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

K. Tamura and T. Yamada

K. Tamura

National Agricultural Research Center for Hokkaido Region, National Agriculture and Food Research Organization, Hitsujigaoka, Toyohira, Sapporo 062-8555, Japan

T. Yamada(✉)

Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita, Sapporo 060-0811, Japan

E-mail: yamada@fsc.hokudai.ac.jp

Tel.: +81-11-7063644

Fax: +81-11-7063644

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 10 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 2.2cM, 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.

Keywords Perennial ryegrass – CBF – Gene expression – Linkage analysis – Conserved synteny – Low-temperature tolerance

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 CBF (C-repeat binding factor) /DREB1 (dehydration-responsive element-binding protein 1) 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 lead to an increased tolerance to freezing and other stresses due to 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 alleic 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. 1997; Francia et al. 2004) in barley. Comparative genomic studies have revealed that homoeologous group 5 in wheat and barley correspond 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. $3x10^5$ recombinant plaques were screened and positive plaques were isolated. After plaque purification, in 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 membrans as the first, except for the use of a 477bp fragment as a probe made from a

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^2s PFD, 25°C) for 3 weeks, and transferred to 4°C under the light and 150 µmol/m^2s PFD condition for a short term experiment (24 hours), or under 8h daylength and 50 µmol/m^2s condition for a long term experiment (35 days). Total RNA was extracted from the shoots of 8-10 seedlings using TRIzol reagent (Invitrogen). After treatment with DNase I (Takara) to remove contaminating DNA, cDNA was synthesized from 1µg 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 sec, 60°C 1min, and 72°C 1min; for LpCBFIa, Ib

and IIIc, cycles of 94°C 30 sec, 58°C 1min, and 72°C 1min. 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 pseudo testcross 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 LpCBF17-1R; for LpCBFIVb, LpCBF27B-2F and LpCBF27B-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, 8 to 10 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 DdeI 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 DdeI, 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 terminal 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 10 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 5 clones has the same sequence as LpCBFIVa, the other 4 clones encoded 3

new genes designated as LpCBFIVb (AB258399), LpCBFVa (AB258400) and LpCBFVb (AB258401). RACE-based cloning of cDNA derived from cold-treated leaf tissue identified a LpCBFIII-like sequence which differs from LpCBFIIIa and LpCBFIIIb, 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 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 increased within 30 min after exposure of perennial ryegrass seedlings to low temperature. Transcript levels reached a high peak after 2 to 4 hours, and then decreased. These expression patterns resemble those of other plant CBFs (Jaglo et al. 2001). Secondly, low-temperature response for a longer period under the 8 hours day length condition was examined (Fig. 3B). Transcript levels of all LpCBFs 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 among 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 were different among 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 location of the LpCBF loci on the perennial ryegrass genetic linkage map, we mapped the LpCBF genes using the p150/112 one-way pseudo-testcross 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 6.7cM 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 25~30cM 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 CBFs because of the homology of the primary structure and the induction of gene expression response to low-temperature 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 correspond 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 to be yet 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 may 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. 2001) 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' x'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^m2 in diploid wheat (Miller et al. 2006). Fr-H2 and Fr-A^m2 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 region on the meadow fescue linkage map. This supports the conserved syntheny between 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' x '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 (Yamada et al. unpublished data). Considering the loci of common markers localized on the linkage maps of p150/112 and 'Aurora' x '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' x '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 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, 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 6.4cM 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 2cM 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 development of specific genetic markers associated with the low-temperature tolerance.

Acknowledgement

We gratefully acknowledge Dr Sohei Kobayashi, Dr Kazuhiro Tase and Mr Yasuharu Sanada for their technical advice, and Miss Mayumi Hata and Miss Mayuko Kawamura for their technical assistance. This work was supported, in part, by Grants-in Aid for Scientific Research (No. 17380163) from the Ministry of Education, Science, Sports and Culture, Japan. The authors thank Prof. Michael Hayward (Rhydgoch Genetics, Aberystwyth, UK) and Dr. Toshinori Komatsu for careful critical reading of the manuscript.

References

Alm V, Fang C, Busso CS, Devos KM, Vollan K, Grieg Z, Rognli OA (2003) A linkage map of meadow fescue (Festuca pratensis Huds.) and comparative mapping with other Poaceae species. Theor Appl Genet 108:25-40

Alm V, Busso CS, Larsen A, Humphreys MW, Rognli OA (2006) Quantitative trait loci for frost tolerance, winter survival and drought tolerance in meadow fescue (Festuca pratensis Huds.), and comparative mapping with cereals. Genetics, in print

Alonso-Blanco C, Gomez-Mena C, Llorente F, Koornneef M, Salinas J, Martinez-Zapater JM (2005) Genetic and molecular analyses of natural variation indicate CBF2 as a candidate gene for underlying a freezing tolerance quantitative trait locus in Arabidopsis. Plant Physiol 139:1304-1312

Armstead IP, Turner LB, Farrell M, Skot L, Gomez P, Montoya T, Donnison IS, King IP, Humphreys MO (2004) Synteny between a major heading-date QTL in perennial ryegrass (Lolium perenne L.) and the Hd3 heading-date locus in rice. Theor Appl Genet 108:822-828

