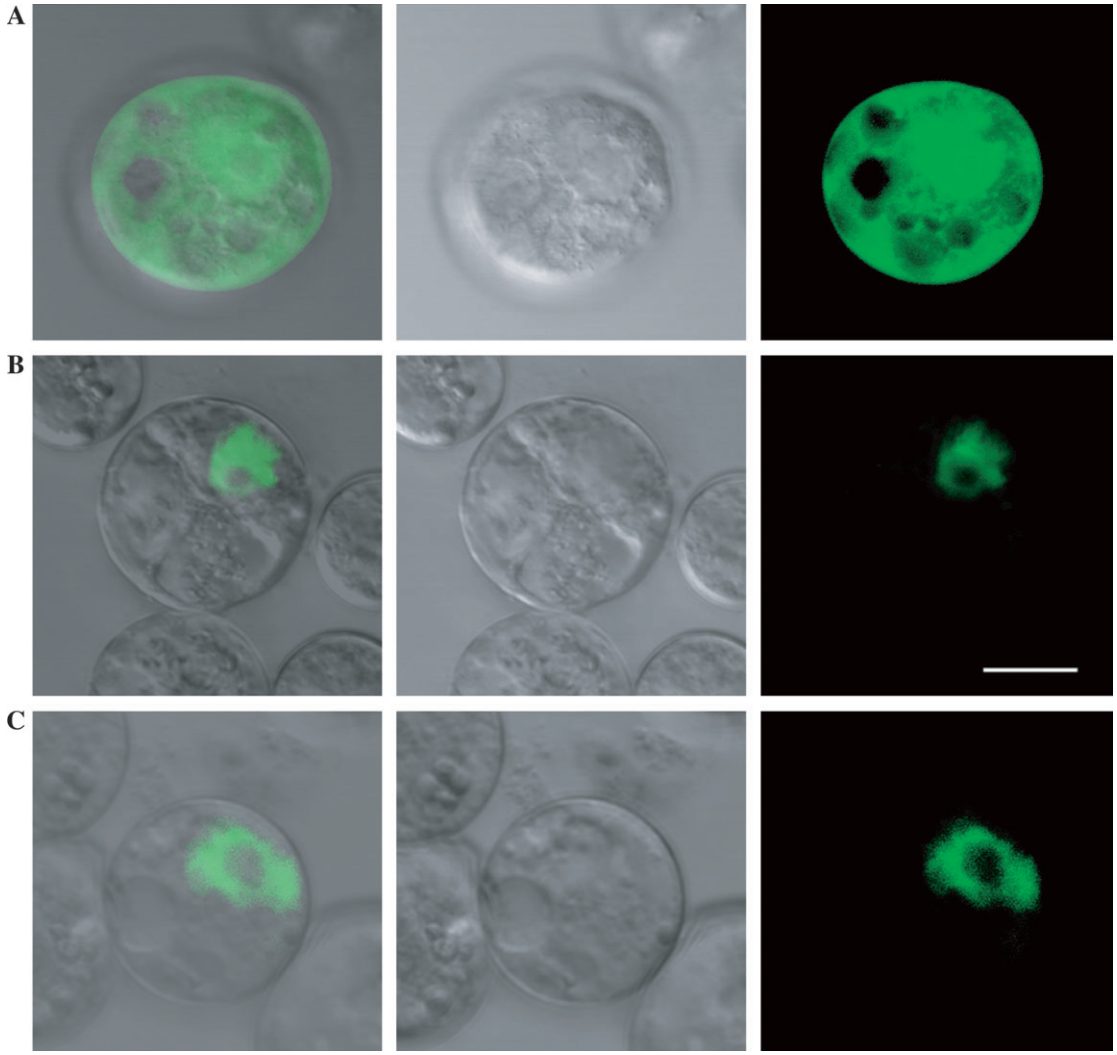


Fig. 6. Nuclear localization of Egu*CBF*1 proteins fused to the GFP tag. Constructs consisting of either the control 35S::GFP, or 35S::Egu*CBF*1a–GFP or 35S::Egu*CBF*1b–GFP were used transiently to transform *Eucalyptus* protoplasts. The subcellular localizations of the GFP protein under the control of 35S (a), the Egu*CBF*1a–GFP (b) or Egu*CBF*1a–GFP fusion protein (c) were analysed using confocal laser scanning microscopy. Light micrographs (medium panel) and fluorescence (right panel) images are merged (left panel) to illustrate the different locations of the two proteins. The length of the bar corresponds to 10 μm.

As previously observed, Egu*CBF*1a showed a stronger response after a 2 h cold exposure compared with Egu*CBF*1b. Egu*CBF*1a and b were also found to respond to salt stress but to a much more limited extent than the cold response (Fig. 9). While Egu*CBF*1b did not appear to be significantly regulated by ABA under the tested conditions, the Egu*CBF*1a transcripts accumulated after 2 h of ABA treatment. This ABA induction was delayed compared with cold or salt stress since no induction could be detected under 2 h, and the maximum induction was observed at 24 h.

Gene expression and development of freezing tolerance in two photoperiodic conditions

The evaluation of the relationship between Egu*CBF*1 expression and the increase of frost tolerance represents one of the main issues of this study to estimate the involvement of CBF1 in *Eucalyptus* cold acclimation. For this purpose, Egu*CBF*1 expression was quantified on two sets of three plants during two specific cold culture programmes resulting in a distinct cold acclimation. The programme used the same light intensity and decrease in temperature but differed in the photoperiod (SD or LD). The measurements of freezing tolerance and gene expression were performed on the leaves which are the sites of photoperiod perception.

