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ORIGINAL PAPER

CBF gene copy number variation at *Frost Resistance-2* is associated with levels of freezing tolerance in temperate-climate cereals

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Abstract *Frost Resistance-1* (*FR-1*) and *FR-2* are two loci affecting freezing tolerance and winter hardiness of the temperate-climate cereals. *FR-1* is hypothesized to be due to the pleiotropic effects of *VRN-1*. *FR-2* spans a cluster of *C-Repeat Binding Factor* (*CBF*) genes. These loci are genetically and functionally linked. Recent studies indicate *CBF* transcripts are downregulated by the *VRN-1* encoded MADS-box protein or a factor in the VRN-1 pathway. Here, we report that barley genotypes 'Dicktoo' and 'Nure' carrying a *vrn-H1* winter allele at *VRN-H1* harbor increased copy numbers of *CBF* coding sequences relative to *Vrn-H1* spring allele genotypes 'Morex' and 'Tremois'. Sequencing bacteriophage lambda genomic clones from these four genotypes alongside DNA blot hybridizations indicate approximately half of the eleven *CBF* orthologs at *FR-H2* are duplicated in individual genomes. One of these duplications discriminates *vrn-H1* genotypes from *Vrn-H1* genotypes. The *vrn-H1* winter allele genotypes harbor tandem segmental duplications through the *CBF2A–CBF4B* genomic region and maintain two distinct *CBF2* paralogs, while the *Vrn-H1* spring allele genotypes harbor single copies of *CBF2* and *CBF4*. An additional *CBF* gene, *CBF13*, is a pseudogene interrupted by multiple non-sense codons in 'Tremois' whereas *CBF13* is a complete uninterrupted coding sequence in 'Dicktoo' and 'Nure'. DNA blot hybridization with wheat DNAs reveals greater copy numbers of *CBF14* also occurs in winter wheats than in spring wheats. These data indicate that variation in *CBF* gene copy numbers is widespread in the Triticeae and suggest selection for winter hardiness co-selects winter alleles at both *VRN-1* and *FR-2*.

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Introduction

Wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*) must be capable of surviving freezing temperatures if autumn-sown in temperate-climate regions. Equally important, they must also be capable of surviving prolonged exposure to freezing temperatures and the fluctuating environmental conditions that occur over the course

of winter, a trait known as winter hardiness. Spring-sown genotypes in contrast do not require the same level of freezing tolerance or winter hardiness. Indeed, autumn-sown genotypes are generally more freezing tolerant than their spring-sown counterparts (Limin and Fowler 2006; Wilen et al. 1996).

A fundamental genetic difference discriminating the autumn-sown from spring-sown genotypes is the allelic state at *VRN-1*, a locus affecting reproductive development and the vernalization requirement (Takahashi and Yasuda 1971; Trevaskis et al. 2007). Spring genotypes carry a *Vrn-1* allele while the autumn-sown winter and facultative genotypes carry a *vrn-1* allele (von Zitzewitz et al. 2005). Molecular isolation of *VRN-1* revealed the primary difference between *Vrn-1* spring and *vrn-1* winter alleles is in transcript accumulation not in structural differences of the coding sequences (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003). Genotypes carrying a *Vrn-1* spring allele constitutively express *VRN-1* to high levels, which confers an intrinsic reproductive competence to these genotypes (Takahashi and Yasuda 1971; Trevaskis et al. 2007). In contrast winter and facultative genotypes carrying a *vrn-1* allele delay *VRN-1* transcript accumulation, which results in a prolonged vegetative growth phase. The *cis*-elements affecting the regulatory difference between *vrn-1* winter allele and *Vrn-1* spring alleles reside both within the *VRN-1* promoter, and within the *VRN-1* first intron (Distelfeld et al. 2009; Fu et al. 2005). As the winter growth habit is the primary form occurring in the wild relatives of modern cereals it is also thought to be the ancestral form (Yan et al. 2003). Multiple spring allele variants occur in the cultivated germplasm, most of which are deletions in the *VRN-1* first intron, the promoter, or both (Distelfeld et al. 2009). These mutations have independently arisen through a mechanism involving non-homologous end joining (NHEJ) (Cockram et al. 2007).