Bert PF, Charmet G, Sourdille P, Hayward MD, Balfourier F (1999) A high-density molecular map for ryegrass (Lolium perenne) using AFLP markers. Theor Appl Genet 99:445–452

Choi DW, Rodriguez EM, Close TJ (2002) Barley Cbf3 gene identification, expression pattern, and map location. Plant Physiol 129:1781–1787

Cogan NO, Ponting RC, Vecchies AC, Drayton MC, George J, Dracatos PM, Dobrowolski MP, Sawbridge TI, Smith KF, Spangenberg GC, Forster JW (2006) Gene-associated single nucleotide polymorphism discovery in perennial ryegrass (Lolium perenne L.). Mol Gen Genomics 276: 101-112

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. Plant J 33:751–763

Faville MJ, Vecchies AC, Schreiber M, Drayton MC, Hughes LJ, Jones ES, Guthridge KM, Smith KF, Sawbridge T, Spangenberg GC, Bryan GT, Forster JW (2004) Functionally associated molecular genetic marker map construction in perennial ryegrass (Lolium perenne L.). Theor Appl Genet 110:12-32

Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Tóth B, Hayes PM, Skinner JS, Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map. Theor Appl Genet 108:670–680

Galiba G, Quarrie SA, Sutka J, Morgounov A, Snape JW (1995) RFLP mapping of vernalization (Vrn1) and frost resistance (Fr1) on chromosome 5A of wheat. Theor Appl Genet 90:1174-1179

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

Plant J 16:433–442

Gilmour SJ, Fowler SG, Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol 54:767–781

Hayes PM, Blake T, Chen THH, Tragoonrung S, Chen F, Pan A, Liu B (1993) Quantitative trait loci on barley (Hordeum vulgare L.) chromosome 7 associated with components of winter hardiness. Genome 36:66-71

Jaglo-Ottosen KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280:104–106

Jaglo-Ottosen 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 Physiol 12:910–917

Jensen LB, Andersen JR, Frei U, Xing Y, Taylor C, Holm PB, Lübberstedt T (2005) QTL mapping of vernalization response in perennial ryegrass (Lolium perenne L.) reveals co-location with an orthologue of wheat VRN1. Theor Appl Genet 110:527–536

Jones ES, Mahoney NL, Hayward MD, Armstead IP, Jones JG, Humphreys MO, Kishida T, Yamada T, Balfourier F, Charmet G, Forster JW (2002) An enhanced molecular marker-based genetic map of perennial ryegrass (Lolium perenne L.)

reveals comparative relationships with other Poaceae genomes. Genome 45:282–295

Kobayashi F, Takumi S, Kume S, Ishibashi M, Ohno R, Murai K, Nakamura C (2005) Regulation by Vrn-1/Fr-1 chromosomal intervals of CBF-mediated Cor/Lea gene expression and freezing tolerance in common wheat. J Exp Bot 56:887–895

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Laurie DA, Pratchett N, Bezant JH, Snape JW (1995) RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter spring barley (Hordeum vulgare L.) cross. Genome 38:575–585

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10:1391–1406

Miller AK, Galiba G, Dubcovsky J (2006) A cluster of 11 CBF transcription factors is located at the frost tolerance locus Fr-A^{m}2 in Triticum monococcum. Mol Gen Genomics 275:193-203

Murray MG, Thompson WF (1980) The isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321-4325

Nakashima K, Yamaguchi-Shinozaki K (2006) Regulons inovolved in osmotic stress-responsive and cold stress-responsive gene expression in plants. Physiol Plant 126:62-71

Nakayama S, Tsurumi Y, Larsen A, Takai T, Iriki N (2001) Breeding forage grasses for winter survival, In: Iriki N et al. (eds) Low Temperature Plant Microbe Interactions Under Snow, Hokkaido National Agricultural Experiment Station, Sapporo, pp 169-180

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

Qin F, Sakuma Y, Li J, Liu Q, Li YQ, Shinozaki K, Yamaguchi-Shinozaki K (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in Zea mays L. Plant Cell Physiol 45:1042–1052

Sim S, Chang T, Curley J, Warnke SE, Barker RE, Jung G (2005) Chromosomal rearrangements differentiating the ryegrass genome from the Triticeae, oat, and rice genomes using common heterologous RFLP probes. Theor Appl Genet 110:1011-1019

Skinner JS, von Zitzewitz J, Szűcs P, Marquez-Cedillo L, Filichkin T, Amundsen K, Stockinger EJ, Thomashow MF, Chen THH, Hayes PM (2005) Structural, functional, and phylogenetic characterization of a large CBF gene family in barley. Plant Mol Biol 59:533–551

Skinner JS, Szűcs P, von Zitzewitz J, Marquez-Cedillo L, Filichkin T, Stockinger EJ, Thomashow MF, Chen THH, Hayes PM (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in Arabidopsis. Theor Appl Genet 112:832-842

