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ORIGINAL PAPER

Comparative expression of *Cbf* genes in the *Triticeae* under different acclimation induction temperatures

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Abstract In plants, the C-repeat binding factors (*Cbf*s) are believed to regulate low-temperature (LT) tolerance. However, most functional studies of *Cbf*s have focused on characterizing expression after an LT shock and have not quantified differences associated with variable temperature induction or the rate of response to LT treatment. In the *Triticeae*, rye (*Secale cereale* L.) is one of the most LT-tolerant species, and is an excellent model to study and compare *Cbf* LT induction and expression profiles. Here, we report the isolation of rye *Cbf* genes (*ScCbf*s) and compare their expression levels in spring- and winter-habit rye cultivars and their orthologs in two winter-habit wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) cultivars. Eleven *ScCbf*s were isolated spanning all four major phylogenetic groups. Nine of the *ScCbf*s mapped to 5RL and one to chromosome 2R. *Cbf* expression levels were variable, with stronger expression in winter- versus spring-habit rye cultivars but no clear relationship with cultivar differences in LT, down-stream cold-regulated gene expression and *Cbf* expression were detected. Some *Cbf*s were expressed only at warmer acclimation temperatures in all three species and their expression was repressed at the end of an 8-h dark period at warmer temperatures, which may reflect a temperature-dependent, light-regulated diurnal response. Our work indicates that *Cbf* expression is regulated by complex genotype by time by induction–temperature interactions, emphasizing that sample timing, induction–temperature and light-related factors must receive greater consideration in future studies involving functional characterization of LT-induced genes in cereals.

Keywords *Secale cereale* · Rye · Cold acclimation · *Cbf* · *Triticeae* · Induction temperature

Abbreviations

PAR	Photosynthetically active radiation
LT	Low temperature
*Cbf*s	C-repeat/dehydration-responsive binding factors
Cor	(Cold regulated) genes
ICE1	Inducer of *Cbf* expression 1

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the following accession numbers: EU194240 (*ScCbfIa-11*), EU194241 (*ScCbfII-5*), EU194242 (*ScCbfIIIa-6*), EU194243 (*ScCbfIIIc-10*), EU194244 (*ScCbfIIIc-3A*), EU194245 (*ScCbfIIIc-3B*), EU194246 (*ScCbfIIId-12*), EU194247 (*ScCbfIIId-15*), EU194248 (*ScCbfIIId-19*), EU194249 (*ScCbfIVa-2A*), EU194250 (*ScCbfIVa-2B*), EU525891 (*ScVrn-1*), EU525892 (*ScActin*).

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VRN	Vernalization
Fr	Frost resistance
LT_{50} value	Lethal temperature for 50% of the plants
CDS	Complete coding sequence

Introduction

In plants, low-temperature (LT) tolerance is induced upon exposure to low, non-freezing temperatures. This phenomenon, known as cold acclimation, is a cumulative process that is activated once temperatures fall below a critical threshold temperature (Fowler 2008). Once acclimation starts, the differences in genetic potentials are quickly magnified such that genotypes with warmer acclimation induction temperatures respond more rapidly to LT treatment. The cold acclimation process is complex, but involves the induction of cold-regulated (*Cor*) genes that code for the cryoprotective proteins necessary for protection against LT stress.

It has been established that an important component of cold acclimation is the C-repeat binding factor (*Cbf*)-induced cold response pathway (Thomashow 2001). The *Cbf*s are transcriptional activators that belong to the AP2/EREBP transcription factor family and are known to induce *Cor* transcription by binding to a C-repeat/dehydration-responsive element (CRT/DRE) in the regulatory regions of those genes (Yamaguchi-Shinozaki and Shinozaki 1994). All reported *Cbf*s lack introns and possess an AP2 DNA-binding domain which is required for *Cor* gene activation (Jaglo et al. 2001). In all *Cbf*s, the AP2 DNA-binding domain is flanked by two signature motifs (CMIII-3-PKK/RPAGRxKFxETRHP and CMIII-1-DSAWR). *Cbf* expression is induced by one or more abiotic stresses (cold, drought and salt) (Xue 2003; Skinner et al. 2005), and in the case of cold, their induction may be regulated in part by an inducer of *Cbf* expression (*ICE*) gene (Guo et al. 2002; Chinnusamy et al. 2003).

Large *Cbf* families are present in cereals and are subdivided into four phylogenetic groups, each with two or more sub-groups (Badawi et al. 2007; Skinner et al. 2005; Miller et al. 2006). In the *Triticeae*, which includes economically important cereals such as wheat (*Triticum* spp.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.), several *Cbf*s have been characterized, including 20 from barley (Skinner et al. 2005), 13 from *T. monococcum* (Miller et al. 2006) and 37 from common wheat (*T. aestivum* L.) (Badawi et al. 2007). The *Cbf* subgroups IVb and IVc appear to be unique within the *Triticeae* tribe (Badawi et al. 2007). Current information indicates that the *Cbf* genes cluster on the homeologous group 5 chromosomes of the *Triticeae*, and coincide with quantitative trait loci (QTL) for LT tolerance (Båga et al. 2007; Francia et al. 2004; Tondelli et al. 2006, Miller et al. 2006; Vágújfalvi et al. 2003). However, it is still not clear which *Cbf* gene(s) most influence LT tolerance or whether they have an additive effect. Recently, Knox et al. (2008) identified a deletion in the AP2 domain of *T. monococcum Cbf12* that prevented the coded protein from binding to the CRT/DRE motif of *Cor* genes, and this molecular variation was linked to phenotypic differences in frost survival.

The *Triticeae* form a homogeneous genetic system and comparative genetic studies suggest that the key components of LT tolerance are conserved among members. However, there is large variation in the ability to survive freezing temperatures, with winter-habit rye cultivars having greater LT tolerance compared to winter common wheat and barley cultivars (Fowler and Carles 1979). Although the genetic factors conferring the superior LT tolerance of rye are not known, acclimation is induced at a higher temperature (Fowler 2008) and the rate of response to cold is more rapid than in wheat and barley (Fowler et al. 1996). This suggests that rye has a superior cold responsive pathway that could be due in part to more rapid induction of one or more *Cbf* genes at higher acclimation temperatures. However, few rye *Cbf*s have been sequenced and characterized (Jaglo et al. 2001). In addition most functional studies of *Cbf*s have characterized *Cbf* expression after a single LT treatment at a constant acclimation temperature and differences associated with variable temperature induction or the rate of response to LT treatment have not been quantified. To better understand the role of this gene family in determining LT tolerance in the *Triticeae*, we initiated a study to identify and characterize *Cbf* genes from the cold hardy rye cultivar “Puma” and to compare *Cbf* gene expression in rye, wheat and barley cultivars in response to differing acclimation times and induction temperatures.