Genetic studies of freezing tolerance and winter hardiness in the Triticeae generally involve populations derived from winter × spring crosses because these two genotypic classes exhibit robust differences for these traits. As the chromosomes of wheat, barley, and rye are syntenic across much of their genomes genetic loci revealed in one of these cereals also often applies to the other members of the Triticeae. Most genetic studies reveal association between a *vrn-1* winter allele and greater freezing tolerance and winter hardiness, and association between a *Vrn-1* spring allele and lesser freezing tolerance and winter hardiness (Francia et al. 2004; Hayes and Aamodt 1927; Quisenberry 1931; Roberts 1990; Skinner et al. 2006; Snape et al. 2001). The locus designation for this phenotype is *Frost Resistance-1* (*FR-1*) (Sutka and Snape 1989). Because freezing tolerance and winter hardiness cosegregate with the *vrn-1* winter allele *FR-1* is hypothesized to be the pleiotropic effect of *VRN-1*, although this remains an unresolved issue. Characterization of *FR-1* is also complicated by fact that the level of freezing tolerance and winter hardiness exhibited by *vrn-1* genotypes diminishes over the course of winter (Fowler et al. 1996a, b; Fowler and Limin 2004; Wilen et al. 1996).

In addition to *FR-1* a second distinct chromosome 5 locus affecting freezing tolerance is revealed in a number of mapping populations (Båga et al. 2007; Francia et al. 2004, 2007; Roberts 1990; Vágújfalvi et al. 2003). This second locus, *Frost Resistance-2* (*FR-2*), is about 30 cM centromere-proximal of *VRN-1* (Båga et al. 2007; Francia et al. 2004, 2007; Vágújfalvi et al. 2003). *FR-2* is coincident with a cluster of genes encoding C-repeat binding factors (CBFs). CBFs are DNA-binding transcriptional activator proteins that regulate pathways affecting cold acclimation and freezing tolerance (Fowler and Thomashow 2002; Stockinger et al. 1997; Vogel et al. 2005). In *Arabidopsis thaliana* three *CBF*s form a cluster of head to tail tandemly linked genes in a 10 kb region on chromosome 4 (Gilmour et al. 1998). Increasing *CBF* levels through overexpression increases freezing tolerance (Jaglo-Ottosen et al. 1998; Jaglo et al. 2001; Liu et al. 1998), and decreasing *CBF* levels through mutations results in decreased freezing tolerance (Alonso-Blanco et al. 2005; Chinnusamy et al. 2003).

CBF genes also occur in clusters in other plants. Both tomato and rice have three *CBF*s that form a cluster of head-to-tail tandemly linked genes in a 10 kb region (Yu et al. 2002; Zhang et al. 2004). In the Triticeae there are 11 different *CBF* gene orthologs at *FR-H2* of barley and *FR-A^{m}2* of einkorn wheat, *T. monococcum* (Miller et al. 2006; Skinner et al. 2006). Unlike the structural organization in Arabidopsis, tomato, and rice the Triticeae cereal *CBF*s are dispersed over a nearly 1 cM genetic interval and a 1 Mb physical interval (Francia et al. 2007; Miller et al. 2006). In the spring einkorn wheat line DV92 the *CBF12* gene harbors a deletion in the DNA-binding domain rendering the protein incapable of binding to target site sequences while the winter G3116 allele encodes a protein that binds target sites (Knox et al. 2008). In barley, transcript profiling indicates that *CBF2* and *CBF4* transcript levels are significantly higher in the more freezing tolerant 'Nure' winter genotype than in the less freezing tolerant 'Tremois' spring genotype (Stockinger et al. 2007). Moreover, differences in *CBF* expression levels between 'Nure' and 'Tremois' cosegregate with the allelic state at *FR-H2* in 'Nure' × 'Tremois' recombinants. However, there is no qualitative difference between 'Nure' and 'Tremois' in the *CBF*s expressed; the *CBF*s expressed in 'Nure' are also expressed in 'Tremois', just at lower levels (Stockinger et al. 2007). These data suggest the possibility that *FR-H2* in the 'Nure' × 'Tremois' population may be due to gene expression level differences (Stockinger et al. 2007).