Snape JW, Semikhodskii A, Fish L, Sarma RN, Quarrie SA *et al.* (1997) Mapping of frost tolerance loci in wheat and comparative mapping with other cereals. Acta Agron Hung 45:265-270

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. Proc Natl Acad Sci USA 94:1035–1040

Stockinger EJ, Cheng H, Skinner JS (2006) Structural organization of barley CBF genes coincident with a QTL for cold hardiness. In: Chen THH, Uemura M, Fujikawa S (eds) Cold Hardiness in Plants. CABI Publishing, Wallingford, pp 53-63

Sutka J, Snape JW (1989) Location of a gene for frost resistance on chromosome 5A of wheat. Euphytica 42:41-44

Tang MJ, Lu SY, Jing YX (2005) Isolation and identification of a cold-inducible gene encoding a putative DRE-binding transcription factor from Festuca arundinacea. Plant Physiol Biochem 43:233-239

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571-599

Tondelli A, Francia E, Barabaschi D, Aprile A, Skinner JS, Stockinger EJ, Stanca AM, Pecchioni N (2006) Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. Theor Appl Genet 112:445-454

Toth B, Galiba G, Feher E, Sutka J, Snape JW (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. Theor Appl Genet 107:509-514

Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) The cold regulated transcriptional activator Cbf3 is linked to the frost tolerance locus Fr-A2 on wheat chromosome 5A. Mol Gen Genome 269:60–67

Wilkins PW (1991) Breeding perennial ryegrass for agriculture. Euphytica 52:201-214

Xiong Y, Fei SZ (2006) Functional and phylogenetic analysis of a DREB/CBF-like gene in perennial ryegrass (Lolium perenne L.). Planta 224: 878-888

Xue GP (2002) Characterisation of the DNA-binding profile of barley HvCBF1 using an enzymatic method for rapid, quantitative and high-throughput analysis of the DNA-binding activity. Nucl Acids Res 30:e77

Yamada T, Jones ES, Cogan NOI, Vecchies AC, Nomura T, Hisano H, Shimamoto Y, Smith KF, Hayward MD, Forster JW (2004) QTL analysis of morphological, developmental, and winter hardiness-associated traits in perennial ryegrass. Crop Sci 44:925-935

Zhao TJ, Sun S, Liu Y, Liu JM, Liu Q, Yan YB, Zhou HM (2006) Regulating the drought-responsive element (DRE)-mediated signaling pathway by synergic functions of trans-active and trans-inactive DRE binding factors in Brassica

napus. J Biol Chem 281:10752-10759

Figure Legends

Figure 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.

Figure 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).

Figure 3

Expression profile of LpCBF genes in response to low temperature (4°C) during a short term (24 hours, under the light condition) (A) and a long term (35 days under the 8h 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.

Figure 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).

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

Electronic supplementary material

S1

Comparison between two allele sequences of LpCBFIb (A), LpCBFII (B), LpCBFIIIc (C) and LpCBFVb (D) from the heterozygous parent (HP) of p150/112 mapping population and cDNA sequences from cv. Aberystwyth 23. Arrows indicate the primer sequence region used for PCR-based markers. Black boxes indicate the polymorphisms between two alleles of HP. White boxes indicate the restriction enzyme recognition sites used for CAPS or dCAPS markers. The Numerical values described left of cDNA sequences indicate the base numbers from the first ATG