In both photoperiod conditions, the results of freezing tolerance measured at −6 °C as well as −8 °C (Fig. 10A, B) showed a significant and gradual increase starting from the end of the first period of the cold programme (day 4=4 d at 12 °C/day and 8 °C/night). At the end of the second period (day 10=6 d at 4 °C), the increase of freezing tolerance reached 60% for the SD (Fig. 10A) and 46% for the LD programme (Fig. 10B), showing the positive influence of

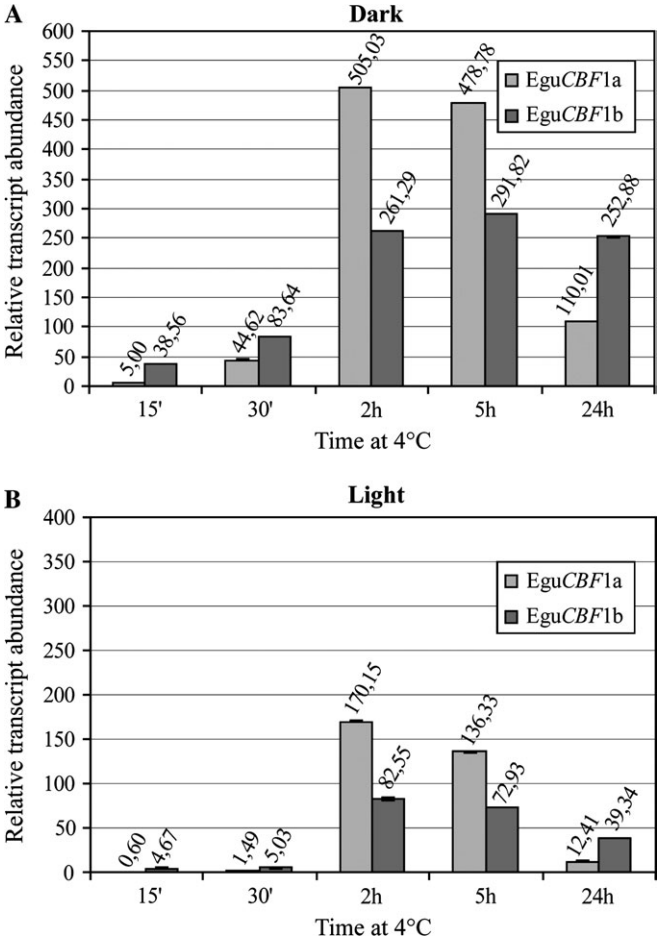


Fig. 7. Time-course of Egu*CBF*1 gene expression at 4 °C under dark (A) and light (B) culture. The relative abundance of Egu*CBF*1a and Egu*CBF*1b transcripts was quantified in comparison with the RNA 18S transcript level using real-time RT–PCR with gene-specific primers (Table 1). Total RNA was extracted from a pool of leaves harvested before cold exposure (control), after 15 min, 30 min, 2 h, 5 h, and 24 h at 4 °C in the dark or under continuous light (45 μmol m^{-2} s^{-1}). The results correspond to the mean value of three assay replicates compared with the mean of the three control values. After normalization of the RNA steady-state level using 18S as an internal control, the Egu*CBF*1 transcript level of control tissues (25 °C) was used as a calibrator to determine the fold change of transcript abundance during cold treatment.

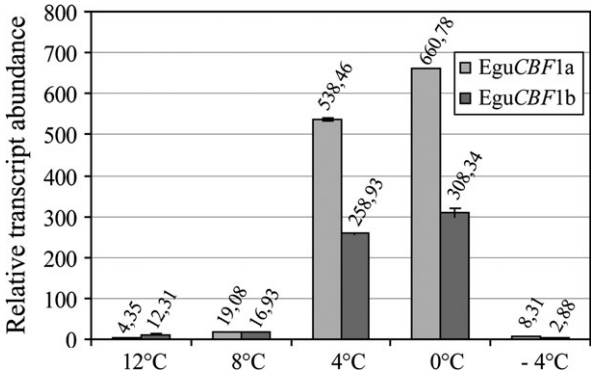


Fig. 8. Effect of cold shock intensity on the Egu*CBF*1 transcription rate. After direct transfer from standard conditions at 22 °C/night used as a control, plantlets were exposed for 2 h in the dark to each indicated temperature (12, 8, 4, 0, or − 4°C). The relative Egu*CBF*1 transcript abundances were determined as described in the legend of Fig. 7.

