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ORIGINAL PAPER

A perennial ryegrass *CBF* gene cluster is located in a region predicted by conserved synteny between Poaceae species

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Abstract CBF/DREB1 proteins are the most important regulators of the cold temperature signaling pathway in many plants. *CBF* genes are candidates for low-temperature tolerance QTL in wheat and barley. Ten novel putative *CBF* cDNAs of perennial ryegrass (*Lolium perenne* L.) have been isolated from cold-treated leaf tissue. Their primary structures contain some conserved motifs, characteristic of the gene class. Phylogenetic analysis revealed that *LpCBF* genes were attributable to the HvCBF3-, and HvCBF4-subgroups following the previously proposed classification of barley *CBF* genes. RT-PCR analysis revealed that the expression of *LpCBF* genes was rapidly induced in response to low temperature and that the expression pattern under the low-temperature conditions for a long period was different between the various *LpCBF* genes. Five of the ten *LpCBF* genes were assigned to the genetic linkage map using the p150/112 reference mapping population. *LpCBFIb, LpCBFII, LpCBFIIIb* and *LpCBFIIIc* were mapped on LG5 forming a cluster within 2.2 cM, while *LpCBFVb* was located on LG1. Based on comparative genetic studies, conserved synteny for *CBF* gene family was observed between the Triticeae cereals and perennial ryegrass. Information on the perennial ryegrass *CBF* genes at both the molecular and genetic level obtained in this study would be useful for the further study on the role of CBF genes and low-temperature tolerance in grasses.

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage grasses in temperate regions because of its high yield of digestible nutrients and ability to tolerate to high grazing pressures (Wilkins 1991). However, perennial ryegrass is generally less winter hardy than other temperate grasses, such as timothy and meadow fescue, because of a lack of freezing tolerance (Nakayama et al. 2001). Improvement of freezing tolerance is one of the most important targets of breeding programs, to expand the cultivated area of perennial ryegrass to regions with severe winter climates.

Many plants, including perennial ryegrass, exhibit an increase in freezing tolerance in response to low, non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow 1999). A number of genes respond to cold and condition the plant cell against the effects of freezing temperature during cold acclimation. It has been suggested that the C-repeat binding factor (CBF)/ dehydration-responsive element-binding protein 1 (DREB1) regulon is the most important transcription unit involved in cold acclimation in

plants (Nakashima and Yamaguchi-Shinozaki 2006). In *Arabidopsis thaliana*, *CBF/DREB1* transcription factor genes are induced in response to low temperature and other stresses at the transcriptional level, and their products activate the expression of multiple stress-inducible genes containing CRT/DRE *cis*-acting elements at their promoter region (Gilmour et al. 1998; Liu et al. 1998; Stockinger et al. 1997). Overexpression of *Arabidopsis CBF/DREB1* genes leads to an increased tolerance to freezing and other stresses due to an induction of multiple-stress responsive genes under non-stress condition (Gilmour et al. 2004; Jaglo-Ottosen et al. 1998; Liu et al. 1998). These reports indicate that *CBF/DREB1* genes are involved in freezing tolerance in *Arabidopsis*.

In monocot plants, *CBF/DREB1*-like genes have been identified in rice (Dubouzet et al. 2003), wheat (Jaglo et al. 2001; Kobayashi et al. 2005), barley (Choi et al. 2002; Xue 2002; Skinner et al. 2005) and maize (Qin et al. 2004). In the Triticeae, large *CBF/DREB1* gene families have been described, many of which are clustered tandemly within the genome. In barley, 20 *CBF* genes were identified (Skinner et al. 2005), and 11 of which were assigned to two tandem clusters on 5HL (Skinner et al. 2006). In diploid wheat (*Triticum monococcum* L.), Miller et al. (2006) reported that 11 *CBF* genes form a cluster within a 0.8 cM region located on chromosome $5A^m$. Individual *CBF* homologue genes were recently identified in the temperate forage grass species perennial ryegrass (Xiong and Fei 2006) and tall fescue (*Festuca arundinacea* Schreb.) (Tang et al. 2005). However, the existence of *CBF* families in forage grasses was not reported so far.

In the Triticeae, two conserved QTLs related to low-temperature tolerance, *Fr-1* [*Fr-A1* (Sutka and Snape 1989; Galiba et al. 1995), *Fr-D1* (Snape et al. 1997), *Fr-B1* (Toth et al. 2003) in wheat and *Fr-H1* (Hayes et al. 1993; Francia et al. 2004) in barley] and *Fr-2* [*Fr-A2* (Vágújfalvi et al. 2003) in wheat and *Fr-H2* (Francia et al. 2004) in barley], have been assigned to the long arms of homoeologous group 5 chromosomes [reviewed by Stockinger et al. (2006)]. Linkage mapping studies revealed that the *CBF* cluster was linked to the *Fr-2* low-temperature tolerance and cold-regulated (*COR*) gene product accumulation QTLs, in the Triticeae (Francia et al. 2004; Tondelli et al. 2006; Vágújfalvi et al. 2003; Miller et al. 2006). These results indicate that *CBF* gene allelic diversity may affect variation for low-temperature tolerance in the Triticeae cereals. On the other hand, the majority of studies have revealed that the *Fr-1* loci were located distal to the teromeric region compared to *Fr-2*, and they were localized near the *Vrn-1* loci, QTLs for vernalization response corresponding to *Vrn-A1* (Galiba et al. 1995) in wheat and *Vrn-H1* (Laurie et al. 1995; Francia et al. 2004) in barley. Comparative genomic studies have revealed that homoeologous group 5 in wheat and barley corresponds to the whole of LG5 and the upper part of LG4 in perennial ryegrass (Sim et al. 2005). The perennial ryegrass orthologue of wheat *VRN1* gene, which is a candidate for *Vrn-1* trait, was mapped on LG4 of perennial ryegrass linkage genetic map (Jensen et al. 2005). However, the location of perennial ryegrass *CBF* gene loci is currently unknown.