Figure 1

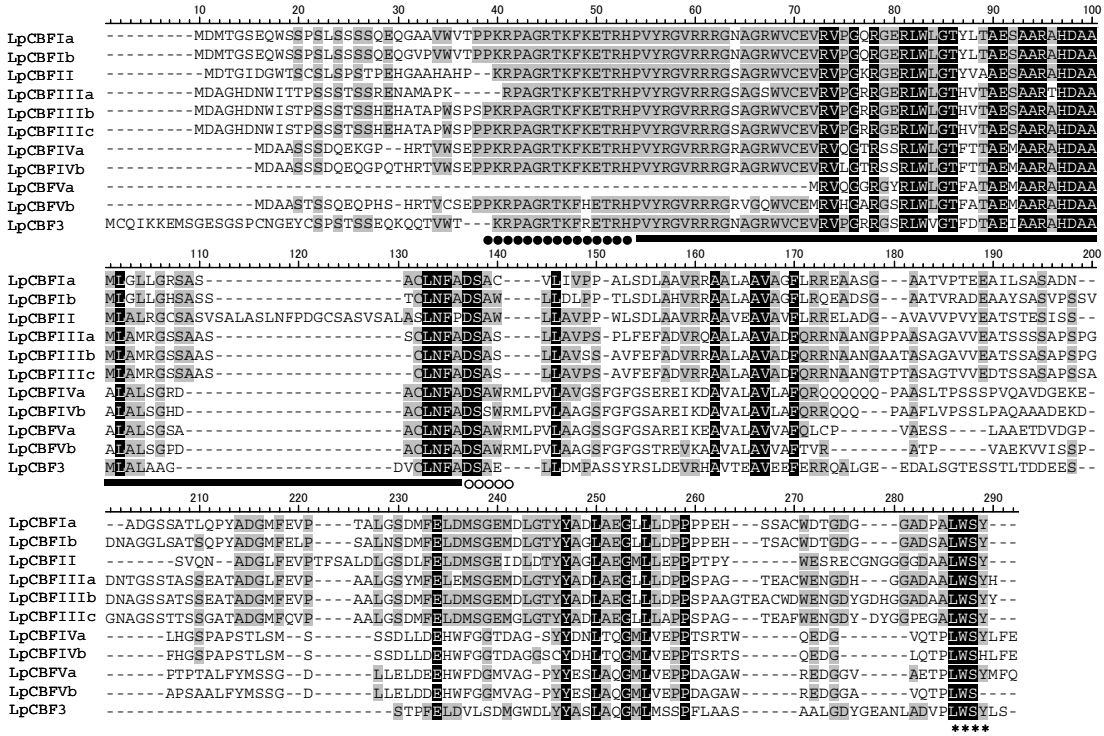

LpCBFIa
LpCBFIb
LpCBFII
LpCBFIIIa
LpCBFIIIb
LpCBFIIIc
LpCBFIVa
LpCBFIVb
LpCBFVa
LpCBFVb
LpCBF3