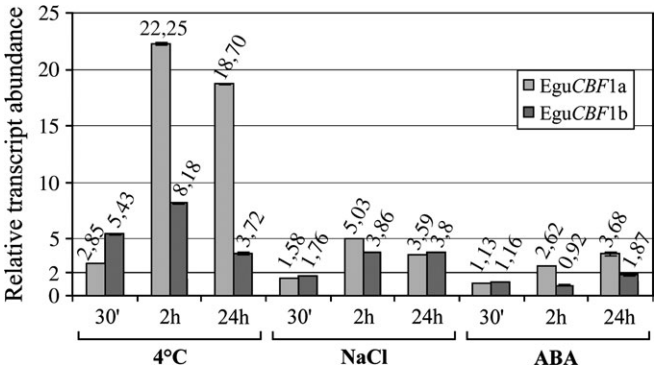
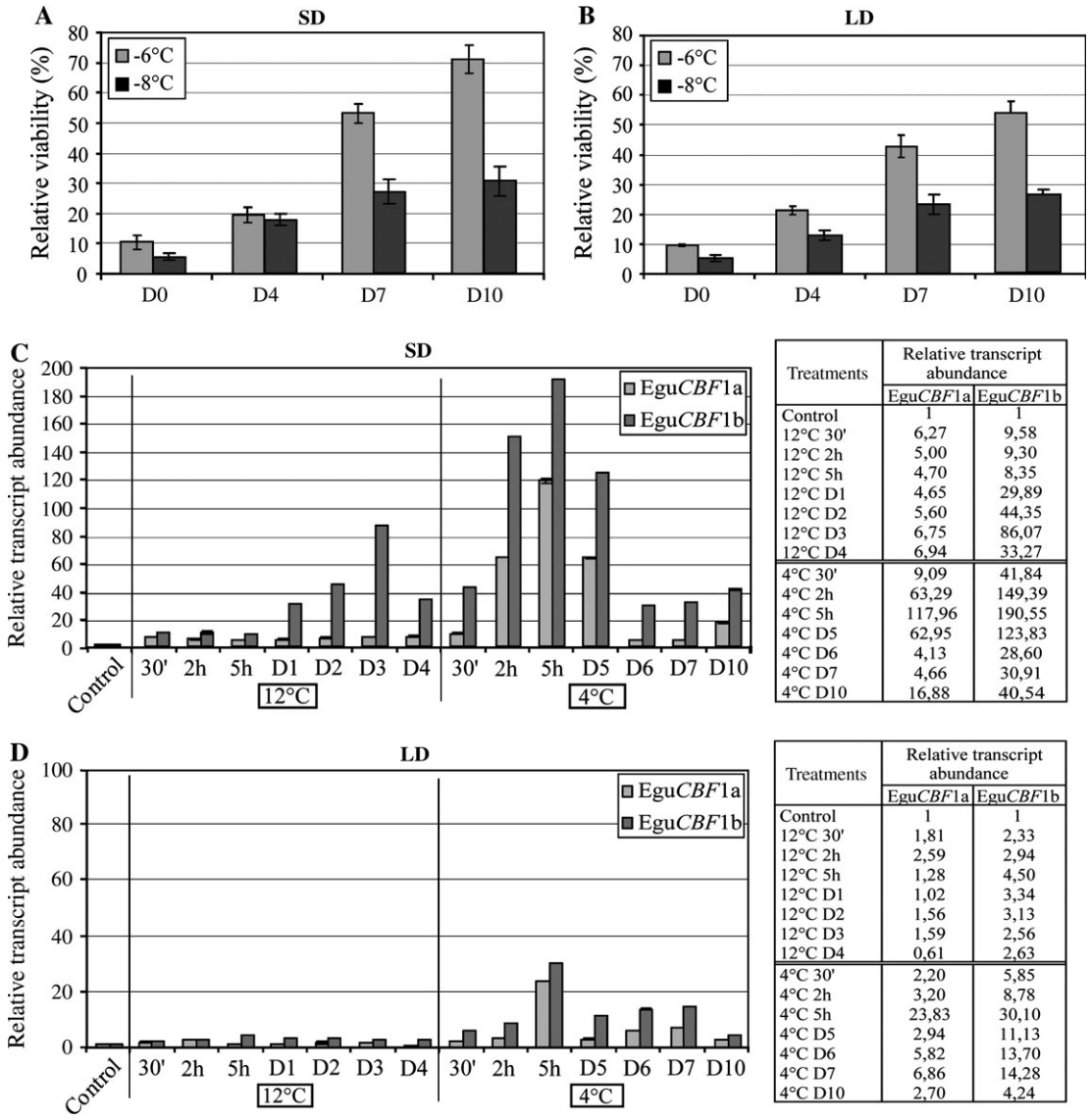


Fig. 9. Response of Egu*CBF*1a and b to different environmental stimuli. The total RNA was extracted from leaf discs of E. gundal exposed to cold (4 °C), NaCl (200 mM), or exogenous ABA (100 μM) during 30 min, 2 h, or 24 h. Leaf discs were incubated with the same time course in distilled water as a control for cold and salt treatments, or in DMSO as a control for ABA application. The Egu*CBF*1 relative transcript abundances were measured using real-time RT–PCR as described in the legend of Fig. 7. The black line at 2-fold change for relative transcript abundance indicates the minimum level considered as significant.

an SD photoperiod on the cold acclimation efficiency. The results show only a 10 d cold-culture programme because at this stage the acclimation was found to reach its maximum and no significant improvement of freezing tolerance could be observed after an additional 4 d period at 4 °C (data not shown).

The Egu*CBF*1 expression patterns observed in these two acclimation programmes (Fig. 10C, D) provide some important information.