One means to increase expression of a gene is to simply increase its copy number. Approximately 12% of the human genome is comprised of regions referred to as copy number variants (CNVs) (Redon et al. 2006). CNVs are a form of genome structural variation thought to underlie major genetic variation across humans and other mammalian species (Korbel et al. 2008). CNVs are unit segments of the genome increased or decreased in number relative to a reference genome; they range in size from 1 kb to 1 Mb, and can encompass many coding sequences (Korbel et al. 2008; Scherer et al. 2007). CNVs are thought to underlie numerous conditions, ailments, diseases, susceptibly to cancer, and neuropsychiatric conditions (Campbell et al. 2008; Cook and Scherer 2008; Hollox et al. 2008; McCarroll and Altshuler 2007; Perry et al. 2007; Reymond et al. 2007). Classes of genes encompassed by CNV regions are also skewed towards those involved with environmental response (Korbel et al. 2008). One gene having striking differences in copy numbers across individuals is the human *AMY1* gene (Iafrate et al. 2004). In situ hybridization of *AMY1* to DNA fibers (fiber FISH) reveal ten *AMY1* copies in one allelic form, a single copy in another allelic form, and intermediate numbers in other allelic forms (Perry et al. 2007). Moreover, allelic forms having increased *AMY1* copy numbers are more frequent in human populations whose diets are traditionally high in starch while the allelic forms having fewer copies are more frequent in populations whose diets are typically low in starch (Perry et al. 2007).

We hypothesized that the structure of the *CBF* genes at *FR-H2* might differ between 'Nure' and 'Tremois'. To test this hypothesis we sequenced the *CBF* genes of 'Nure' and 'Tremois'. We also determined portions of the *CBF* genomic sequences for two additional genotypes, 'Dicktoo' and 'Morex', which greatly facilitated structural comparisons. 'Dicktoo' and 'Nure' are winter hardy genotypes possessing a *vrn-H1* winter allele, while 'Morex' and 'Tremois' are non-winter hardy genotypes possessing a *Vrn-H1* spring allele. A key finding is that these two winter hardy genotypes having a *vrn-1* winter allele harbored increased *CBF* gene copy numbers relative to the two genotypes carrying *Vrn-1* alleles. We hypothesize *CBF* gene copy number differences might be the underlying molecular basis of *FR-H2*.

Materials and methods

Plant material

The source of barley (*Hordeum vulgare*) genotypes used in this study is provided in Table SI alongside information about their origin, end use, spike type, and horticultural classification. Additional genotypes (not listed in Table SI) used in the pedigree analysis of 88Ab536-B are listed in Table SII. Pedigrees of 'Dicktoo', 'Morex', 'Nure', and 'Tremois' have been described (Francia et al. 2004; Johnson 1953; Rasmusson and Wilcoxson 1979). The wheat (*Triticum aestivum*) genotypes used for DNA blot hybridization are described (Stockinger et al. 2007).