This study has obtained molecular genetic information on perennial ryegrass *CBF* genes as part of a study of low-temperature and freezing tolerance in grasses. *CBF* homologue genes were identified and shown to be expressed in response to low temperature conditions. The positions of *CBF* loci on the perennial ryegrass genetic map were determined. The comparative relationships of *CBF* cluster loci and low-temperature tolerance QTLs between forage grasses and other Poaceae species are discussed.

Materials and methods

Isolation of perennial ryegrass *CBF* clones

Plants of perennial ryegrass cultivar (cv.) Aberystwyth S23 were grown in a controlled climate chamber (16 h day length, 22°C/18°C day/night) for 30 days, and transferred to 4°C for 2 h. A cDNA library was constructed from mRNA extracted from the leaf of cold-treated plants, using a ZAP Express cDNA Synthesis Kit and a ZAP Express cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. Partial fragments of putative *CBF* genes were obtained by PCR amplification from cDNA library, using the primer pair combination HvCBF-1 and HvCBF-4 designed on the basis of the DNA sequence of barley *Cbf3* cDNA [accession no. AF239616, Choi et al. (2002)]. All primer sequences used in this study are listed in Table 1. Amplified fragments were cloned to T-vector using pGEM®-T Easy Vector System (Promega) and nucleotide sequences of the fragments were determined using the CEQ 8000 Genetic Analysis System (Beckman Coulter). The 364 bp fragment corresponding to an internal sequence of *LpCBFIb* was labeled and used for the first cDNA library screening by plaque hybridization using a PCR DIG Probe Synthesis Kit and DIG Luminescent Detection Kit for Nucleic Acid (Roche). A total of ca. 3×10^5 recombinant plaques were screened and positive plaques were isolated. After plaque purification, in

Table 1 Primer sequence used in this study

Primer name	Primer sequence (5′ to 3′)
HvCBF-1	GCGAACGACGCTGCCATGCTC
HvCBF-4	TGTAGTACGAGCCCAGGTCCAT
LpCBF1-1F	CTCATCAAGCCATGGACATG
LpCBF1-2F	CTCACAGTCCACAGTCCACC
LpCBF1-1R	TGAGGATGGCCTCCTCTGTT
LpCBF1-2R	TGGAGTCAAAGCGCGCAGCA
LpCBF1-4R	GAACTGCATCTGCTTGCATG
LpCBF1-5R	TCTCCTTGAACTTGGTCCTCC
LpCBF3-1F	AGCATCCAGAGCTTACCAAG
LpCBF3-1R	GCACTATCACATCACATAACCAG
LpCBF12-1F	ACATGTTCGAGCTGGACATGTCCGG
LpCBF12-1R	GAATCTACAGGCAGATCTTCCAGC
LpCBF13-1R	AATCATAGAGTCAAAGCAGCAGTA
LpCBF16-1F	ACACTCCATACTCGAGCTCG
LpCBF17-1F	TACCTGCACCAAGCGATCTA
LpCBF17-1R	CGACAGTGACGTCAGTAGGTCCGT
LpCBF17-2R	TGGTTTCTCGTCGATGTCATCA
LpCBF17-dCAPS-1F	TTTCAGGTTCCGGCTGCACTGGGCAGGGAC
LpCBF20-2F	TCTCGCCGTCTTGGCTTTCCA
LpCBF21-1R	GTAATCACCGTTCTCCCAGT
LpCBF23-1F	TCGATCGGCACACTTCGATCG
LpCBF23-1R	CAAGTGCTCGACGATTCTCA
LpCBF27-1F	GTTCTACATGTCATCCGGAG
LpCBF27-1R	GATGGTCCATGTTCTTTCCG
LpCBF27B-2F	TTCACCGGGCATCTCCACTAG
LpCBF27B-1R	CCGTTGCATCATAAGTTGCA
LpCBF36-dCAPS-1F	CCAAATACAGGAAGAAACAGAGTAGGCTGA
LpCBF38-1F	CTCGACGACGAGCACTGGTT
LpCBF38-1R	CTTTGCCATCAACAGTGACG
LpCBF3′RACE4	TTCCGCTGGCACCGTCGTGGA
α-tubulin-1F	GTCGAACTTGTGGTCGATGC
α-tubulin-1R	GCTTACCATGAGCAGCTCTC
LpVRN1-1F	CTTCAGCTCGCACGGTGCTT
LpVRN1-1R	ATCACGGAGACAAAGGTCCA

vivo excisions of the pBluescript SK-phagemid vector were performed in the *E. coli* XLOLR strain. The second cDNA library screening was performed using the same method and membranes as the first, except for the use of a 477 bp fragment as a probe made from an *LpCBFIa* gene sequence amplified with LpCBF1-1F/LpCBF1-1R primer set. Positive plaques other than those identified in the first screening were identified. For RACE (random amplification of cDNA ends) cloning, cDNA was synthesized from the leaf of cold-treated plants using the GeneRacer™ kit (Invitrogen) according to the manufacture's instructions. The 3′-RACE procedure was conducted using the gene-specific LpCBF3′RACE4 primer designed to one of the putative *CBF* gene fragments. The 5′-RACE procedure failed to amplify any fragment using the gene-specific LpCBF17-1R primer specific to 3′-UTR sequence of one of the 3′-RACE products and 5′-RACE primer supplied in the kit, but a putative 5′ region fragment was amplified by PCR using the gene-specific LpCBF16-1F primer specific to *LpCBFIIIa* 5′-UTR sequence, and LpCBF17-1R primer. RACE products were extracted from agarose gels after electrophoresis and inserted into T-vector as described above. The nucleotide sequences of the inserts were determined with sequencing of the both strands.