1. Gene expression differs according to the acclimation programme: the maximum gene induction rate for the two genes was observed in SD conditions during the 4 °C period. The gene induction rate reached a 118/191-fold change for Egu*CBF*1a and b, respectively, in an SD photoperiod and 24/30 for the same genes during the same time course (5 h) in LD conditions. A significant difference was observed in all the kinetic points, in accordance with the higher magnitude of cold hardening obtained in SD conditions.
2. The intensity of the response at 4 °C during the acclimation programme is lower than the induction observed after a direct shock at the same temperature. During the initial transfer at 12 °C, the induction ratios (ranging ∼5- or 10-fold depending on the gene) are similar to those observed after a 2 h direct exposure at 12 °C (Fig. 8). In interesting contrast, after the transfer from the 12 °C phase to 4 °C, Egu*CBF*1a is 10-fold less induced and Egu*CBF*1b is 2-fold less induced than after a 2 h direct treatment at 4 °C (Fig. 8).
3. The two *CBF*1 genes respond differently to these acclimation programmes: Egu*CBF*1b is always more



Treatments	Relative transcript abundance	
	Egu*CBF*1a	Egu*CBF*1b
Control	1	1
12°C 30'	6,27	9,58
12°C 2h	5,00	9,30
12°C 5h	4,70	8,35
12°C D1	4,65	29,89
12°C D2	5,60	44,35
12°C D3	6,75	86,07
12°C D4	6,94	33,27
4°C 30'	9,09	41,84
4°C 2h	63,29	149,39
4°C 5h	117,96	190,55
4°C D5	62,95	123,83
4°C D6	4,13	28,60
4°C D7	4,66	30,91
4°C D10	16,88	40,54

Treatments	Relative transcript abundance	
	Egu*CBF*1a	Egu*CBF*1b
Control	1	1
12°C 30'	1,81	2,33
12°C 2h	2,59	2,94
12°C 5h	1,28	4,50
12°C D1	1,02	3,34
12°C D2	1,56	3,13
12°C D3	1,59	2,56
12°C D4	0,61	2,63
4°C 30'	2,20	5,85
4°C 2h	3,20	8,78
4°C 5h	23,83	30,10
4°C D5	2,94	11,13
4°C D6	5,82	13,70
4°C D7	6,86	14,28
4°C D10	2,70	4,24

Fig. 10. Cold culture and Egu*CBF*1 transcript accumulation during the two acclimation programmes of plantlets in short (SD) or long (LD) daylength. Three plantlets cultivated for 3 d at the appropriate photoperiod at 25 °C day/22 °C night were used as control and then transferred under chilling conditions for 4 d at 12 °C day/8 °C night (D4), then 6 d at 4 °C (D5 to D10). The freezing tolerance was evaluated on D4, D7, and D10, by ion leakage measurements on leaf discs randomly harvested and frozen to −6 or −8 °C. The results for the SD (A) or LD (B) photoperiod were expressed as the percentage of cell viability after freezing at a rate of −2 °C h^{-1} compared with the viability of unfrozen leaf discs, and are given as the mean of the three plantlet measurements. On the same plants, total RNA was extracted from *Eucalyptus* leaves randomly harvested at different times during the cold culture programme: 30 min, 2 h, and 5 h after transfer to 12 °C (D1) and to 4 °C (D5), and at the end of each day (except D8 and D9). The transcript accumulation of Egu*CBF*1a and Egu*CBF*1b during the SD (C) or LD (D) acclimation programme of plantlets was quantified using real-time RT–PCR as described in the legend to Fig. 7. The fold changes in the transcript level of both genes are shown in the right panels of the (C) and (D).

significantly induced than Egu*CBF*1a (often around twice as much) and is the only one to exhibit a significant response at 12 °C (Fig. 10D). It is important to note that the differential response of the two Egu*CBF*1 genes is mostly inverse for the two conditions of cold treatment since induction is usually higher for Egu*CBF*1a after a shock while Egu*CBF*1b is the more up-regulated during the acclimation. However, the differential behaviour observed during the acclimation is in agreement with the responses previously observed in cold shock at 4 °C (15 min, 30 min, and 24 h, Fig. 7) or 12 °C (2 h, Fig. 8). In total, the expression patterns of the two genes show some complementarity: Egu*CBF*1a exhibits an efficient short-term response to abrupt and drastic cold conditions whereas Egu*CBF*1b shows a better and longer response to more moderate or progressive temperature changes.

4. The time-course of gene induction during the 10 d experiment is very unexpected. Except for the predictable

main peaks corresponding to 5 h at 4 °C, induction under the best conditions (SD) is significant (a >2-fold rate) for all the kinetic points measured, including those measured several days after a temperature change (days 6, 7, and 10). In order to check whether the Egu*CBF*1 transcripts are unusually stable or produced anew as a reaction to repeated cues, the transcription rate was more precisely quantified for the most induced gene (Egu*CBF*1b). The time-course was studied during an SD acclimation programme and in particular 15 min before and 2 h after the thermoperiod transition (12 °C day/8 °C night). This detailed kinetics (Fig. 11) confirmed the likely occurrence of subsequent gene inductions at least every day as accumulation of transcripts is always higher after the phase transition. This temperature change (12/8 °C) combined with switching off the light could represent a daily trigger to induce Egu*CBF*1. This result agrees with the slight negative influence on gene regulation by cold already observed for the photoperiod effect and for the 4 °C cold shock in the dark or in continuous light. However, on the 4th day of the acclimation programme, while the thermoperiod transition still had a positive influence, the induction level was significantly lower than on the previous days, suggesting a reduction in the impact of this daily trigger.

Overall, the transcriptional response of sensitive genes such as Egu*CBF*1s seems highly dependent both on the conditions of cold occurrence and on the associated photoperiod. Therefore, if the short exposure to 4 °C seems to be an efficient system for evaluating the different parameters of regulation quickly and qualitatively, the culture leading to acclimation that mimics natural conditions is more representative of the response in the field.