Materials and methods

Plant material, growth conditions and low-temperature tolerance measurements

For these studies, two winter-habit rye cultivars “Puma” and “Sangaste”, “Gazelle” spring rye, “Norstar” winter-habit wheat and “Kold” winter-habit barley were used. For gene expression studies, plants were grown hydroponically. Imbibed seeds were held in the dark for 2 days at 4°C and then incubated at 25°C for 1 day. Actively germinating seeds were transferred, embryos down, into white light-blocking plastic trays with holes backed by a 1.6-mm mesh screen. Trays were incubated at 25°C for 2 days to allow for further root growth. The seedlings were then placed in hydroponics tanks filled with continuously aerated one-half strength modified Hoagland’s solution in a controlled environment chamber at 20°C with a 16 h light and a photosynthetically active radiation (PAR) of 250 µmol m^{-2} s^{-1}. Once seedlings had developed two to three fully expanded leaves and visible crowns, trays were transferred to LT-acclimation chambers set at 6, 10, 15 and 18°C (as measured at the crown level) for 2, 4, 8 and 48 h and 16 h light at a PAR of 230 µmol m^{-2} s^{-1}. The experiment was replicated three times in time and space.

The procedure outlined by Fowler (2008) was used to determine the LT_{50} of each cultivar acclimated at 6°C for 0, 2 and 49 days. Threshold induction temperatures, defined as the warmest temperature at which plants achieved an LT_{50} of −3°C after 2 days acclimation, were also determined for each cultivar using the method outlined by Fowler (2008). Briefly, plants were grown hydroponically as outlined above. At each sampling date, five crowns per cultivar were frozen for each of five freezing temperatures previously established for each cultivar (Fowler 2008). The crowns were placed in aluminum weighing cans, covered in moist sand and then loaded into a programmable freezer that was held at −3°C for 12 h. After 12 h, they were cooled at a rate of 2°C h^{-1} down to −17°C and then cooled at a rate of 8°C h^{-1} until the established temperatures in each treatment were reached. The crowns were thawed overnight at 3°C and then transplanted into 52 × 26 × 6 cm black plastic trays (Kord Products, Bramalea, ON, Canada) containing "Sunshine" artificial soil medium for re-growth at 20°C with a 16 h day and 8 h night. Plant recovery was rated (alive vs. dead) after 3 weeks and the LT_{50} was calculated for each treatment within each replicate. The experiment was repeated three times in time and space.

Isolation of *Scvrn-1* sequence, *Cbf* sequences and their 3′ UTR regions

Primers (Supplementary Table Ia, Sigma-Genosys Canada, Oakville, ON, Canada) designed based on wheat *TaVRT-1* (AY280870, Danyluk et al. 2003) were used to amplify a 699 bp fragment of its rye ortholog from Puma rye cDNA. First-strand cDNA was synthesised from 5 µg of RNA (extracted from leaf samples of plants cold-acclimated for 70 days at 6°C) using 0.5 µg of poly T primer, and 200 U of M-MLV Reverse Transcriptase (Invitrogen Canada Inc., Burlington, ON, Canada). Subsequently 2 µl of the synthesised cDNA was amplified in a 25 µl PCR reaction volume containing 0.15 µM of each primer, 1 U of *Taq* DNA polymerase (Invitrogen Canada Inc., Burlington, ON, Canada), 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 5% DMSO. Amplification conditions were as follows: 95°C for 5 min, 35 cycles of 95°C (1 min), the established annealing temperature (1 min) and 72°C (1 min), followed by an extension step at 72°C (15 min). The annealing temperatures for the primers used are listed in supplementary Table Ia. Amplification products were separated at 150 V for 1 h on a 1% (w/v) agarose gel, fragments of the expected size were gel-purified and cloned using pCR®4-TOPO® vector cloning kit (Invitrogen Canada Inc., Burlington, ON, Canada). Five positive clones were identified using colony-PCR and sequenced using DNA sequencing services at Plant Biotechnology Institute-National Research Council, Saskatoon, Saskatchewan, Canada.

Nine primer pairs, designed based on wheat *Cbf* orthologous sequences, were used to clone 11 rye *ScCbf*s (Table 2) from Puma. *Cbf* fragments were amplified from 100 ng of genomic DNA using the PCR conditions indicated above. The primers used and their annealing temperatures and expected fragment lengths are listed (Supplementary Table Ia). All primer sets amplified the complete coding sequences, except those used to amplify *ScCbf*IVa. Amplification products were separated at 150 V for 1 h on a 1% agarose gel and fragments of the expected size were cloned and sequenced. The 3′ UTR sequence of *ScCbf*IIId-12 was cloned with a 3′ RACE approach as follows: cDNA was synthesised from 5 µg of RNA (Puma rye, 4 h treated at 6°C) as indicated in the previous section but using 0.5 µg of T17-ADAPTER primer. Subsequently 2 µl of the synthesised cDNA was amplified in a reaction containing a forward gene-specific primer and the ADAPTER reverse primer. The 3′ UTR of the remaining *Cbf*s were amplified as described above except using reverse primers designed on the 3′ UTR of their wheat orthologs. Partial 3′ UTR sequences of *ScCbf*IVa-2A and *ScCbf*IVa-2B were cloned directly from gDNA. Amplification products were run on a 1% agarose gel and fragments of the expected size were cloned and sequenced. Primers, annealing temperatures and expected fragment sizes are listed in Supplementary Table Ib. Sequence alignments, in silico translations, and protein isoelectric point and molecular weight calculations were performed using programs within VectorNTI Advance 10 (Invitrogen Canada Inc., Burlington, ON, Canada. MEGA software version 4 (Tamura et al. 2007) and ClustalW alignment of the nucleotide sequences used to coding for the three conserved domains of *Cbf*s were used to generate a phylogenetic dendrogram.

All *ScCbf* sequences isolated in this study were named based on the nomenclature proposed by Badawi et al. (2007). For example, *ScCbf*IIIc-10 indicates a rye (Sc) *Cbf* sequence belonging to group/subgroup IIIc that shares the highest homology with wheat ortholog *Cbf*10. For clarity, we have re-designated the three previously reported *ScCbf*s (Jaglo et al. 2001) using the same nomenclature such that AF370728, AF370729 and AF370730 are now designated as *ScCbf*IVb-20, *ScCbf*IVd-9A and *ScCbf*IVd-9B, respectively. All sequences presented here have been deposited in the DDBJ/EMBL/GenBank databases and the accession numbers are reported.