Molecular phylogenetic analysis

For the phylogenetic analysis, the perennial ryegrass CBF proteins were compared with previously published CBF proteins from barley, wheat, rice, maize and tall fescue. Their accession numbers are detailed in Fig. 2. Multiple sequence alignments and phylogenetic trees were constructed by the neighbor-joining method, using the CLUSTALW program in the DNASIS®Pro ver. 2.0 software (Hitachi). Calculations of protein pI values were also performed using DNASIS®Pro ver. 2.0. For acidic C-terminal domain pI calculations, the region from the first acidic amino acid occurring after the second CBF signature motif DSAWR through to the last amino acid was utilized.

Gene expression analysis

Seedlings from perennial ryegrass cv. Aberystwyth S23 were grown in a controlled climate chamber (12 h day length, 150 μmol/m^2 s PFD, 25°C) for 3 weeks, and transferred to 4°C under the light and 150 μmol/m^2 s PFD condition for a short-term experiment (24 h), or under 8 h day length and 50 μmol/m^2 s condition for a long-term experiment (35 days). Total RNA was extracted from the shoots of eight to ten seedlings using TRIzol reagent (Invitrogen). After the treatment with DNase I (Takara) to remove contaminating DNA, cDNA was synthesized from 1 μg of RNA using oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). Semi-quantitative RT-PCR was performed using gene-specific primer sets as follows: for *LpCBFIa*, LpCBF12-1F and LpCBF1-2R; for *LpCBFIb*, LpCBF12-1F and LpCBF13-1R; for *LpCBFII*, LpCBF12-1F and LpCBF12-1R; for *LpCBFIIIc*, LpCBF3′RACE4 and LpCBF17-2R; for *LpCBFIVb*, LpCBF20-2F and LpCBF27B-1R. As an internal control, a fragment from the perennial ryegrass α-tubulin gene was amplified using the α-tubulin-1F and -1R primers. PCR was performed in a gene-specific manner as follows: for *LpCBFII*, *IVb* and α-tubulin, cycles of 94°C 30 s, 60°C 1 min, and 72°C 1 min; for *LpCBFIa*, *Ib* and *IIIc*, cycles of 94°C 30 s, 58°C 1 min, and 72°C 1 min. Amplified fragments were detected by electrophoresis using 1.5% (w/v) agarose gels and Tris–acetic acid–EDTA (TAE) buffer. The DNA fragments were visualized by staining with 0.5 μg/ml ethidium bromide.

Linkage analysis

The p150/112 one-way pseudotestcross genetic mapping population (Bert et al. 1999; Jones et al. 2002) was used for the linkage mapping of the perennial ryegrass *CBF* genes. Genomic DNA was extracted from the multiple heterozygous parent and the 108 F_1 genotypes using the modified CTAB method (Murray and Thompson 1980). Each PCR amplicon generated from the multiple heterozygous parent genomic DNA using the primer pair specific to each *CBF* gene was cloned. Primer sets are as follows: for *LpCBFIa* and *LpCBFIb*, LpCBF1-2F and LpCBF1-4R; for *LpCBFII*, LpCBF23-1F and LpCBF23-1R; for *LpCBFIIIb*, LpCBF17-1F and LpCBF21-1R; for *LpCBFIIIc*, LpCBF17-1F and Lp CBF17-1R; for *LpCBFIVb*, LpCBF27B-2F and LpCB F27B-1R; for *LpCBFVa*, LpCBF27-1F and LpCBF27-1R; for *LpCBFVb*, LpCBF38-1F and LpCBF38-1R; for *LpCBF3* (accession no. AY960831), LpCBF3-1F and LpCBF3-1R. Amplicons of *LpCBFIIIa* and *LpCBFIVa* could not be obtained using any primer sets tried in this study. For each gene, eight to ten of the cloned PCR amplicons were sequenced and compared by multiple sequence alignment to detect polymorphism between the two alleles of the heterozygous parent. Sequencing of amplicons amplified using LpCBF1-2F and LpCBF1-4R demonstrated that all amplicons sequenced were of the *LpCBFIb* sequence, not of *LpCBFIa*. For the mapping of the *CBF* genes, PCR-based markers showing segregation in the F_1 mapping population were developed. For *LpCBFIb*, an insertion–deletion (indel) polymorphism of the PCR amplicons generated using a primer pair of LpCBF1-2F and LpCBF1-5R was used. For the *LpCBFII*, cleaved amplified polymorphisms (CAPS) of the amplicons generated using a primer pair of LpCBF12-1F and LpCBF12-1R digested with *Dde*I were used for detection of single nucleotide polymorphisms (SNPs). For *LpCBFIIIb*, allele-specific dominant PCR markers were generated using a primer pair of LpCBF3′ RACE4 and LpCBF21-1R. For *LpCBFIIIc*, a degenerated cleaved amplified polymorphisms (dCAPS) marker was generated by digestion of the PCR amplicons produced using a primer pair of LpCBF17-dCAPS-1F and LpCBF17-2R with AvaII. For *LpCBFVb*, the dCAPS marker using a primer pair of LpCBF36-dCAPS-1F and LpCBF38-1R digested with *Dde*I was used. For the *LpVRN1* gene mapping, an indel PCR marker was developed using a primer set of LpVRN1-1F and LpVRN1-1R designed from the sequence of the perennial ryegrass *LpVRN1* gene (accession no. AY198326). These PCR-based markers were detected by electrophoresis using 1–4% (w/v) agarose gels and visualized as previously described. These markers were mapped within the context of a framework set of genetic markers from the p150/112 based reference map (Jones et al. 2002) using the MAPMAKER 2.0 application (Lander et al. 1987).