Discussion

This study presents a quantitative transcriptional analysis of two *CBF*-like genes in a woody plant in response to different environmental conditions. These genes exhibit all the major structural characteristics of a *CBF* transcription activator gene, including the absence of an intron also reported for *Arabidopsis* (Medina *et al*., 1999) or tomato (Zhang *et al*., 2004). In agreement with the predicted identity as a transcriptional regulator, the nuclear location of the protein was proved by the *in vivo* targeting experiment. Based on these characteristics and the highest level of homology to *CBF*1, the two genes were designated Egu*CBF*1a and Egu*CBF*1b. These new genes are paralogues because they are clearly different; the polymorphism between the two sequences is even higher than between the well-known *Arabidopsis* members *CBF*1–*CBF*3.

Several promoter *cis*-acting elements common to the known *CBF* genes were predicted in Egu*CBF*1a, in particular the MYC recognition sequences that would be involved in CBF transcriptional activation by cold (Chinnusamy *et al*., 2003). This cold regulation was evidenced by the expression analysis showing a strong and fast response to cold, typical of a *CBF* gene. Induction of Egu*CBF*1a by cold is ~10 times higher than the accumulation of transcripts measured by the same method for At*CBF*1–2 in similar cold conditions (Rohde *et al*., 2004). According to the hypothesis of Chinnusamy *et al.* (2003), this higher induction level could be related to the number of MYC elements, eight for Egu*CBF*1a and only one for At*CBF*1–2 (Shinwari *et al*., 1998). Moreover, the five MYB transcription factor recognition sites predicted in Egu*CBF*1a, as well as At*CBF*1–2 (four or two sites, respectively), could be involved in the CBF activation by cold, as suggested by the same authors (Chinnusamy *et al*., 2003).

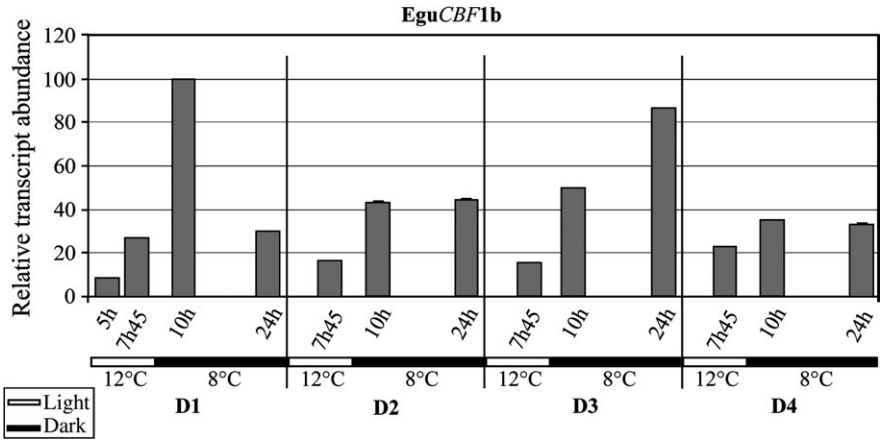


Fig. 11. Effect of the daily thermoperiod transition on the expression pattern of Egu*CBF*1b during the first step (12 °C day/8 °C night) of the short daylength (SD) cold acclimation programme. RNA was extracted from a pool of leaves randomly harvested at the following times: 5 h after the transfer to 12 °C, 15 min (7 h 45) before and 2 h (10 h) after the thermoperiod transition, and finally at the end of each day (24 h). The fold changes in transcript levels were quantified as previously described

Overall, since CBF transcript quantifications have rarely been reported, it is difficult to compare the Egu*CBF*1 induction levels with data from the literature. The comparative transcriptional analysis of two CBF members in multiple conditions as described in this report was only made possible by the use of the powerful real-time RT–PCR technique. Benefiting from the quantitative and highly sensitive measurements of the transcriptional regulation and the possible multiplication of assays, the presented data have proved highly significant and reproducible. They underline the importance of applying such a method to further studies on a weakly expressed transcriptional activator gene such as *CBF*.

In addition to these MYC and MYB recognition sites, three ABRE *cis*-elements were predicted in the Egu*CBF*1a promoter in accordance with a detected response to ABA treatment. In *Arabidopsis*, a potential ABRE *cis*-acting element was predicted in At*CBF*1–3 promoters (Busk and Pages, 1998; Medina *et al*., 1999), but the situation still remains unclear concerning the participation of this hormone in CBF regulation (Medina *et al*., 1999; Knight *et al*., 2004). Surprisingly, in addition to cold, the Egu*CBF*1 response to salt was significant even though it was impossible to compare the two regulations quantitatively in the absence of any optimization of the experimental conditions for NaCl. This response is rather uncommon since At*CBF*1–3 as well as Le*CBF*1 are usually described to have cold-specific responses (Medina *et al*., 1999; Zhang *et al*., 2004). However, during a global survey through microarray analysis of the transcriptome changes in response to different environmental cues, Kreps *et al*. (2002) observed a strong induction by cold and a slight but significant up-regulation by salt or mannitol for At*CBF*1. In addition, Choi *et al.* (2002), by using real-time RT–PCR, were able to reveal a small transient induction of Hv*CBF*3 after ABA treatment, although nothing was detected by standard reverse transcription analysis (semi-quantitative), which confirms that these data regarding response specificity may vary according to the method of measurement. It is therefore difficult to draw any conclusions about potential differences or similarities for the specificity of response to environmental stimuli in the comparison of *CBF* genes from *Eucalyptus* and other species.