Figure 2

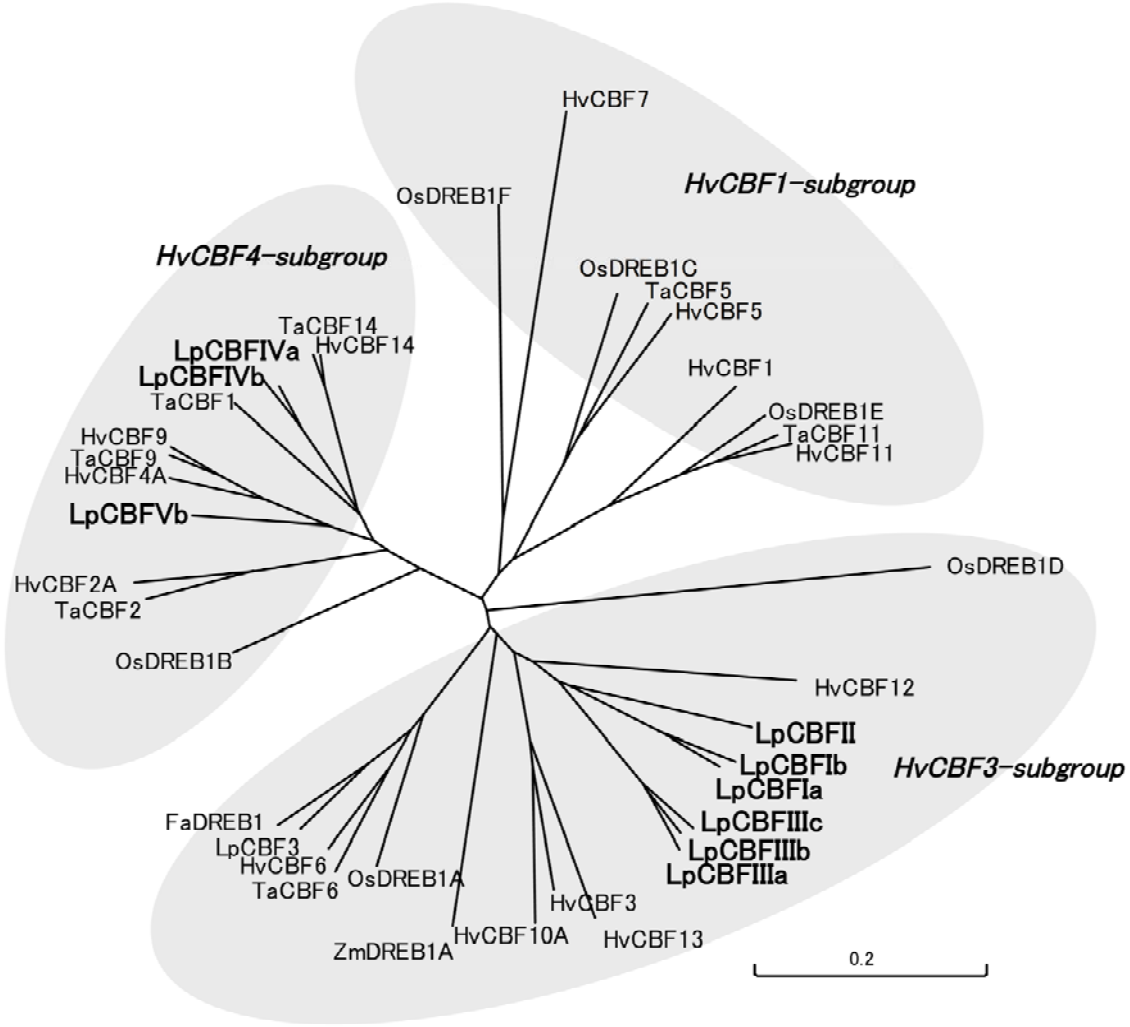

HvCBF7
HvCBF1-subgroup
OsDREB1F
HvCBF4-subgroup
OsDREB1C
TaCBF5
HvCBF5
TaCBF14
HvCBF14
LpCBFIVa
LpCBFIVb
TaCBF1
HvCBF1
OsDREB1E
TaCBF11
HvCBF11
HvCBF9
TaCBF9
HvCBF4A
LpCBFVb
OsDREB1D
HvCBF2A
TaCBF2
OsDREB1B
HvCBF12
LpCBFII