Results

Identification of *CBF* homologues in perennial ryegrass

Ten novel perennial ryegrass *CBF* homologue genes were identified and classified into five gene groups: *LpCBFI*, *II*, *III*, *IV* and *V*, based on amino acid sequence homology (Fig. 1). These sequences differed from the previously identified perennial ryegrass *LpCBF3* gene (Xiong and Fei 2006). Hybridization-based screening of the perennial ryegrass cDNA library was performed using a probe corresponding to the partial sequence of the *LpCBFIb* gene encoding the termi-

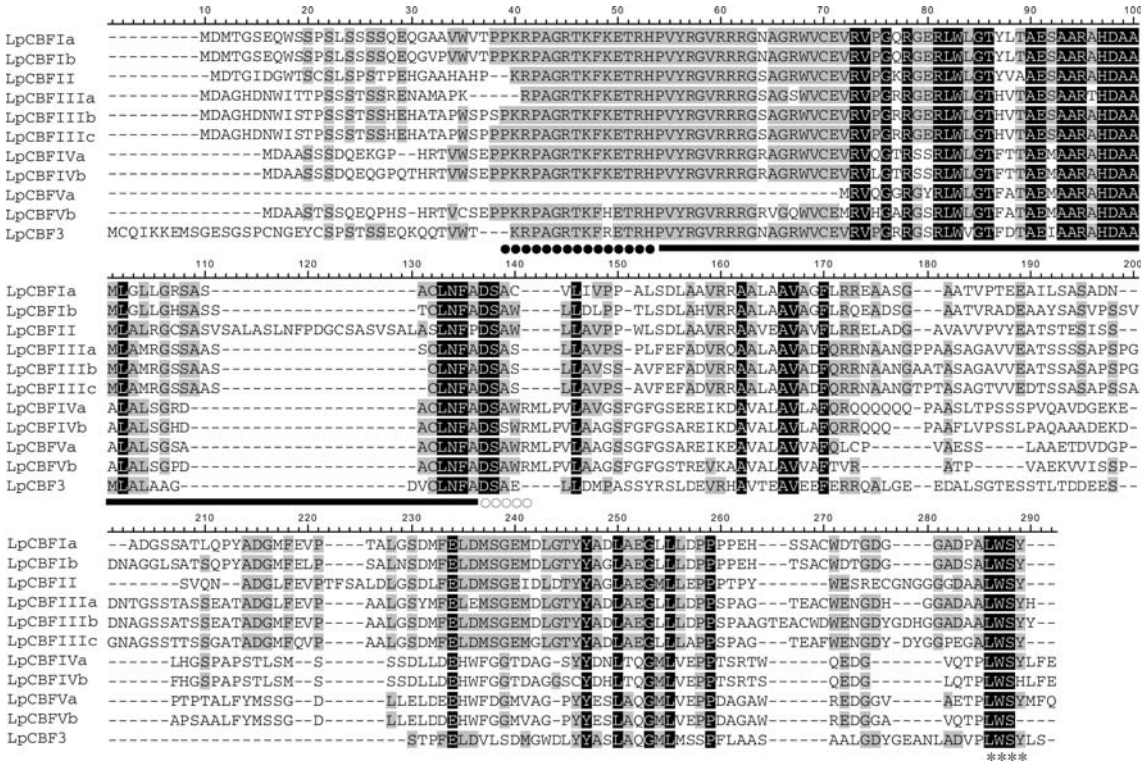

Fig. 1 Alignment of the amino acid sequences of *CBF* homologues cloned from perennial ryegrass. The conserved AP2 domain is *underlined*. *Filled circles* indicate the CBF signature conserved motif including the nuclear localization sequence. *Open circle* indicates the DSAWR motif. *Asterisk* indicates the LWSY motif

nal part of the AP2 domain and the acidic region. This procedure led to the identification of 22 positive plaques. Following sub-cloning, DNA sequence analysis, and BLASTN-based sequence similarity search, 14 clones encoding proteins with high similarity with known *CBF/DREB1* genes were identified, including 6 different genes designated as *LpCBFIa* (AB258392), *LpCBFIb* (AB258393), *LpCBFII* (AB258394), *LpCBFIIIa* (AB258395), *LpCBFIIIb* (AB258396) and *LpCBFIVa* (AB258398). The secondary screening of the cDNA library using the probe corresponding to the *LpCBFIa* gene partial sequence encoding the entire AP2 domain resulted in the identification of ten positive plaques which differed from the plaques identified in the first round of screening. Five clones derived from the second screening of positive plaques were demonstrated to encode CBF-like proteins. Although one of the five clones has the same sequence as *LpCBFIVa*, the other four clones encoded three new genes designated as *LpCBFIVb* (AB258399), *LpCBFVa* (AB258400) and *LpCBFVb* (AB258401). RACE-based cloning of cDNA derived from cold-treated leaf tissue identified an *LpCBFIII*-like sequence, which differs from *LpCBFIIIa* and L*pCBFIIIb*, designated *LpCBFIIIc* (AB258397).

Multiple alignment of amino acid sequence revealed these proteins to share extensive peptide similarity at the putative AP2 DNA-binding domain and the CBF signature sequence (PKK/RPAGRxKFxETRHP) including a putative *N*-localization signal sequence, as shown for other CBF/DREB1s (Jaglo et al. 2001; Dubouzet et al. 2003). The exception is *LpCBFVa*, which encodes a truncated amino acid sequence at the N-terminal region in comparison with other *LpCBF* genes (Fig. 1). The absence of an N-terminal amino acid for the LpCBFVa protein was attributable to nucleotide substitutions at the start codon, or the failure to clone the complete 5′-cDNA. LpCBF proteins also contain two CBF/DREB1 characteristic motifs, DSAWR at the end of the AP2 domain (Jaglo et al. 2001), and LWSY at the C-terminal region (Dubouzet et al. 2003), although there are some exceptions (Fig. 1). Moreover, they have acidic C-terminal domains (pI 3.51-4.47), which are believed to function as transcription activation regions. These suggested that *LpCBF* genes are members of a *CBF/DREB1* gene family.