However, clearer differential features were observed regarding the effect of light on CBF regulation by cold. In contrast to the data on At*CBF* (Kim *et al*., 2002), darkness during cold shock, as well as the application of an SD photoperiod during the acclimation programme, has a strong positive effect on Egu*CBF*1 expression. In neither case did light act as an independent and adequate signal. Instead, it acted by quantitatively modulating the Egu*CBF*1 gene response, which is primarily regulated by low temperature. This predominance of temperature over light has already been reported for growth and a number of other physiological responses in *Eucalyptus* when 12 different species, grown under different combinations of temperature and photoperiod, showed greater response to temperature than to photoperiod (Paton, 1978). The data presented on the photoperiodic response of *Eucalyptus* are also fully in agreement with the literature for adaptive traits associated with overwintering on many perennial woody plants (Thomas and Vince-Prue, 1997; Welling *et al*., 2002).

The involvement of light in fine-tuning plant molecular responses to cold has often been described and, as in *Eucalyptus*, the positive effect of dark or an SD photoperiod on the regulation of cold-responsive genes has been reported for other plant species including barley (Grossi *et al*., 1998; Fowler *et al*., 2001) and poplar (Zhu and Coleman, 2001). The very fast effect of darkness on the Egu*CBF*1 cold response (already significant after 15 min) as well as the influence of photoperiod on gene expression strongly suggests that a photoreceptor is involved rather than the photosynthetic electron chain observed in this regulation. In *Arabidopsis*, phytochrome B appears to act primarily as a light signalling mediator for CBF regulation (Kim *et al*., 2002) while in *Populus*, phytochrome A appears to be the main intermediary for dehydrin gene regulation (Zhu and Coleman, 2001).

The contrasting response to light between *Arabidopsis* and *Eucalyptus* suggests that although they exhibit many similarities with At*CBF* genes, the Egu*CBF* genes differ significantly both structurally and in their regulation. These differences may be related to the very characteristic biology and physiology of this persistent woody plant compared with an annual species such as *Arabidopsis*, which survives winters through seed storage. These first data on the expressional analysis of *CBF* genes from a forestry species highlight the utility of in-depth study of this predominant cold response pathway. Such a study needs to be carried out on a complex perennial model like the present one in order to reveal the molecular mechanisms underlying many of its unique developmental features.

One of the most useful results of this study is the apparent relationship between the development of freezing tolerance and the induction level of the two Egu*CBF*1 genes highlighted by the comparison of the two acclimation programmes. The involvement of *CBF* genes in cold hardening is now well documented, most commonly through the genetic modulation of gene expression on model species. The involvement of a *CBF* gene from a woody plant (sweet cherry) in freezing tolerance was demonstrated for the first time by Kitashiba *et al.* (2004) by Pa*CBF* expression in *Arabidopsis*. However, such heterologous approaches remain limited and beg the question of the efficacy of the CBF pathway in the species under study. For example, due in particular to a limited regulon or reduced induction by cold, the Le*CBF* gene did not work for tomato cold acclimation whereas it was found to enhance freezing tolerance when transferred to *Arabidopsis* (Zhang *et al*., 2004). In a broader range of plant species, the

comparison of cold-tolerant or cold-sensitive genotypes is now an emerging approach to investigate similarities and differences in the CBF cold response pathway. The definition of the two acclimation experiments on *Eucalyptus* and the use of a very efficient and sensitive transcript level measurement provided a first approach to this gene regulation in a woody plant associated with the level of frost tolerance. The extremely strong and rapid Egu*CBF*1 induction by cold, in agreement with the tolerance level in leaves, suggests the involvement of the two genes in *Eucalyptus* cold hardening. This must be confirmed by genetic modulation in an available homologous system (Tournier *et al*., 2003; Valério *et al*., 2003).

Among the main data from this quantitative expressional analysis, the two Egu*CBF*1 genes were found to be differentially induced depending on the speed and intensity of the temperature drop. Such a distinct regulation was also established for the At*CBF* members, for example when At*CBF*2 induction was found to be delayed compared with At*CBF* and At*CBF*3 (Novillo *et al*., 2004). When the three At*CBF* genes were found to show redundant functional activity in At*CBF*1–3-overexpressing plants, Gilmour *et al.* (2004) raised the question of whether they are functionally equivalent *in planta*. As a first step towards an answer, the presented data on *Eucalyptus* strongly suggest that the two studied Egu*CBF* members may be regulated in the field in a complementary manner.