LpCBFIb
LpCBFIa
HvCBF3-subgroup
FaDREB1
LpCBF3
HvCBF6
TaCBF6
OsDREB1A
LpCBFIIIc
LpCBFIIIb
LpCBFIIIa
HvCBF3
HvCBF10A
ZmDREB1A
HvCBF13
0.2

Figure 3

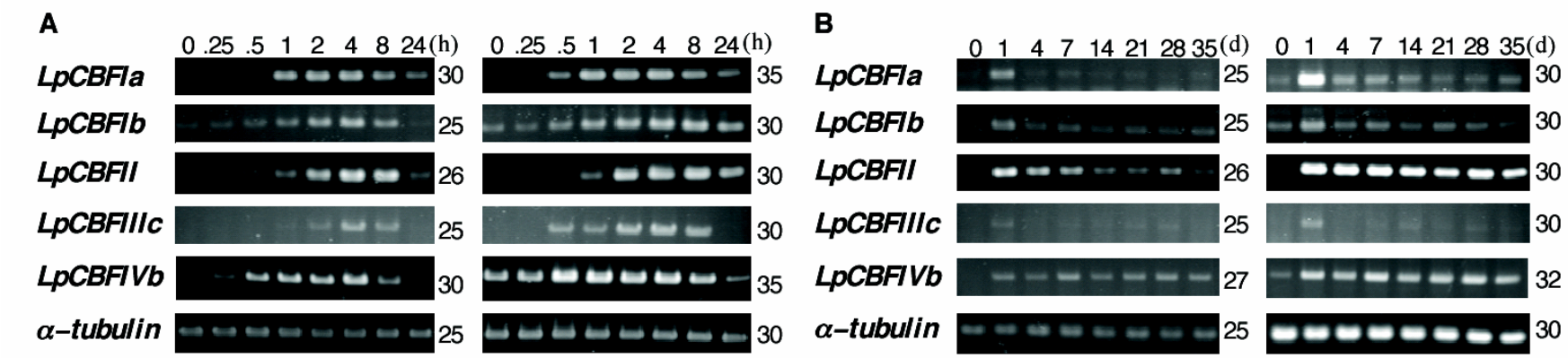
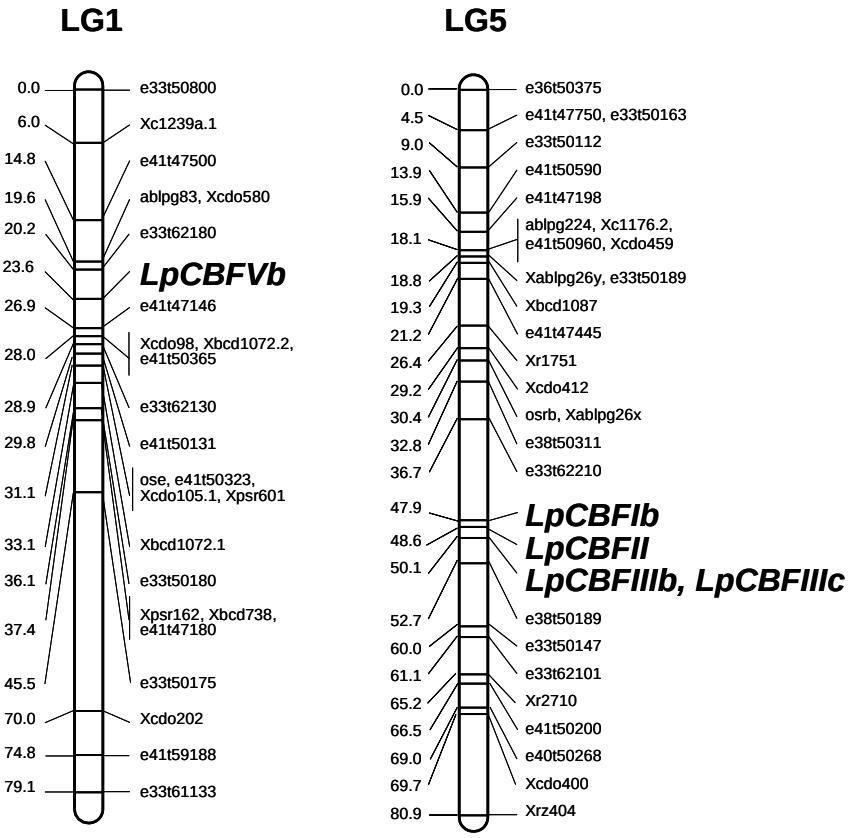
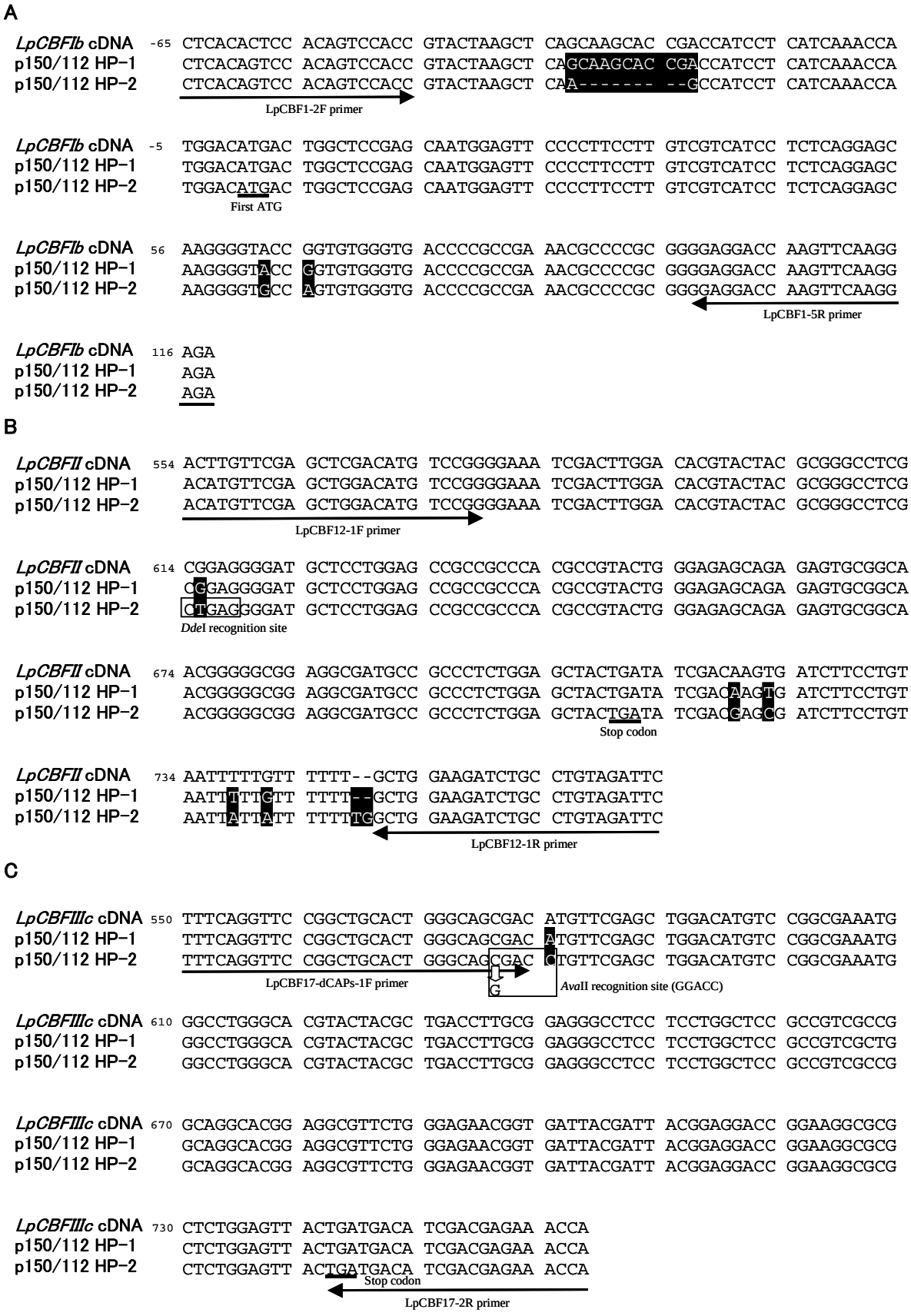