Phylogenetic relationship between perennial ryegrass and other Poaceae CBFs

Phylogenetic relationships between perennial ryegrass, rice, barley, wheat, maize and tall fescue CBFs were determined (Fig. 2). Poaceae genes could be classified

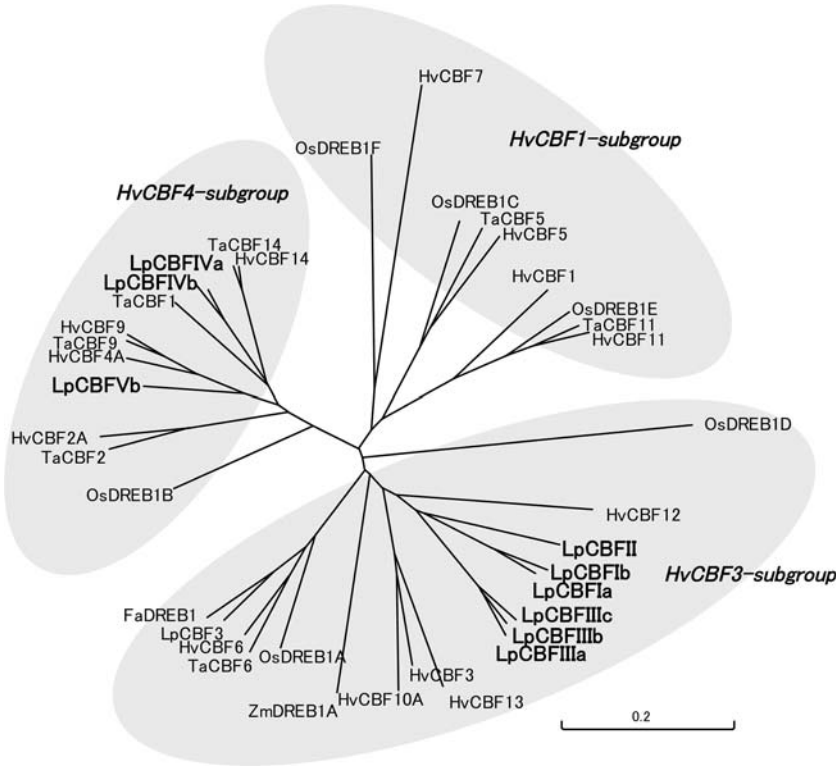

Fig. 2 Phylogenetic relationship of monocot CBF proteins. The full length of each protein was used for phylogenetic tree analysis. *Scale* indicates branch length. LpCBFVa was excluded from this analysis due to the lack of N-terminal amino acid sequence. The Genbank accession numbers of proteins other than LpCBF used in this analysis are: OsDREB1A (AF300970), OsDREB1B (AY785894), OsDREB1C (AP001168), OsDREB1D (AY785895), OsDREB1E (AY785896), OsDREB1F (AY785897), HvCBF1 (AY785837), HvCBF2A (AY785841), HvCBF3 (AY785845), HvCBF4A (AY785849), HvCBF5 (AY785855), HvCBF6 (AY785860), HvCBF7 (AY785864), HvCBF9 (AY785878), HvCBF10A (AY785882), HvCBF11 (AY785890), HvCBF12 (DQ095157), HvCBF13 (DQ095158), HvCBF14 (DQ095159), TaCBF1 (AF376136), TaCBF2 (AY785900), TaCBF5 (AY785902), TaCBF6 (AY785903), TaCBF9 (AY785905), TaCBF11 (AY785906), Ta CBF14 (AY785901), ZmDREB1A (AF450481), FaDREB1 (AY423713)

into the three subgroups: HvCBF1-, HvCBF3- and HvCBF4-subgroup, on the basis of phylogenetic analysis (Skinner et al. 2005). The affinities of Poaceae CBFs assigned LpCBF proteins to the HvCBF3- and HvCBF4-subgroups, HvCBF1-subgroup members being absent from perennial ryegrass CBFs. LpCBFI, LpCBFII and LpCBFIII were attributed to the HvCBF3-subgroup and formed a separate cluster to other Poaceae CBFs. LpCBF3 protein was classified into the HvCBF3-subgroup, but not within the LpCBF-specific phylogenetic cluster in this subgroup. The LpCBFIV and LpCBFV were assigned to the HvCBF4-subgroup. Like other Poaceae CBFs (Skinner et al. 2005), the perennial ryegrass HvCBF4-subgroup members, apart from LpCBFIVb, contain a consensus DSAWR motif, while the HvCBF3-subgroup members are missing the C-terminal arginine residue, and some family members even lack the tryptophan. In Poaceae CBFs of the HvCBF1- and HvCBF3-subgroups have an overall acidic character, while HvCBF4-subgroup members contain various pI characteristics from acidic to basic (Skinner et al. 2005). In perennial ryegrass, over all amino acid sequence of HvCBF3-subgroup members are acidic (4.62–5.63); on the other hand, HvCBF4-subgroup members excluding LpCBFVa have neutral pI (7.34–7.37). This finding demonstrates that the grouping proposed by Skinner et al. (2005) also applies to perennial ryegrass.