Chromosomal localization of *Scvrn-1* and *ScCbf* genes

The chromosomal localization of the isolated *ScCbf*s were performed using nine Chinese Spring wheat × Imperial rye [CS/IMP-1R, -2R, -3R, -4R, -5R, -5RL (long arm only), -5RS (short arm only), -6R, -7R] disomic addition lines, kindly provided by Dr. J.P. Gustafson (USDA, ARS, University of Missouri). Rye chromosome-specific microsatellite markers (RMS markers, Chebotar et al. 2003; WRM, Bolibok et al. 2006; REMS and GWM, Khlestkina et al. 2004; and SCM, Saal and Wricke 1999) were used to confirm the presence of each single rye chromosome in each wheat/rye addition line -1R (RMS10, SCM9), -2R (SCM75), -3R (RMS28), -4R (WRM216), -5R (SCM138, SCM268), -5RL (RMS115, REMS1205, REMS1218, REMS1237, REMS1264; GWM1059, SCM109, SCM120), -6R (GWM1103, SCM180) and -7R (SCM86) as previously described (Silkova et al. 2006). Cleaved amplified polymorphic markers (CAPS) were used to map many of the *ScCbf*s. The amplification products for *ScCbf*IIIc-3A/B, *ScCbf*IIId-15 and *ScCbf*IVa-2A/B were digested with restriction endonucleases *Bsr*I, *Xho*I and *Rsa*I, respectively, which cut only within wheat fragments. Amplified fragments of *ScCbf*IIId-12 and *ScCbf*IIId-19 were digested with *Mbo*I, while the *ScCbf*II-5 product was digested with *Tse*I. Amplification products for the remaining *Cbf*s (*ScCbf*Ia-11, *ScCbf*IIIa-6, *ScCbf*IIIc-10, *ScCbf*IVb-20, *ScCbf*IVd-9A and *ScCbf*IVd-9B) and Sc*vrn-1* did not require restriction because the primers were rye specific. Chinese spring and Puma gDNA were used as controls. Primers, restriction enzymes, annealing temperatures, fragment sizes and restriction patterns after digestion are listed (Supplementary Table Ic).

Expression analysis of *Cbf* and *Cor* genes

Whole plant samples, excluding the roots, were collected before LT treatment (0 h = control sample) and at five time points (0, 2, 4, 8 and 48 h) after induction of acclimation at 6, 10, 15 and 18°C. The 0 and 48 h tissue samples were collected within 15 min after dawn at the start of a 16 h day. Samples were immediately frozen in liquid nitrogen and stored at −80°C until required for molecular analyses. For each treatment, total RNA was extracted from 100 mg of tissue using TRIZOL® reagent (Invitrogen) following the manufacturer's instructions. First-strand cDNA synthesis was performed on 5 μg of total RNA, as described above, but a poly T primer was used instead. Semi-quantitative RT-PCRs were performed on cDNA samples using gene-specific primers (sequences, annealing temperatures and expected fragment sizes are listed in Supplementary Table Id). A rye actin fragment was amplified from 1 μl cDNA (Puma rye, 4 h treated at 6°C) using primers designed from a wheat actin sequence (AB181991). A 120 bp amplification product was cloned and sequenced (submitted as GenBank accession number EU525892). The sequenced fragment was 98.3% identical to the AB181991 and was designated as *Scactin*. All samples were normalized to this gene. Expression analyses were performed by amplifying 1 μl of cDNA for 18–34 cycles, using gene-specific primers and PCR conditions described above except that 5% DMSO (v/v) was used for *Cbf* amplification. Primer annealing temperatures are listed in Supplementary Table Id.

Results

Low-temperature tolerance

There were significant differences ($P < 0.05$) in temperatures at which plants began to acclimate (Table 1). At 6°C, all cultivars acclimated rapidly and large differences in LT tolerance were obvious by 2 days. Gazelle spring rye reached maximum LT tolerance after 2 days of acclimation at 6°C, whereas winter-habit genotypes continued to acclimate, with the greatest differences in LT tolerance observed after 49 days. The winter rye cultivar Puma began acclimating at 17°C, compared to 15°C for Sangaste and 14.5°C for Norstar winter wheat. Acclimation in Kold and Gazelle was induced at approximately 10°C (Table 1).

Rye *Cbf* gene isolation

Prior to conducting expression studies, 11 *ScCbf* genes were isolated and their characteristics are summarized in Table 2. The isolated 3′ UTR of *ScCbf* sequences ranged from 23 bp (*ScCbf*IVa-2A) to 172 bp (*ScCbf*Ia-11) in length (see supplementary information for 3′ UTR sequences) and showed a sequence similarity of 50–100% with their wheat orthologs. The translated proteins ranged from 204 to 232 amino acids in length and shared

Table 1 Threshold induction temperature and LT_{50} (°C) after 0, 2 and 49 days acclimation at 6°C for Kold winter barley, Norstar winter wheat, Puma winter rye, Sangaste winter rye and Gazelle spring rye

Cultivar	Threshold induction temperature (°C)	Acclimation time (days)		
		0	2	49
Kold	10.5b	−2.0a	−5.3a	−11.7b
Gazelle	10.0b	−2.0a	−6.3a	−5.0a
Sangaste	15.0c	−2.0a	−7.7ab	−16.5c
Norstar	14.5c	−2.7a	−9.3b	−21.7d
Puma	17.0d	−3.3a	−13.3c	−25.0e

Within columns, means followed by the same letter are not different as determined by Duncan's new multiple range test ($P < 0.05$)

Table 2 Nomenclature and characteristics of rye *Cbf* genes cloned from the cultivar Puma rye

Gene name	Genbank accession number	Group	Chromosome location	Sequence length (bp)	Protein length (aa)	p*I*	MW (kDa)	Best BLAST	Identity (%)
*ScCbf*Ia-11	EU194240	Ia	2R	614	204	5.60	23.8	*TaCbf*Ia11	92.9
*ScCbf*II-5	EU194241	II	–	641	213	6.06	23.8	*TaCbf*II-5.2	86.0
*ScCbf*IIIa-6	EU194242	IIIa	5RL	689	229	4.92	25.9	*TaCbf*IIIa-D6	91.2
*ScCbf*IIIc-10	EU194243	IIIc	5RL	666	221	4.80	24.8	*TmCbf*10	92.4
*ScCbf*IIIc-3A	EU194244	IIIc	5RL	686	228	4.57	25.7	*TaCbf*IIIc-D3	94.6
*ScCbf*IIIc-3B	EU194245	IIIc	5RL	695	231	4.61	25.8	*TaCbf*IIIc-D3	94.0
*ScCbf*IIId-12	EU194246	IIId	5RL	698	232	5.70	26.2	*TaCbf*IIId-B12	93.7
*ScCbf*IIId-15	EU194247	IIId	5RL	662	220	5.11	24.8	*TaCbf*IIId-A15	93.4
*ScCbf*IIId-19	EU194248	IIId	5RL	665	221	5.13	25.2	*TaCbf*IIId-D19	94.4
*ScCbf*IVa-2A[a]	EU194249	IVa	5RL	605	>193	(5.76)	(22.0)	*TaCbf*IVa-A2	94.7
*ScCbf*IVa-2B[a]	EU194250	IVa	5RL	651	>193	(5.98)	(22.0)	*TaCbf*IVa-A2	94.3
*ScCbf*IVb-20[b]	AF370728	IVc	5RL	850	212	8.53	23.3	*TaCbf*IVb-A20	91.4
*ScCbf*IVd-9A[b]	AF370729	IVd	5RL	1040	268	9.47	28.9	*TaCbf*IVd-B9	90.9
*ScCbf*IVd-9B[b]	AF370730	IVd	5RL	1145	270	9.00	29.2	*TaCbf*IVd-B9	92.8