The present study also provides a range of information about the conditions of cold treatments affecting the accumulation of transcripts. In agreement with the findings on *Arabidopsis* (Zarka *et al*., 2003), the intensity of the cold obviously proved to be critical since, during a direct cold exposure in a positive temperature range, lower temperature results in higher induction. However, the Egu*CBF*1 transcript accumulation level was not as proportional to the temperature decrease as observed for At*CBF* and, above all, a negative temperature only results in a low induction in *Eucalyptus*, whereas it is the best stimulus for *Arabidopsis*. These data are consistent with the evidence about lower temperature applied for acclimating *Arabidopsis* (4 °C), which was too stressful to harden *Eucalyptus* directly. Together they indicate the best compromise between an efficient stimulus and moderate stress ranges at higher temperatures for this woody plant. More importantly, the rapidity or progression of cold exposure proved to be relevant to the Egu*CBF*1 response since, for the same temperature (4 °C), the induction level during the two-step acclimation programme was lower than after a direct shock. This observation suggests that the cold response of Egu*CBF*1 is not only limited to a direct monitoring of absolute temperature but also takes the temperature difference into account. It could also reflect the desensitization phenomenon described by Zarka *et al*. (2003) for At*CBF* who observed a loss of cold response after 14 d at 4 °C. This apparent loss of response, also shown by the study of the detailed kinetics at 12 °C, was already detected in our hands for cold acclimation (unpublished data). Therefore, like *Arabidopsis*, *Eucalyptus* cells probably keep a memory of chilling exposure by adjusting the CBF response in particular. The dynamism of this Egu*CBF*1 response over numerous temperature and light changes which occur permanently in natural conditions was also shown by the study of Egu*CBF*1a and b expression over 10 d. As expected, subsequent decreases of temperature were found to lead to corresponding increases in transcript level. However, considering the CBF RNA half-life, 7.5 min for *Arabidopsis* (Zarka *et al*., 2003), the basic induction level remained surprisingly high for several days, even at 4 °C in the absence of any new temperature stimulus. In addition, new CBF induction peaks were observed every day at each photoperiod/thermoperiod transition during the 12 °C culture step. Such a long-term regulation of a *CBF*1 gene was only recently suggested for the first time by Hannah *et al.* (2005) in an overview of gene regulation during cold acclimation in *Arabidopsis*. The authors mentioned that although At*CBF*1 and At*CBF*3 were supposed to be only transiently expressed because they were tightly negatively controlled by At*CBF*2 (Novillo *et al*., 2004), the data indicated the likelihood that they were up-regulated in the long term (>48 h). In addition, the same authors found that 12 other short-term responsive transcription factor genes continue to be induced during a long-term cold acclimation. The present work provides a possible explanation for this apparently 'permanent' long-term induction with the occurrence of repeated inductions in response to environmental changes (temperature, light, etc.). Altogether, the results underline how complex and dynamic this gene regulation is. It is affected not only by various environmental factors but also by the history of the cells, which have an apparent memory for cold.

An original feature of this study is the monitoring of *CBF* expression in controlled conditions closely corresponding to the natural environment (day and night temperatures, light intensity, and photoperiod) and compatible with cold acclimation and long-term plant survival. The differences observed in the CBF response between the 'shock experiments' and the 'acclimation experiments' confirmed that this approach is crucial for assessing the real involvement of *CBF* in cold tolerance and its regulation during autumn and winter. These interesting new data on the transcription factor gene suggest that, in the field, it is permanently subjected to positive and negative regulation and can be quickly induced in response to each temperature decrease. However, due to the memory effect, this response would show a higher amplitude in non-acclimated conditions than in an acclimated plant state (late autumn and winter).

In the near future, the isolation of at least three other members of the Egu*CBF* family, coupled with a quantitative analysis of their transcriptional pattern both in controlled

conditions and in the field, should provide an overview of the CBF regulation during overwintering of this perennial woody plant.

Acknowledgements

We would like to thank AFOCEL for providing the Eucalyptus cuttings. Thanks to Helene San Clemente for bioinformatic analyses and Yves Martinez for confocal microscopy analysis. This work was supported by the 'Midi Pyrénées French Council', TEMBEC SA R&D KRAFT (St GAUDENS, France), and European funds for development (FEDER).

References

Busk PK, Pages M. 1998. Regulation of abscisic acid-induced transcription. *Plant Molecular Biology* **37,** 425–435.

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes and Development* **17,** 1043–1054.

Choi D-W, Rodriguez EM, Close TJ. 2002. Barley Cbf3 gene identification, expression pattern, and map location. *Plant Physiology* **129,** 1781–1787.

Cook D, Fowler S, Fiehn O, Thomashow MF. 2004. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **101,** 15243–15248.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2003. OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *The Plant Journal* **33,** 751–763.

Fowler DB, Breton G, Limin AE, Mahfoozi S, Sarhan F. 2001. Photoperiod and temperature interactions regulate low-temperature-induced gene expression in barley. *Plant Physiology* **127,** 1676–1681.

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

Fowler S, Thomashow MF. 2002. *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *The Plant Cell* **14,** 1675–1690.

Gilmour SJ, Fowler SG, Thomashow MF. 2004. Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Molecular Biology* **54,** 767–781.

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. *The Plant Journal* **16,** 433–442.

Grossi M, Giorni E, Rizza F, Cattivelli L. 1998. Wild and cultivated barleys show differences in the expression pattern of a cold-regulated gene family under different light and temperature conditions. *Plant Molecular Biology* **38,** 1061–1069.

Hannah MA, Heyer AG, Hincha DK. 2005. A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana. The Public Library of Science Genetics* **1,** e26.

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM. 2000. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42,** 819–832.

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

Hong J-P, Kim WT. 2005. Isolation and functional characterization of the *Ca-DREBLP1* gene encoding a dehydration-responsive element binding-factor-like protein 1 in hot pepper (*Capsicum annuum* L. cv. Pukang). *Planta* **220,** 875–888.

Hsieh TH, Lee JT, Charng YY, Chan MT. 2002. Tomato plants ectopically expressing *Arabidopsis* CBF1 show enhanced resistance to water deficit stress. *Plant Physiology* **130,** 618–626.

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF. 2001. Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiology* **127,** 910–917.

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. 1998. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280,** 104–106.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnology* **17,** 287–291.