Expression of *LpCBF* genes induced by cold treatment

In many plant species, *CBF* gene expression is induced by cold treatment. To examine the response of perennial ryegrass *CBF* genes to a cold treatment, semi-quantitative RT-PCR analysis was performed using primer sets designed to specific *LpCBF* genes. First, low-temperature response under the light condition during a short period was assessed (Fig. 3a). Under the unstressed conditions, *LpCBF* transcripts were detected at low or negligible levels, and their levels

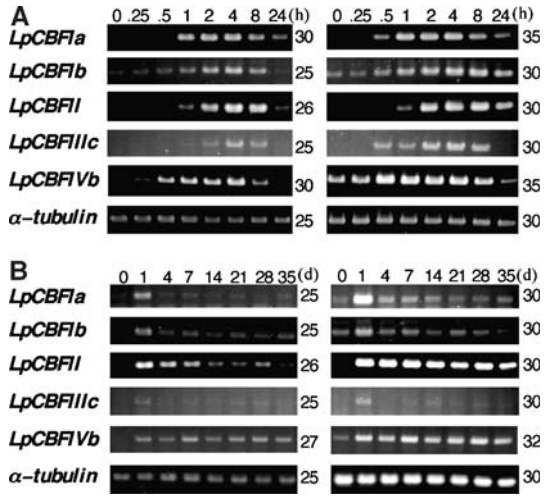

Fig. 3 Expression profile of *LpCBF* genes in response to low temperature (4°C) during a short term (24 h, under the light condition) (**a**) and a long term (35 days under the 8 h day length) (**b**). Transcript accumulation of *LpCBF* genes was monitored by semi-quantitative RT-PCR analysis using gene-specific primers. α-tubulin gene was used as a control. The *numerical value* described to the right of each electrophoresis photo indicates the PCR cycles

increased within 30 min after exposure of perennial ryegrass seedlings to low temperature. Transcript levels reached a high peak after 2–4 h, and then decreased. These expression patterns resemble those of other plant *CBF*s (Jaglo et al. 2001). Secondly, low-temperature response for a longer period under the 8 h day length condition was examined (Fig. 3b). Transcript levels of all *LpCBF*s examined increased for 1 day after the low-temperature treatment, but afterwards different expression patterns were observed for distinct genes. Transcript levels of HvCBF3-subgroup genes: *LpCBFIa*, *LpCBFIb*, *LpCBFII* and *LpCBFIIIc* decreased after 1 day, although the rate of decrease was different between the genes; on the other hand, *LpCBFIVb* transcript levels remained constant from 1 day until 35 days. These results showed that the low-temperature response of *LpCBFs* at the transcriptional levels was different between the genes.

LpCBF genes are clustered on LG5 of perennial ryegrass

In the Triticeae, multiple *CBF* genes form clusters near the low-temperature tolerance QTL on the genetic linkage maps. To confirm the location of the *LpCBF* loci on the perennial ryegrass genetic linkage map, we mapped the *LpCBF* genes using the p150/112 one-way pseudotestcross mapping population. Sequencing of PCR amplicons amplified from genomic DNA of the multiple heterozygous parent using primer sets specific to each *LpCBF* gene showed polymorphisms among each amplicon corresponding to the *LpCBFIb*, *LpCBFII*, *LpCBFIIIc* and *LpCBFVb* genes (S1). PCR-based markers detecting allelic variation within the mapping population were developed. Sequencing analysis did not detect polymorphism within the *LpCBFIIIb* gene, but the PCR amplicon segregated as a dominant feature. Linkage genetic analysis of 108 individuals of the mapping population allowed assignment of the five LpCBF markers on the linkage map. Four markers: LpCBFIb, LpCBFII, LpCBFIIIb and LpCBFIIIc were located on LG5 between the markers e33t62210 and e38t50189 within a cluster 2.2 cM across (Fig. 4). Two markers: LpCBFIIIa and LpCBFIIIc co-segregated in this population. Segregation between markers LpCBFIb and LpCBFII was observed in one out of 108 plants, and between markers LpCBFII and LpCBFIIIs was observed in two plants. On the other hand, the LpCBFVb marker was located within the framework of the reference genetic map to the 6.7 cM interval between the markers e33t62180 and e41t47146 on LG1 (Fig. 4).

In the Triticeae, *CBF* gene clusters were located near the low-temperature tolerance QTL *Fr-2* on homoeologous group 5. Meanwhile, another major cold tolerant QTL *Fr-1* are located near the *Vrn-1* loci about 25–30 cM away from *Fr-2* on chromosome 5 of the Triticeae (Stockinger et al. 2006). A segregating indel marker in the p150/112 mapping population was developed for the *LpVRN1* gene. Genetic linkage analysis showed that LpVRN1 marker was located on LG4 as reported by Jensen et al. (2005), co-segregating with the markers e41t50710 and Xr1538 (Jones et al. 2002). This result indicated that two candidate gene loci for low-temperature tolerance QTL, *CBF* genes and *VRN1* gene, on the chromosome 5 of wheat and barley, were separated into two chromosomes of perennial ryegrass.

Discussion

Since the initial characterization of *CBF/DREB1* gene from *A. thaliana* (Gilmour et al. 1998; Liu et al. 1998; Stockinger et al. 1997), many *CBF/DREB1* homologue genes have been cloned from various plant species (Nakashima and Yamaguchi-Shinozaki 2006). A total of ten *CBF* homologue genes were isolated from perennial ryegrass, in addition to a previously characterized family member (Xiong and Fei 2006). These novel genes are supposed to function as other *CBF*s because of the homology of the primary structure and the induction of gene expression response to low-tem-