A
0 .25 .5 1 2 4 8 24(h)
0 .25 .5 1 2 4 8 24(h)
LpCBFIa
LpCBFIb
LpCBFII
LpCBFIIIc
LpCBFIVb
α–tubulin
30
25
26
25
30
25
35
30
30
30
35
30
B
0 1 4 7 14 21 28 35(d)
0 1 4 7 14 21 28 35(d)
LpCBFIa
LpCBFIb
LpCBFII
LpCBFIIIc
LpCBFIVb
α–tubulin
25
25
26
25
27
25
30
30
30
30
32
30

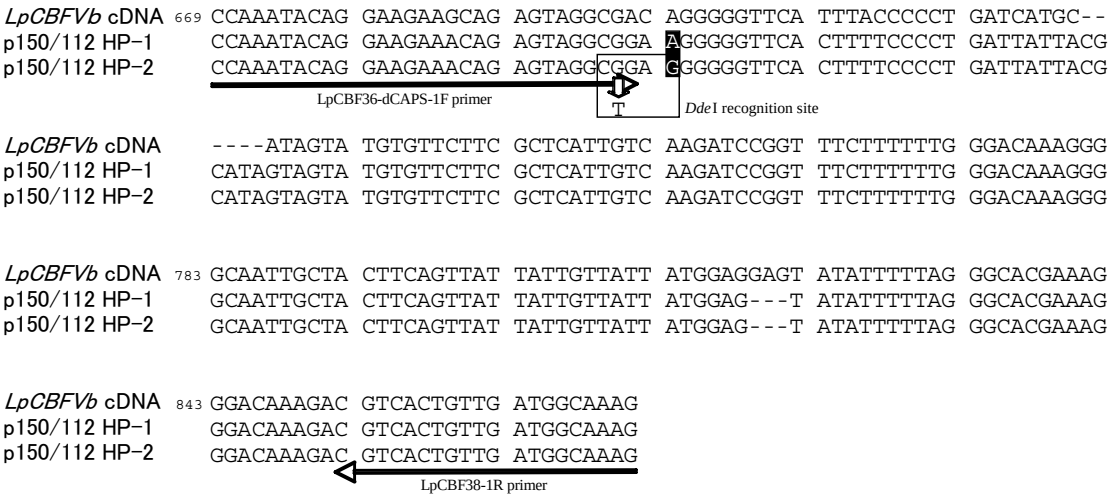
Figure 4


LG1
0.0 e33t50800
6.0 Xc1239a.1
14.8 e41t47500
19.6 ablpg83, Xcdo580
20.2 e33t62180
23.6 LpCBFVb
26.9 e41t47146
28.0 Xcdo98, Xbcd1072.2, e41t50365
28.9 e33t62130
29.8 e41t50131
31.1 ose, e41t50323, Xcdo105.1, Xpsr601
33.1 Xbcd1072.1
36.1 e33t50180
37.4 Xpsr162, Xbcd738, e41t47180
45.5 e33t50175
70.0 Xcdo202
74.8 e41t59188
79.1 e33t61133
LG5
0.0 e36t50375
4.5 e41t47750, e33t50163
9.0 e33t50112
13.9 e41t50590
15.9 e41t47198
18.1 ablpg224, Xc1176.2, e41t50960, Xcdo459
18.8 Xablpg26y, e33t50189
19.3 Xbcd1087
21.2 e41t47445
26.4 Xr1751
29.2 Xcdo412
30.4 osrb, Xablpg26x
32.8 e38t50311
36.7 e33t62210
47.9 LpCBFIb
48.6 LpCBFII
50.1 LpCBFIIIb, LpCBFIIIc
52.7 e38t50189
60.0 e33t50147
61.1 e33t62101
65.2 Xr2710
66.5 e41t50200
69.0 e40t50268
69.7 Xcdo400
80.9 Xrz404