[a] Cloned sequences were truncated at their 5′ ends (lacked the first 78 bp based on their wheat ortholog). Partial protein sequences were used to calculate p*I* and MW values (in brackets)

[b] Previously reported by Jaglo et al. (2001)

conserved motifs typical of other *Cbf*s (Fig. 1), including the AP2 DNA-binding domain flanked by CMIII-3 (PKK/RPAGRxKFxETRHP) and CMIII-1 (DSAWR, Jaglo et al. 2001) and the LWSY sequence near the C-terminal (Dobouzet et al. 2003). The majority of isolated rye sequences from groups I to III coded for proteins with a predicted acidic character, with p*I* values ranging from 4.57 to 6.06 (Table 2). In contrast, the group IV *ScCbf*s possessed a predicted basic character, with p*I* values ranging from 8.53 to 9.47. *ScCbf*IVa-2A and 2B could not be evaluated for this feature because the complete CDS' were not isolated.

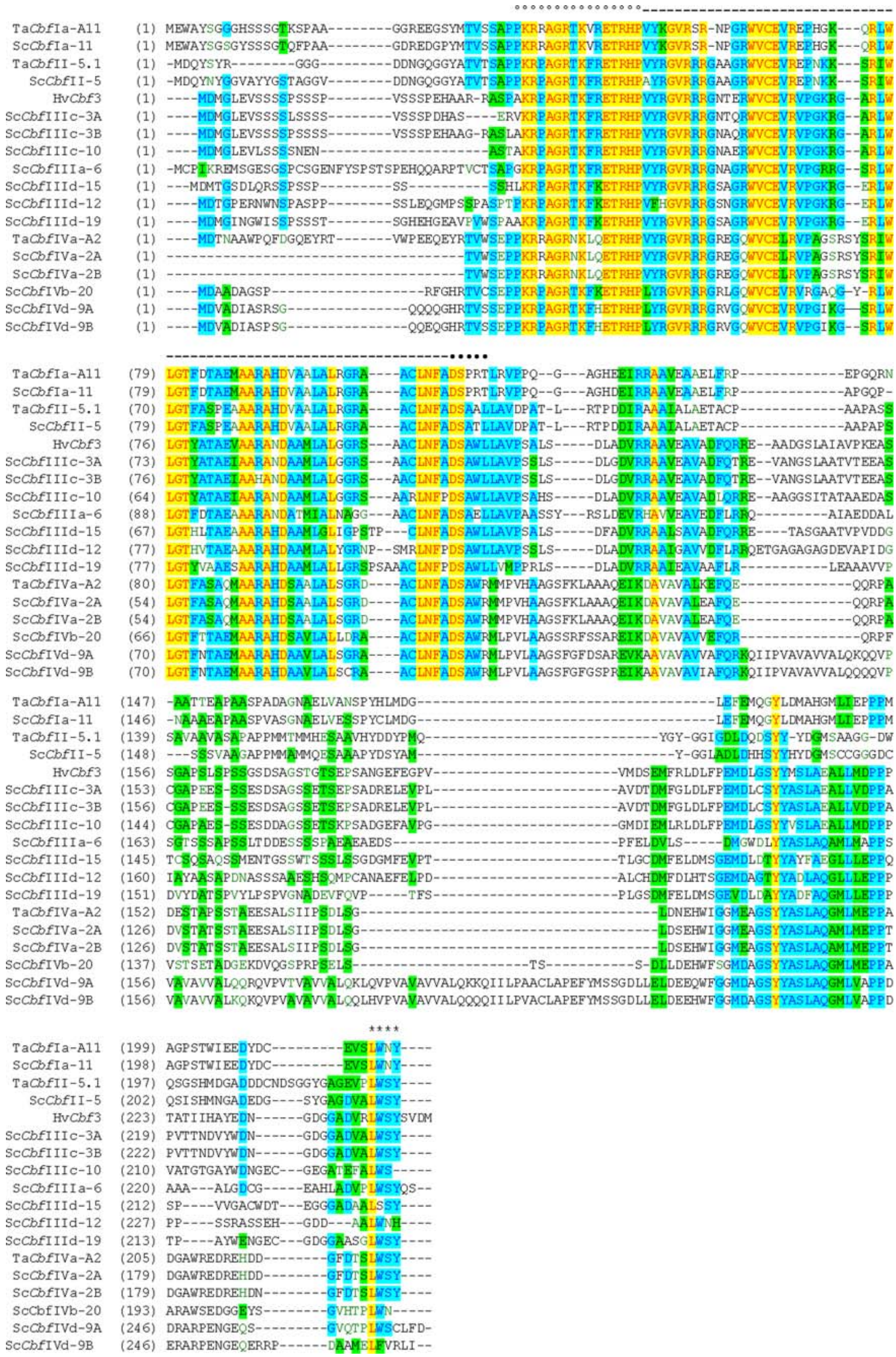
The nucleotide sequences coding for the three conserved domains of the *ScCbf* genes were aligned with reported hexaploid wheat (Ta, $n = 15$), barley (Hv, $n = 12$), diploid wheat (Tm, $n = 13$) to develop a phylogenetic tree. All of the *ScCbf*s clustered into one of four phylogenetic groups (Fig. 2). Seven group III *ScCbf* genes were identified and were 91.2–94.6% identical to their wheat orthologs (Table 2). *ScCbf*IIIc-3A and *ScCbf*IIIc-3B were 97.3% identical and likely represent gene duplications when a species cut-off of 95% identity is used to separate homeologous copies and recently duplicated genes. Group IV *Cbf*s *ScCbf*IVd-9A and *ScCbf*IVd-9B had identical protein sequences and likely also represent gene duplications. Most *ScCbf*s showed greater similarity to their wheat orthologs (Table 2) except for *ScCbf*IIIc-10 which showed a higher identity with a *T. monococcum Cbf* gene (94.0%). A *ScCbf* was not found from subgroup IVc, and no rye orthologs to Hv*Cbf*1 and Hv*Cbf*13 were isolated.

ScCbf genes and *Scvrn-1* map on long arm of chromosome 5R

The Chinese Spring/Imperial wheat/rye addition lines (CS/IMP 1R-7R, 5RL and 5RS) were used to determine the chromosomal location of the *ScCbf*s (Table 2). The vernalization gene Sc*vrn-1* has been mapped to 5R in rye (Plaschke et al. 1993) and was isolated to confirm the chromosome 5 substitutions. The 699 bp partial cDNA sequence of *Scvrn-1* (EU525891) was 97% identical to *TaVRT-1* (AY280870, Danyluk et al. 2003). Rye-specific primers were designed for Sc*vrn-1* and localized to 5RL (Fig. 3), confirming the CS/IMP 5R addition. All group III (seven sequences) and IV (five sequences) *ScCbf*s also mapped to 5RL (data not shown), whereas *ScCbf*Ia-11 mapped to 2R (Fig. 3). It was not possible to assign a chromosomal localization to *ScCbf*II-5 using the addition lines as the CAPS marker was not polymorphic between Chinese spring (wheat parent) and Imperial (rye parent).