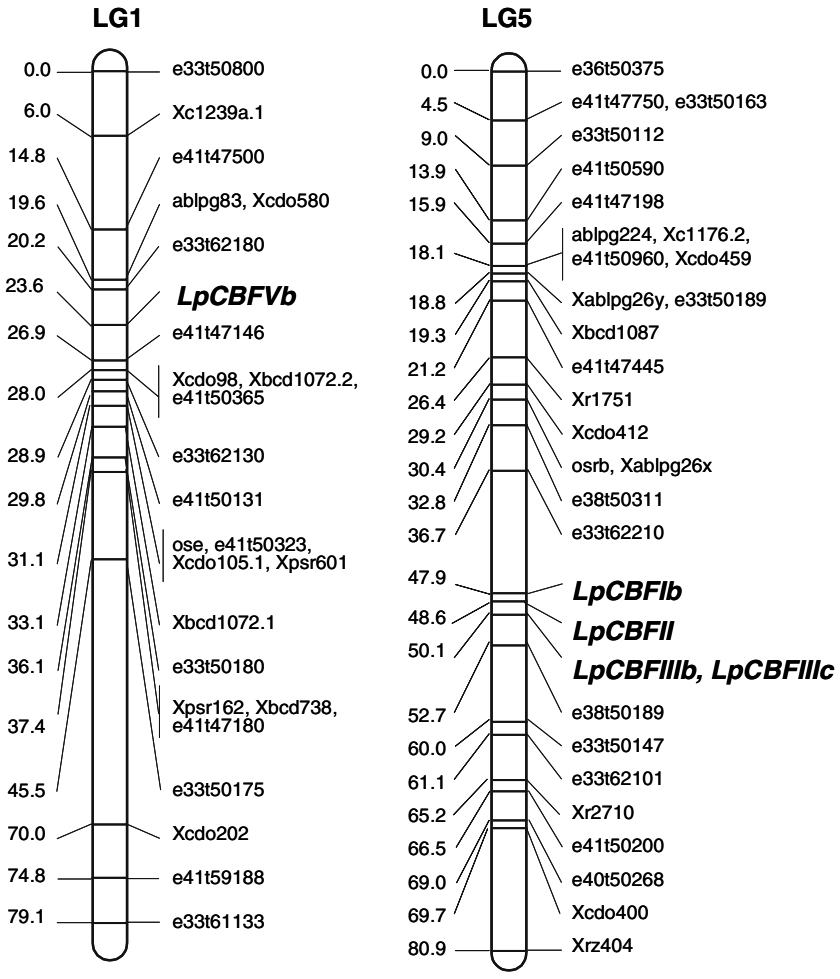

Fig. 4 Loci of the *LpCBF* genes on linkage group 1 and 5 of the p150/112 perennial ryegrass mapping population. Nomenclature of AFLP loci and RFLP loci is as described by Jones et al. (2002). The *numerical value* described to left of each marker indicates genetic distance (cM)

perature treatment, although binding of the LpCBF products to the CRT/DRE sequence was not confirmed. Since these putative *CBF* genes were cloned from a cDNA pool, which originated from heterozygous plants with different genotypes, some of them might be alleles. However, at least, *LpCBFIb*, *II*, *IIIb*, *IIIc* and *Vb* were apparently corresponding to different gene family members. We confirmed the two different sequences of each gene from the heterozygous diploid plant for *LpCBFIb*, *II*, *IIIc* and *Vb* by genomic sequencing. Concerning *LpCBFIIIb*, only one sequence was confirmed, but a dominant PCR marker specific to *LpCBFIIIb* sequence segregated in the mapping population. A large gene family has been observed for *CBF* in monocot species. For example, in barley, 20 *CBF* genes were identified (Skinner et al. 2005). Other *CBF* homologue genes are likely yet to be identified in perennial ryegrass.

Phylogenetic analysis revealed that LpCBFs were classified to HvCBF3- and HvCBF4-subgroups (Fig. 2). The characteristic primary structures of each subgroup, such as the DSAWR motif and pI value, were also seen in perennial ryegrass. These demonstrated that the grouping scheme proposed by Skinner et al. (2005) also applied to perennial ryegrass. In barley the HvCBF1- and HvCBF3-subgroup members could bind to the CRT motif under both low and warm temperature conditions. In contrast, HvCBF4-subgroup members would only bind to the CRT motif under low-temperature conditions (Skinner et al. 2005). This indicated that the functional role of CBFs might be different among the CBF subgroups. In perennial ryegrass, gene expression patterns in response to a long period of low temperature were different between the HvCBF3- and HvCBF4-subgroup genes (Fig. 3). Different functional roles of each CBF family member have been reported in *Arabidopsis* (Novillo et al. 2004) and *Brassica* (Zhao et al. 2006). Further detailed characterization of each subgroup members would clarify the relationships between each *CBF* gene and low-temperature tolerance in monocots.

Genetic linkage analysis has revealed that four of the 5 *LpCBF* genes are located on LG5, forming a gene cluster similar to those in the Triticeae (Fig. 4). *CBF* gene clusters of barley and wheat were located on the homoeologous group 5 chromosome (Skinner et al.

2006; Tondelli et al. 2006; Miller et al. 2006), corresponding to the perennial ryegrass LG5 and the upper region of LG4 as revealed by comparative genetic studies (Sim et al. 2005). The location of the *LpCBF* gene cluster showed conserved synteny with the Triticeae. In rice, three *CBF/DREB1* genes, *OsDREB1A*, *OsDREB1B* (Dubouzet et al. 2003) and *OsDREB1H* (Skinner et al. 2005) are believed to be located tandemly within about 10 kbp on chromosome 9 of rice, based on the BLAST search of the release 4 of the TIGR rice pseudomolecules (TIGR Rice Genome Annotation, http://www.tigr.org/tdb/e2k1/osa1/index.shtml), corresponding to the perennial ryegrass LG5. This observation confirms the predicted syntenic relationships between perennial ryegrass and rice *CBF* genes. On the other hand, *LpCBFVb* was mapped on LG1. Some *CBF/DREB1* genes are mapped to a different locus other than the *CBF* cluster. For example, in barley, five HvCBF genes were mapped or localized to different chromosomes other than chromosome-5H, although the loci were not located on 1H, which corresponds to perennial ryegrass LG1 (Skinner et al. 2006).