Kim H-J, Kim Y-K, Park J-Y, Kim J. 2002. Light signalling mediated by phytochrome plays an important role in cold-induced gene expression through the C-repeat/dehydration responsive element (C/DRE) in *Arabidopsis thaliana. The Plant Journal* **29,** 693–704.

Kim S, An CS, Hong YN, Lee KW. 2004. Cold-inducible transcription factor, CaCBF, is associated with a homeodomain leucine zipper protein in hot pepper (*Capsicum annuum* L.). *Molecules and Cells* **18,** 300–308.

Kitashiba H, Ishizaka T, Isuzugawa K, Nishimura K, Suzuki T. 2004. Expression of a sweet cherry DREB1/CBF ortholog in Arabidopsis confers salt and freezing tolerance. *Journal of Plant Physiology* **161,** 1171–1176.

Knight H, Zarka DG, Okamoto H, Thomashow MF, Knight MR. 2004. Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiology* **135,** 1710–1717.

Kreps JA, Wu Y, Chang H-S, Zhu T, Wang X, Harper JF. 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. *Plant Physiology* **130,** 2129–2141.

Leborgne N, Teulières C, Travert S, Rols MP, Teissie J, Boudet AM. 1995. Introduction of specific carbohydrates into *Eucalyptus gunnii* cells increases their freezing tolerance. *European Journal of Biochemistry* **229,** 710–717.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25,** 402–408.

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K. 2004. Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. *The Plant Journal* **38,** 982–993.

Medina J, Bargues M, Terol J, Pérez-Alonso M, Salinas J. 1999. The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiology* **119,** 463–469.

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. Proceedings of the National Academy of Sciences, USA* **101,** 3985–3990.

Paton D. 1978. Eucalyptus physiology. I. Photoperiodic responses. *Australian Journal of Botany* **26,** 633–642.

Prestridge DS. 1991. SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. *Computer Applications in the Biosciences* **7,** 203–206.

Riechmann JL, Meyerowitz EM. 1998. The AP2/EREBP family of plant transcription factors. *Journal of Biological Chemistry* **379,** 633–646.

Rohde P, Hincha DK, Heyer AG. 2004. Heterosis in the freezing tolerance of crosses between two *Arabidopsis thaliana* accessions (Columbia-0 and C24) that show differences in non-acclimated and acclimated freezing tolerance. *The Plant Journal* **38,** 790–799.

Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Huner NP, Shinozaki K, Singh J. 2005. The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus. Plant and Cell Physiology* **46,** 1525–1539.

Seki M, Kamei A, Satou M, Sakurai T, Fujita M, Oono Y, Yamaguschi-Shinozaki K, Shinozaki K. 2003. Transcriptome analysis in abiotic stress conditions in higher plants. In: Hirt H, Shinozaki K, eds. *Topics in current genetics*, Vol. 4. Berlin/ Heidelberg: Springer, 271–295.

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K. 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *The Plant Cell* **13,** 61–72.

Shinozaki K, Yamaguchi-Shinozaki K, Seki M. 2003. Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology* **6,** 410–417.

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. *Biochemical and Biophysical Research Communications* **250,** 161–170.

Stockinger EJ, Gilmour SJ, Thomashow MF. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Sciences, USA* **94,** 1035–1040.

Teulières C, Feuillet C, Boudet AM. 1989. Differential characteristics of cell suspension cultures initiated from *Eucalyptus gunnii* clones differing by their frost tolerance. *Plant Cell Reports* **8,** 407–410.

Thomas B, Vince-Prue D. 1997. *Photoperiodism in plants.* London: Academic Press.

Thomashow MF. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 571–599.

Thomashow MF. 2001. So what's new in the field of plant cold acclimation? Lots! *Plant Physiology* **125,** 89–93.

Tournier V, Grat S, Marque C, El Kayal W, Penchel R, de Andrade G, Boudet A-M, Teulières C. 2003. An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis*×*Eucalyptus urophylla*). *Transgenic Research* **12,** 403–411.

Vagujfalvi A, Aprile A, Miller A, Dubcovsky J, Delugu G, Galiba G, Cattivelli L. 2005. The expression of several Cbf genes at the Fr-A2 locus is linked to frost resistance in wheat. *Molecular Genetics and Genomics* **274,** 506–514.

Valério L, Carter D, Rodrigues JC, Tournier V, Gominho J, Marque C, Boudet A-M, Maunders M, Pereira H, Teulières C. 2003. Down-regulation of cinnamyl alcohol dehydrogenase, a lignification enzyme, in *Eucalyptus camaldulensis. Molecular Breeding* **12,** 157–167.

Welling A, Moritz T, Palva ET, Junttila O. 2002. Independent activation of cold acclimation by low temperature and short photoperiod in hybrid aspen. *Plant Physiology* **129,** 1633–1641.

Welling A, Rinne P, Vihera-Aarnio A, Kontunen-Soppela S, Heino P, Palva ET. 2004. Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.). *Journal of Experimental Botany* **55,** 507–516.

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. *The Plant Cell* **6,** 251–264.

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

Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, Thomashow MF. 2004. Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant Arabidopsis. *The Plant Journal* **39,** 905–919.

Zhu B, Coleman GD. 2001. Phytochrome-mediated photoperiod perception, shoot growth, glutamine, calcium, and protein phosphorylation influence the activity of the poplar bark storage protein gene promoter (bspA). *Plant Physiology* **126,** 342–351.

Zhu JK. 2002. Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* **53,** 247–273.