Expression levels of *Cbf*s genes during cold acclimation

The expression profiles of ten *ScCbf* genes were evaluated in three rye cultivars at five time points (0, 2, 4, 8 and 48 h) after induction of acclimation at 6, 10, 15 and 18°C (Fig. 4). The expressions of *ScCbf*IVa-2B and *ScCbf*IIIc-3B were excluded because primers could not be designed to distinguish them from *ScCbf*IVa-2A and *ScCbf*IIIc-3A, respectively. *ScCbf*II-5 and *ScCbf*IIIa-6 were tested but their expression levels were below visible detection in all



◀ **Fig. 1** Alignment of the predicted amino acid sequences of the *Cbf* sequences cloned from the rye cultivar Puma. The Genbank accession numbers of the sequences used in this analysis are listed in Supplementary Table I.e and rye *Cbf* Genbank accession numbers are reported in Table 2. Designations are as follows: AP2/EREBP domain (*over line*), signature sequences PKK/RPAGRxKFxETRHP (*open circles*) and DSAWR (*filled circles*), conserved C-terminal region LWSY (*asterisks*)

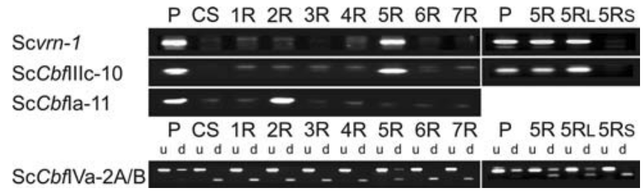


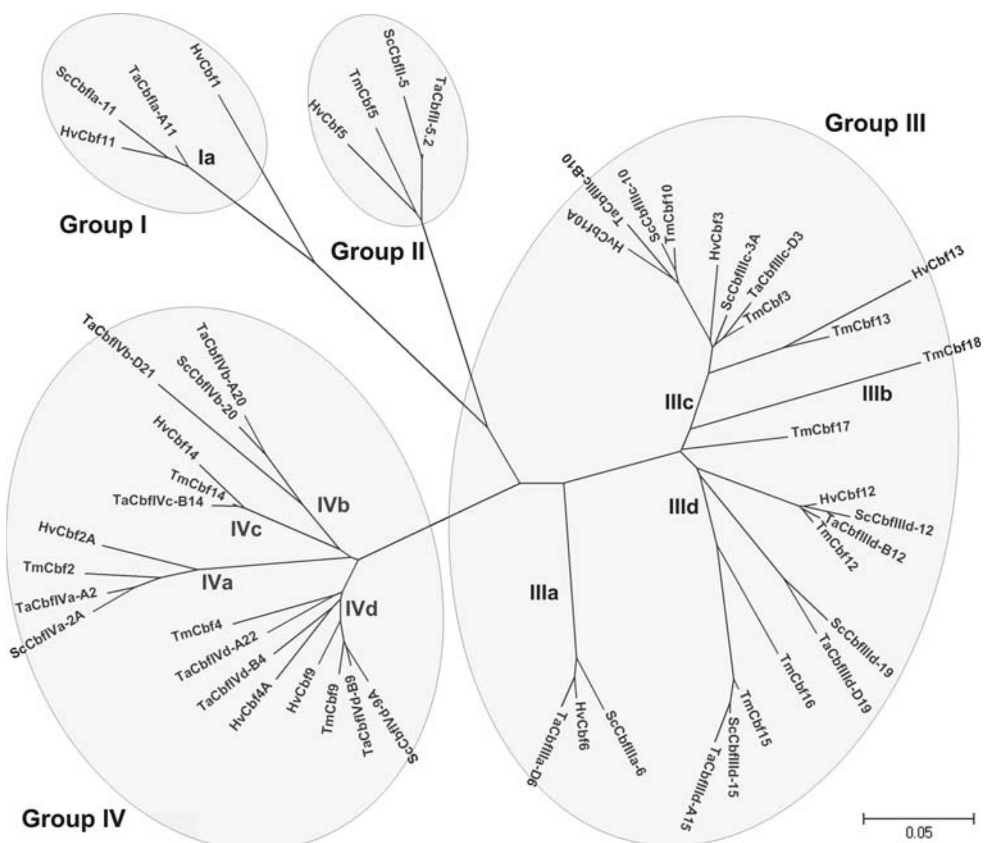
Fig. 3 Chromosomal localization of *Scvrn-1* and three *ScCbf*s using Chinese Spring (CS) wheat × imperial rye addition lines (1R-7R, 5RL and 5RS). *Scvrn-1* is known to map to 5R in rye and was used as a control sequence. The undigested (*u*) and digested (*d*) fragments for the CAPS marker used to map *ScCbf1Va-2A/B* are presented

three rye genotypes, regardless of temperature (data not shown).

Comparison of *ScCbf* expression in Gazelle spring rye with the two winter-habit rye cultivars showed comparable expression of *ScCbf*IIId-12, *ScCbf*IIId-19 and *ScCbf*IVd-9B among the three genotypes at 6 and 10°C, while at higher temperatures the expression level was greater in the winter-habit cultivars (Fig. 4). In contrast, delayed induction of *ScCbf*IVa-2A and *ScCbf*IVb-20 was observed in Gazelle, with maximum expression only after 8 h of cold treatment. The expression of *ScCbf*IIId-15 and *ScCbf*IVd-9A generally increased as temperature decreased and was similar in all three rye cultivars. *ScCbf*IIIc-10 was detectable at low levels only in Puma and after 2 h at 6°C (data not shown for Sangaste and Gazelle). The expression of *ScCbf*IVb-20 was pronounced after 8 h at 18°C in Puma compared to no detectable expression in the less hardy cultivar Sangaste. *ScCbf*IIId-19, *ScCbf*IVa-2A and *ScCbf*IVb-20 were the only genes that were expressed at high levels at 18°C, but only at 4 and 8 h after treatment and only in the winter cultivars. In the winter-habit rye cultivars, most of the group III and group IV *ScCbf*s showed strong initial LT induction that peaked by 8 h and then decreased at 48 h, which was the end of an 8-h dark period.