In the barley 'Nure' × 'Tremois' population, the *CBF* cluster consisting of 6 *CBF* genes was located around the cold tolerance QTL, *Fr-H2* (Tondelli et al. 2006). In addition, a cluster of 11 *CBF* genes was mapped at the frost-tolerance locus *Fr-A* ^{m}*2*in diploid wheat (Miller et al. 2006). *Fr-H2* and *Fr-A*^{m}*2* were also QTLs for the level of transcription of the cold-regulated gene *COR14b* containing the CRT/DRE motif in its promoter region (Francia et al. 2004; Vágújfalvi et al. 2003). The *CBF* gene family was consequently thought to provide candidate genes for the *Fr-2* loci. Recently, in meadow fescue (*Festuca pratensis* Huds.), whose genome is related closely to *Lolium* spp, some QTLs for the frost tolerance and winter survival in the field were mapped on LG5 (Alm et al. 2006). Based on the comparative map, Alm et al. (2006) proposed that the two QTLs, QFt5F-2 for frost tolerance and QWs5F-1 for winter survival, correspond to the *Fr-2* loci in barley and wheat. Comparison of the genetic map of meadow fescue LG5 with that of perennial ryegrass LG5 using common RFLP markers, loci of the marker Xcdo412 and Xcdo400 flanking the *CBF* cluster on the perennial ryegrass linkage map are near the QFt5F-2 and QWs5F-1 regions on the meadow fescue linkage map. This supports the conserved syntheny between the loci of QFt5F-2 and QWs5F-1 in meadow fescue and the Fr-2 loci linked to the *CBF* clusters in the Triticeae. In the p150/112 mapping population, a QTL for electrical conductivity corresponding to frost tolerance was located on the upper region of LG4, but QTLs related to low-temperature tolerance have not been found near *CBF* gene region (Yamada et al. 2004). In the 'Aurora' × 'Perma' F_2 genetic map population of perennial ryegrass (Armstead et al. 2004), QTL for freezing tolerance in tillers was observed on the upper region of LG5 (T. Yamada et al. unpublished data). Considering the loci of common markers localized on the linkage maps of p150/112 and 'Aurora' × 'Perma', this QTL for freezing tolerance appears to be located some distance from the *CBF* gene cluster, although there are a few markers localized on LG5 of both linkage maps. Because both the p150/112 and 'Aurora' × 'Perma' mapping populations had not been designed for the analysis of low-temperature tolerance, they may not be adequate material for the QTL analyses related to low-temperature tolerance. To know whether the loci of *CBF* genes are involved in QTLs for low-temperature tolerance in perennial ryegrass, examinations of appropriate populations using parental plants having other genotypes are necessary.

Comparisons of the genetic maps of rice, perennial ryegrass, meadow fescue and the Triticeae have revealed a large-scale chromosomal rearrangement on rice chromosome 3 and LG4 in perennial ryegrass and meadow fescue relative to the Triticeae chromosomes 4 and 5 (Sim et al. 2005; Alm et al. 2003). Rice chromosome 3 and LG4 of perennial ryegrass and meadow fescue correspond to two Triticeae chromosome segments. One segment corresponds to the whole of homoeologous group 4, and the other to a partial region of homoeologous group 5. In this study, two genes located on the Triticeae chromosome-5 were located on different linkage groups of perennial ryegrass: *CBF* genes were mapped on LG5, on the other hand, *LpVRN1*, the candidate gene supposed to be responsible for flowering time QTL (Jensen et al. 2005), was mapped on LG4. Xpsr580, a marker assigned to coordinate position 14.7 cM distal to the *LpVRN1* locus, was mapped on the Triticeae homoeologous group 5, while Xpsr922, a marker localized on the 6.4 cM lower region of *LpVRN1* locus, was mapped on the Triticeae homoeologous group 4 (Jones et al. 2002). The chromosomal arrangement of perennial ryegrass LG4 is supposed to be more of the ancestral form than that of the Triticeae. It is likely that the translocation of the fragment of the chromosome corresponding to perennial ryegrass LG4 upper region including *LpVRN1* gene locus to the terminal region of the chromosome corresponding to perennial ryegrass LG5 in the ancestor of the Triticeae resulted in the present Triticeae LG5.

In the Triticeae, homoeologous group 5 has two low-temperature tolerance QTLs: *Fr-1* and *Fr-2*. The loci of

Fr-1 are near the *Vrn-1* loci. Galiba et al. (1995) reported that *Fr-A1* has been mapped 2 cM distal from *Vrn-A1* in wheat 5A, while Francia et al. (2004) suggested that *Fr-H1*/*Vrn-H1* was a pleiotropic effect of the *HvBM5A* gene, the candidate for the *Vrn-H1* gene in barley. In meadow fescue, it was proposed that the distal QTL for winter survival on 5F corresponds to the *Fr-1* loci, and that the proximal frost tolerance/winter survival QTLs correspond to the *Fr-2* loci (Alm et al. 2006). In perennial ryegrass, the information of the candidate gene loci and the chromosome arrangement among grasses obtained in this study indicate that it would be useful to analyze the QTLs for low-temperature tolerance using other appropriate populations.

The next generation of molecular genetic markers for forage grass breeding will be derived from functionally defined genes associated biochemically and physiologically related to the target phenotypic trait (Faville et al. 2004; Cogan et al. 2006). The *CBF* genes could be useful candidate genes for markers linked to low-temperature tolerance including freezing tolerance. Recently, in *Arabidopsis*, deletion mutation at the promoter region of *CBF2* gene was associated with the phenotype of the reduced freezing tolerance (Alonso-Blanco et al. 2005). In monocots, *CBF* genes form a large gene family. Determination of the functional role of each gene will be necessary for the development of specific genetic markers associated with the low-temperature tolerance.

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