S1


A
LpCBFIb cDNA -65 CTCACACTCC ACAGTCCACC GTACTAAGCT CAGCAAGCAC CGACCATCCT CATCAAACCA
p150/112 HP-1 CTCACAGTCC ACAGTCCACC GTACTAAGCT CAGCAAGCAC CGACCATCCT CATCAAACCA
p150/112 HP-2 CTCACAGTCC ACAGTCCACC GTACTAAGCT CAA------- --GCCATCCT CATCAAACCA
LpCBF1-2F primer
LpCBFIb cDNA -5 TGGACATGAC TGGCTCCGAG CAATGGAGTT CCCCTTCCTT GTCGTCATCC TCTCAGGAGC
p150/112 HP-1 TGGACATGAC TGGCTCCGAG CAATGGAGTT CCCCTTCCTT GTCGTCATCC TCTCAGGAGC
p150/112 HP-2 TGGACATGAC TGGCTCCGAG CAATGGAGTT CCCCTTCCTT GTCGTCATCC TCTCAGGAGC
First ATG
LpCBFIb cDNA 56 AAGGGGTACC GGTGTGGGTG ACCCCGCCGA AACGCCCCGC GGGGAGGACC AAGTTCAAGG
p150/112 HP-1 AAGGGGTACC GGTGTGGGTG ACCCCGCCGA AACGCCCCGC GGGGAGGACC AAGTTCAAGG
p150/112 HP-2 AAGGGGTGCC AGTGTGGGTG ACCCCGCCGA AACGCCCCGC GGGGAGGACC AAGTTCAAGG
LpCBF1-5R primer
LpCBFIb cDNA 116 AGA
p150/112 HP-1 AGA
p150/112 HP-2 AGA
B
LpCBFII cDNA 554 ACTTGTTCGA GCTCGACATG TCCGGGGAAA TCGACTTGGA CACGTACTAC GCGGGCCTCG
p150/112 HP-1 ACATGTTCGA GCTGGACATG TCCGGGGAAA TCGACTTGGA CACGTACTAC GCGGGCCTCG
p150/112 HP-2 ACATGTTCGA GCTGGACATG TCCGGGGAAA TCGACTTGGA CACGTACTAC GCGGGCCTCG
LpCBF12-1F primer
LpCBFII cDNA 614 CGGAGGGGAT GCTCCTGGAG CCGCCGCCCA CGCCGTACTG GGAGAGCAGA GAGTGCGGCA
p150/112 HP-1 CGGAGGGGAT GCTCCTGGAG CCGCCGCCCA CGCCGTACTG GGAGAGCAGA GAGTGCGGCA
p150/112 HP-2 CTGAGGGGAT GCTCCTGGAG CCGCCGCCCA CGCCGTACTG GGAGAGCAGA GAGTGCGGCA
DdeI recognition site
LpCBFII cDNA 674 ACGGGGGCGG AGGCGATGCC GCCCTCTGGA GCTACTGATA TCGACAAGTG ATCTTCCTGT
p150/112 HP-1 ACGGGGGCGG AGGCGATGCC GCCCTCTGGA GCTACTGATA TCGACAAGTG ATCTTCCTGT
p150/112 HP-2 ACGGGGGCGG AGGCGATGCC GCCCTCTGGA GCTACTGATA TCGACGAGCG ATCTTCCTGT
Stop codon
LpCBFII cDNA 734 AATTTTTGTT TTTT--GCTG GAAGATCTGC CTGTAGATTC
p150/112 HP-1 AATTTTTGTT TTTT--GCTG GAAGATCTGC CTGTAGATTC
p150/112 HP-2 AATTATTATT TTTTTGGCTG GAAGATCTGC CTGTAGATTC
LpCBF12-1R primer
C
LpCBFIIIc cDNA 550 TTTCAGGTTC CGGCTGCACT GGGCAGCGAC ATGTTCGAGC TGGACATGTC CGGCGAAATG
p150/112 HP-1 TTTCAGGTTC CGGCTGCACT GGGCAGCGAC ATGTTCGAGC TGGACATGTC CGGCGAAATG
p150/112 HP-2 TTTCAGGTTC CGGCTGCACT GGGCAGCGAC GTGTTCGAGC TGGACATGTC CGGCGAAATG
LpCBF17-dCAPs-1F primer
G
AvaII recognition site (GGACC)
LpCBFIIIc cDNA 610 GGCCTGGGCA CGTACTACGC TGACCTTGCG GAGGGCCTCC TCCTGGCTCC GCCGTCGCCG
p150/112 HP-1 GGCCTGGGCA CGTACTACGC TGACCTTGCG GAGGGCCTCC TCCTGGCTCC GCCGTCGCTG
p150/112 HP-2 GGCCTGGGCA CGTACTACGC TGACCTTGCG GAGGGCCTCC TCCTGGCTCC GCCGTCGCCG
LpCBFIIIc cDNA 670 GCAGGCACGG AGGCGTTCTG GGAGAACGGT GATTACGATT ACGGAGGACC GGAAGGCGCG
p150/112 HP-1 GCAGGCACGG AGGCGTTCTG GGAGAACGGT GATTACGATT ACGGAGGACC GGAAGGCGCG
p150/112 HP-2 GCAGGCACGG AGGCGTTCTG GGAGAACGGT GATTACGATT ACGGAGGACC GGAAGGCGCG
LpCBFIIIc cDNA 730 CTCTGGAGTT ACTGATGACA TCGACGAGAA ACCA
p150/112 HP-1 CTCTGGAGTT ACTGATGACA TCGACGAGAA ACCA
p150/112 HP-2 CTCTGGAGTT ACTGATGACA TCGACGAGAA ACCA
Stop codon
LpCBF17-2R primer

D



LpCBFVb cDNA 669 CCAAATACAG GAAGAAGCAG AGTAGGCGAC AGGGGGTTCA TTTACCCCCT GATCATGC--
p150/112 HP-1 CCAAATACAG GAAGAAACAG AGTAGGCGGA AGGGGGTTCA CTTTTCCCCT GATTATTACG
p150/112 HP-2 CCAAATACAG GAAGAAACAG AGTAGGCGGA GGGGGGTTCA CTTTTCCCCT GATTATTACG

LpCBFVb cDNA ----ATAGTA TGTGTTCTTC GCTCATTGTC AAGATCCGGT TTCTTTTTTG GGACAAAGGG
p150/112 HP-1 CATAGTAGTA TGTGTTCTTC GCTCATTGTC AAGATCCGGT TTCTTTTTTG GGACAAAGGG
p150/112 HP-2 CATAGTAGTA TGTGTTCTTC GCTCATTGTC AAGATCCGGT TTCTTTTTTG GGACAAAGGG

LpCBFVb cDNA 783 GCAATTGCTA CTTCAGTTAT TATTGTTATT ATGGAGGAGT ATATTTTTAG GGCACGAAAG
p150/112 HP-1 GCAATTGCTA CTTCAGTTAT TATTGTTATT ATGGAG---T ATATTTTTAG GGCACGAAAG
p150/112 HP-2 GCAATTGCTA CTTCAGTTAT TATTGTTATT ATGGAG---T ATATTTTTAG GGCACGAAAG

LpCBFVb cDNA 843 GGACAAAGAC GTCACTGTTG ATGGCAAAG
p150/112 HP-1 GGACAAAGAC GTCACTGTTG ATGGCAAAG
p150/112 HP-2 GGACAAAGAC GTCACTGTTG ATGGCAAAG