A subset of *Cbf*s belonging to groups I, II, III and IV were evaluated in winter wheat (Norstar) and barley (Kold) cultivars (Fig. 4) for comparison with rye to determine if variability in expression could be associated with variation in LT induction among species. Interactions among *Cbf* genes, species and induction temperature were evident and the results are summarized in Table 3. In barley, Hv*Cbf*1 showed a stronger expression level than *ScCbf*Ia-11 after exposure at 6°C, while at 10°C the expression of Sangaste

Fig. 2 Phylogenetic relationship of *Poaceae Cbf*s isolated from rye (Sc), wheat (Ta), diploid wheat (Tm) and barley (Hv). The nucleotide sequences corresponding to the conserved AP2 domain and the flanking CMIII-3 and CMIII-1 sequences were used to construct the dendrogram. *Cbf*s belonging to the same group are contoured, while subgroups are indicated near the branch



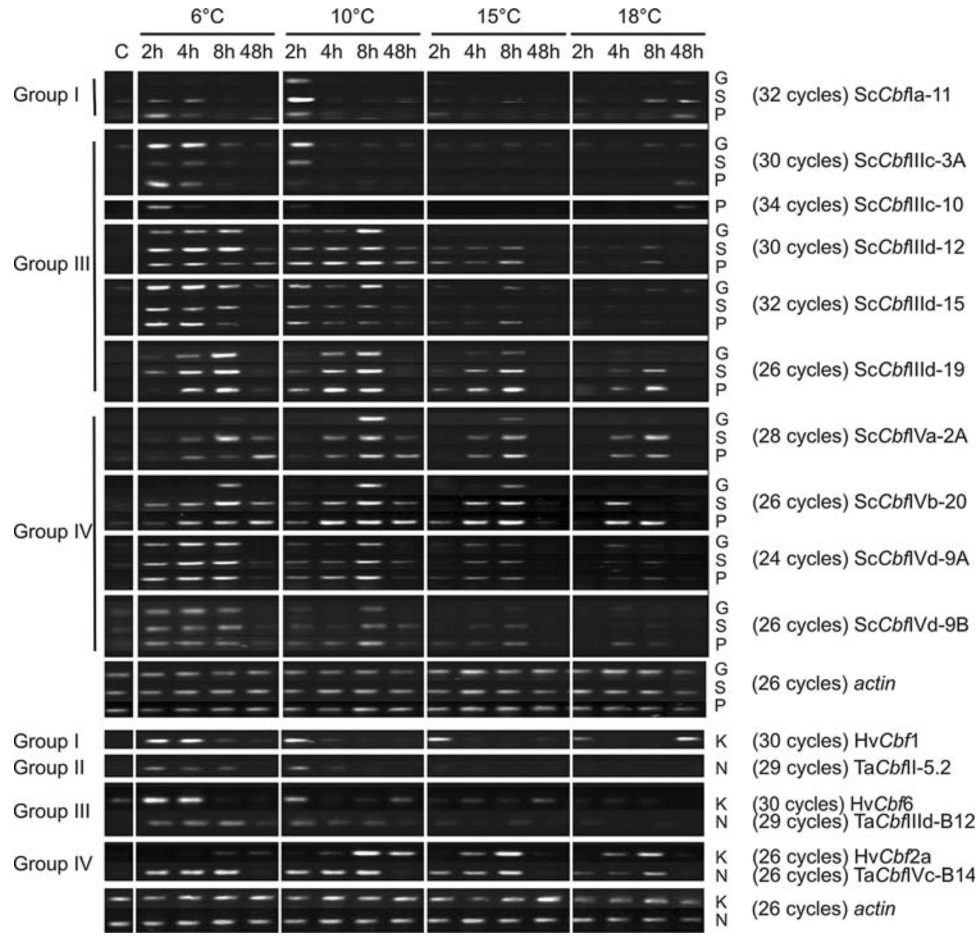


Fig. 4 Expression profile of *Cbf* genes in rye cultivars Gazelle (G), Sangaste (S), Puma(P) and Norstar (N) winter wheat and Kold (K) winter barley in response to varying acclimation temperatures (6, 10, 15 and 18°C) and sampling times [before treatment (C) and after 2, 4, 8 and 48 h]. Actin was used as the reference gene for all cultivars

was comparable to that of Kold. Ta*Cbf*II-5.2 was detectable in Norstar (Fig. 4), while expression of its ortholog *ScCbf*II-5 was not detectable in any of the rye cultivars. In winter wheat and rye cultivars, the group III *Cbf*s showed prolonged expression until 8 h, while in barley Hv*Cbf*6 transcript levels started to decrease after 4 h (6°C) or 2 h (10°C). Ta*Cbf*IIId-B12 and Hv*Cbf*6 showed weaker expression than the Group III *ScCbf*s that were characterized. The expression levels of *ScCbf*IVa-2A, Ta*Cbf*IVc-B14 and Hv*Cbf*2A were comparable in the winter species, while the spring cultivar Gazelle rye showed delayed induction and reduced expression (Fig. 4).

Induction of *Cor* genes during cold acclimation

Expression levels of *Cor* genes were profiled in rye, wheat and barley cultivars to compare the cold induction response of genes downstream from the *Cbf*s (Fig. 5). Under our test conditions, *Cor* genes were up-regulated slightly after 4 h of LT exposure and expression levels were maintained until 48 h at 6°C, but gradually decreased by 48 h, particularly at 15 and 18°C (Fig. 5). Rye *Cor* gene *Rep14* (AF491840; Ndong et al. 2002) is orthologous to wheat *wcs19* (Chauvin et al. 1993) and showed similar induction levels among spring and winter rye cultivars. However, higher expression was observed in Norstar, especially at 18°C. Expression of the rye *Cor* gene *Rep13* (AF491839; Ndong et al. 2002), which is orthologs to wheat *Wcor14b* (Tsvetanov et al. 2000) and barley *Hvcor14b* (Dal Bosco et al. 2003), was higher in Puma at all acclimation temperatures while expression levels were similar in Gazelle and Kold (Fig. 5). The two reported rye ESTs, TC2696 and TC3078, were annotated, respectively, as wheat *Cor39* and wheat/barley *Cor410/Dhn5* homologues (Close et al. 1995; Danyluk et al. 1994) and expression of these was induced at higher acclimation temperatures in the winter cultivars Puma and Sangaste compared to Gazelle spring rye. Wheat *Wcs120* (M93342; Houde et al. 1992) and barley *Dhn8* (AF043093) expression was similar in Norstar and Kold.

Discussion

Cbf gene isolation and chromosomal location

Plant cold acclimation is a complex process and has been studied extensively in *Arabidopsis* and in commercially important cereal species such as wheat and barley. The

Table 3 Comparison of *Cbf* expression levels among different rye cultivar and different species (wheat, barley, rye)

	Rye *Cbf*s	Comparison among rye cultivars	Comparison among species	Wheat/barley *Cbf*s
Group I	*ScCbfIa-11*	Higher expression in winter cv	Higher expression in barley	*HvCbf1*
Group II	*ScCbfII-5*	Not detectable	Detectable only in wheat	*TaCbfII-5.2*
Group III	*ScCbfIIIa-6*	Not detectable	Higher expression in rye	*TaCbfIIId-B12/HvCbf6*
	ScCbfIIIc-3A	Higher expression in spring Gazelle		
	ScCbfIIIc-10	Detectable only in winter Puma		
	ScCbfIIId-12	Comparable expression level at 6–10°C, higher expression level at 15–18°C		
	ScCbfIIId-15	Comparable expression level		
	ScCbfIIId-19	Comparable expression level at 6–10°C, higher expression level at 15–18°C		
Group IV	*ScCbfIVa-2A*	Higher expression in winter cv	Comparable	*TaCbfIVc-B14/HvCbf2a*
	ScCbfIVb-20	Higher expression in winter Puma		
	ScCbfIVd-9A	Comparable expression level		
	ScCbfIVd-9B	Comparable expression level at 6–10°C, higher expression level at 15–18°C		

The Genbank accession numbers of genes presented in the table are listed in Table 2 (rye) and Supplementary table I.e (wheat/barley)

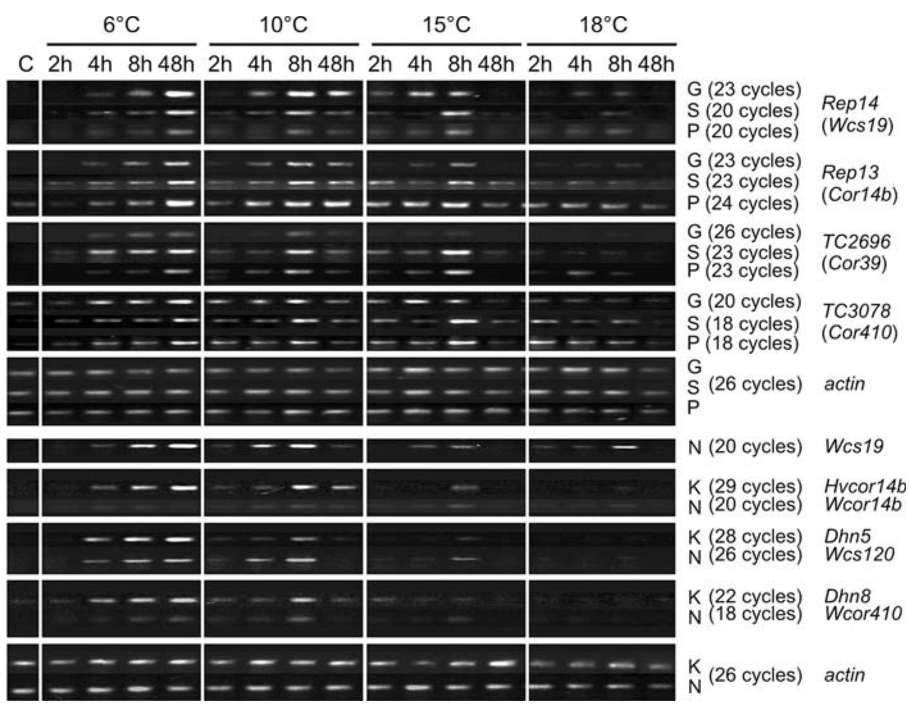


Fig. 5 Expression profile of *Cor* genes in rye cultivars Gazelle (G), Sangaste (S), Puma(P) and Norstar (N) winter wheat and Kold (K) winter barley in response to varying acclimation temperatures (6, 10, 15 and 18°C) and sampling times [before treatment (C) and after 2, 4, 8 and 48 h]. The Genbank accession numbers of genes used in this analysis are: *Rep14* (AF491840), *Rep13* (AF491839), *Cor39hom* (TC2696), *Cor410hom* (TC3078), *Wcs19* (L13437), *Hvcor14b* (AJ512944), *Wcor14b* (AF207546), *Dhn5* (M95810), *Wcs120* (M93342), *Dhn8* (AF181458), *Wcor410* (L29152). Samples were normalized with wheat actin-specific primers (AB181991)

Cbf transcription factors play a central role in plant response to cold stress, and in the present work, 11 *Cbf* genes were cloned and characterized from the LT-tolerant rye cultivar Puma and their expression levels were compared in rye cultivars with different growth habits, acclimation thresholds and LT-tolerance genetic potentials (Table 1). The majority of *ScCbf*s were mapped to 5RL, which is consistent with reports for wheat and barley where clusters of *Cbf* genes have been localized to the LT-tolerance QTL on the group 5 chromosomes. *ScCbf*1a-11 was localized to 2R and is orthologous to *TaCbf1a-11* which has been mapped to 2AL in common wheat (Badawi et al. 2007). We were unable to identify a chromosomal location for *ScCbf*II-5, probably because the single nucleotide polymorphism used to generate the *Tse*I CAPS marker for mapping was specific to the Norstar sequence (EF028752, EF028753, EF028754) and not present in the sequence of Chinese spring, the wheat parent of the Imperial line substitution series. However, the *T. monococcum* and barley orthologs have been mapped on 7A^{m} and 7HS, respectively (Miller et al. 2006; Skinner et al. 2005).

Alignment of the coding sequence for the AP2 and *Cbf* signature-conserved domains allowed us to assign *ScCbf*s to different phylogenetic groups (Fig. 2). Groups I and II contain a reduced number of sequences compared to other *Cbf* groups. Group I is the most ancient monocot *Cbf* group and it shares the highest homology with dicotyledonous *Cbf*s (Badawi et al. 2007). Despite repeated attempts, we could not identify a rye ortholog of Hv*Cbf*1. Perhaps this gene does not exist in rye as it also appears to be absent in common wheat and *T. monococcum*. The absence of this *Cbf* in wheat and rye suggests that it does not play a critical role in determining LT tolerance in the *Triticeae*. Seven out of 11 of the rye *Cbf*s sequences were assigned to *Cbf* group III (Fig. 2). However, no genes belonging to subgroup IIIb were found in rye, which is also the case in wheat and barley. To date, the only subgroup IIIb *Cbf* reported in *Pooideae* is *TmCbf18* identified in *T. monococcum*. The remaining five *ScCbf*s genes were assigned to group IV, but we did not identify a rye orthologs of Ta*CbfIVb-D*21, Ta*CbfIVc-B14* and Ta*CbIVd-A22*, so continued effort is warranted to determine if these are present in rye. In addition, orthologs of barley and *T. monococcum Cbf*13, *Cbf*16 and *Cbf*17 have yet to be identified in rye and common wheat.

Low-temperature induction and expression of *Cbf*s and *Cor* genes during cold acclimation

Rye is very responsive to temperatures in the acclimation range, generating rapid increases in LT acclimation and high levels of expression of LT-associated genes (Fowler et al. 1996). In this study, Puma winter rye displayed the best LT tolerance followed by Norstar winter wheat, Sangaste winter rye and Kold winter barley (Table 1), which is consistent with earlier reports (Fowler and Carles 1979). Once acclimation started, the differences in LT-tolerance genetic potentials were quickly magnified such that cultivars with acclimation induced at warmer temperatures were the most cold hardy. While the most rapid changes in freezing tolerance occurred during the initial stages of acclimation, the largest differences among cultivars were observed after 49 days of acclimation. These observations are consistent with our hypothesis that cold-sensing mechanisms and responses in the early stages of acclimation play a critical role in determining the genetic potential for LT tolerance (Fowler 2008). Full expression of cold hardiness genes only occurs in the vegetative stage (Limin and Fowler 2006) and the inability of Gazelle spring rye to continue accumulating LT tolerance after an early burst is consistent with the theory that the delayed vegetative/reproductive transition allows winter-habit genotypes to be more responsive to extended periods of acclimation (Fowler et al. 1999).

We compared the expression of several rye, wheat and barley *Cbf*s to assess their rate of response to varying acclimation temperatures. We found that the group I *Cbf ScCbf*Ia-11 had a very low-induction level after cold exposure and we were unable to detect mRNA accumulation for group II *ScCbf*II-5 under our test conditions. This was not surprising because *Cbf*s are induced not only by cold, but also by drought and osmotic stress (Haake et al. 2002; Dobouzet et al. 2003). Skinner et al. (2005) found that Hv*Cbf*1 was the only *Cbf* strongly induced after drought exposure in barley, and in this study, its expression was induced following 2 h at lower acclimation temperatures, but only after 48 h at 18°C. In general, the expression patterns of most of group III and IV *Cbf*s were more closely associated with the threshold induction temperatures than the other groups (Table 1; Fig. 4). In particular, the group III *Cbf*s where induced at higher temperatures and had prolonged expression at 6°C in the most cold tolerant winter rye cultivars. However, this was not true of all group III subgroups because *Cbf* subgroups IIIa and IIIc were either not expressed (IIIa-6) or had low-expression levels at warmer temperatures (IIIc-3A, IIIc-10) in rye. *TaCbfIIId-B12* was also expressed at very low levels at all temperatures (Fig. 4), despite high expression of LT tolerance in Norstar (Table 1). However, expression of *TaCbfIVc-B14* was high at 6 and 10°C in Norstar, with reduced expression at 18°C, which reflects the acclimation induction temperature of that cultivar. These observed differences in expression levels among the *Cbf* genes could be due to different upstream regulatory factors that monitor temperature, or to mutations within a common regulatory factor controlling gene response.

Once the threshold induction temperature for cereals is reached, there is an inverse relationship between rate of LT acclimation and exposure temperature (Fowler et al. 1999). There are also genotypic differences in the threshold induction temperature (Fowler 2008) with the result that the warmer the induction temperature, the sooner LT tolerance starts to accumulate. If *Cbf*s are involved in the regulation of plant response to LT, their expression should also be delayed in cultivars with colder induction temperatures and the level of expression should be higher in hardier genotypes. However, we found that differences in *Cbf* expression did not necessarily correspond to the acclimation response of the cultivars considered in this study. After 2 days acclimation at 6°C, the LT_{50} of Gazelle and Sangaste were similar and significantly warmer than Puma (Table 1), but the threshold responses of the *Cbf*s were similar for Sangaste and Puma and generally much warmer than Gazelle (Fig. 4). Furthermore, *ScCbf* genes belonging to the same subgroups showed similar expression in the two winter rye cultivars evaluated despite the large difference in expression of LT tolerance at all three acclimation temperatures (Table 1). These results suggest that *Cbf* expression is being dictated to a greater extent by the winter/spring growth habit than by genetic differences in cold hardiness potential.

Interestingly, for those rye *Cbf*s expressed at higher induction temperatures (15 and 18°C), expression was strongly repressed at 48 h, just as plants came out of the dark. This was also true for rye *ScCbfIVd-9A* and *TaCbfIVc-B14*, where expression at 6°C was repressed at 48 h. These results suggest a diurnal fluctuation in *Cbf* expression in grasses, particularly at higher induction temperatures. These observations are consistent with results in *Arabidopsis*, where endogenous circadian rhythms have been reported to regulate *Cbf* transcripts at higher temperatures (Edwards et al. 2006). In contrast, expression of several *Cbf*s at 48 h was noted at 6 and 10°C, suggesting that their turnover rate is temperature-dependent or induction of these *Cbf*s at low temperatures is not gated by the diurnal response, as is the case in *Arabidopsis* (Fowler et al. 2005).

Although there is a rapidly expanding pool of information on *Cbf* sequencing, mapping and expression analyses in cereals, only *TmCbf-12* and *TmCbf-14* have been correlated with variation in LT tolerance in *T. monococcum* (Knox et al. 2008). In this study the expression patterns of these *Cbf*s were variable, with complex genotype by time by induction–temperature interactions (Fig. 4). In barley, the *CbfIIId-12* ortholog (*HvCbf6*) was highly expressed at 2 and 4 h after treatment at 6°C, with variable expression as temperature increased. In contrast, except for the 48 h sampling time, the rye ortholog was strongly expressed at 15°C in winter-habit cultivars. In Norstar, *TaCbfIIId-B12* was expressed after 2, 4 and 8 h exposure to 6°C and decreased dramatically at 15°C (Fig. 5). *Wcs120* expression in wheat is highly correlated with LT_{50} (Limin et al. 1997) and *Wcs120* expression more closely followed *CbfIIId-B12* than *CbfIVc-14* expression. Puma began to acclimate at temperatures as high as 17°C, and only the *Cor14b* ortholog was expressed in Puma at temperatures greater than 15°C (Fig. 5). However, accumulation of the *Cor14b* ortholog in Puma was not correlated to *ScCbfIIId-12* or *CbfIVc-14* expression. These observations show that *Cbf* gene(s) have different temperature thresholds and that the interaction of several different *Cbf* gene(s) and/or regulatory factors may determine *Cor* gene expression and the superior LT tolerance in rye compared to wheat and barley.

Conclusions

We analyzed the expression of *Cbf*s in rye, the most cold tolerant cereal species, and found LT acclimation induction of winter and spring rye cultivars was most associated with the expression levels of *Cbf* sequences belonging to groups III and IV. However, none of the *Cbf*s considered adequately explained the differences in response to LT temperature tolerance observed in the cultivars examined, and *Cbf* expression was dictated to a greater extent by winter/spring growth habit. Comparison of *Cbf* and *Cor* gene expressions among wheat, barley and rye revealed complex threshold induction temperature by time by LT-induced gene interactions indicating that expression of *Cbf* and *Cor* genes is staged at different temperatures and times during the day, making it difficult to determine cause-and-effect relationships. Our results also suggest that *Cbf* expression in cereals is regulated not only by induction temperature, but also by diurnal rhythm, particularly at higher temperatures. To date, most *Cbf*s expression studies in cereals have focused on sampling at a single time point, usually after treatment with a single LT shock. These methods do not adequately address the variable time/temperature and rate of response that we show to be critical in determining LT tolerance (Table 1). Our results indicate that sample timing, induction temperature and light-related factors must be considered in future studies involving functional characterization of LT-induced genes in cereals.

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