Genomic clone isolation and DNA sequencing strategy

Procedures for construction of the bacteriophage λ genomic libraries and the shotgun subclone sequencing libraries are briefly outlined here. Detailed procedures are available online (http://www.oardc.ohio-state.edu/stockingerlab/). High MW DNA was isolated from 'Dicktoo', 'Morex', 'Nure' and 'Tremois' as described (Stockinger et al. 2006). DNA was partially restricted with *Sau*3AI and ligated to the bacteriophage λ Fix® II vector using the components of the λ FixII Gigapack III XL kits (Stratagene, La Jolla, CA). DNA templates were individually radiolabeled to the same specific activity and then combined for a mixed probe. *CBF2*, *CBF3*, *CBF4*, *CBF6*, *CBF8*, *CBF9*, and *CBF10* probes encompassed the coding sequences (CDSs), *CBF15* and *CBF16* probes encompassed the AP2 domains and CBF signature sequences. λ genomic clones classified as unique and that harbored one or more *CBF* genes were subcloned into the *Not*I site of plasmid vector pGEM®-11Zf(-) (Promega, Madison, WI). *Not*I subclones $\leq$7 kb were sequenced by primer walking, *Not*I subclones >7 kb were sequenced using a shotgun subcloning strategy. Shotgun subclones were sequenced to an approximate sevenfold redundancy. Sequencing was carried out by Amplicon Express (Pullman, Washington) or the Washington University Genome Sequencing Center (Saint Louis, MO). End sequences of *Not*I subclones were determined at Amplicon Express (Pullman, Washington) or the Molecular Cellular and Imaging Center at the Ohio State University, Ohio Agricultural Research and Development Center. In instances where a *Not*I site occurred within the *CBF* CDS, PCR products that spanned the *Not*I site were generated from the λ clones and sequenced. The 'Nure' *CBF3* clone terminated immediately upstream of *CBF13*. The missing segment physically colinear with 'Dicktoo' was obtained through PCR amplification from genomic DNA. The 'Morex' *CBF3* clone terminated in the *CBF3* CDS and was completed using a subclone from 'Morex' BAC clone 790P15. The 'Morex' *CBF6* sequence was also determined using a plasmid subclone of 'Morex' BAC clone 572K24. Shotgun sequence assembly utilized the software package Sequencher (Gene Codes Corp., Ann Arbor MI).

DNA blot hybridization

Approximately 10 μg of genomic DNA was restricted with each enzyme, electrophoresed, and transferred to Hybond N (Amersham Biosciences, Piscataway, NJ) as described (Stockinger et al. 2007) using standard procedures (Ausubel et al. 1993). Overnight hybridizations were carried out at 42°C. Washes were performed at 65°C in 0.2× SSC, 0.05% SDS and 0.01% sodium pyrophosphate. Radiolabeled probes were generated by random priming (Feinberg and Vogelstein 1983) using the Megaprime Random Labeling Kit (Amersham Biosciences, Piscataway, NJ). Images were generated using a Molecular Dynamics Storm840 PhosphorImager (GE Healthcare, Buckinghamshire UK). Unless noted otherwise, DNA blot analyses were conducted using probes representing sequences immediately upstream or downstream of the CDS to eliminate cross-hybridization to other *CBF*s including *CBF* pseudogenes. Primers to amplify these regions are listed in Table SIII.

Genomic sequence annotation

Genomic sequences were annotated using BLAST and tBLASTx searches that queried the NCBI non-redundant nucleotide and EST databases (http://www.ncbi.nlm.nih.gov/), the barley Tentative Consensus Sequences maintained in the Gene Index Database (http://compbio.dfci.harvard.edu/tgi/), and the Triticeae Repeat Database, TREP (http://wheat.pw.usda.gov/ITMI/Repeats/index.shtml). Sequences were also annotated using the gene structure prediction software GENSCAN (Burge and Karlin 1997) and comparative alignments with previously published sequences and expressed sequence tags. Sequence alignments were made using the Clustal X windows interface version of ClustalW (Thompson et al. 1997) and were formatted using BOXSHADE 3.2 (http://www.ch.embnet.org/software/BOX_form.html).

Nomenclature and gene symbols

VRN-1, *VRN-2*, *FR-1*, and *FR-2* are used throughout this work when reference is to the locus. All uppercase italic also specifies the transcript (e.g., *VRN-1*). *Vrn-1* (dominant and spring) and *vrn-1* (recessive and winter) are used to specify the alleles at these loci. When specifically referring to barley or wheat loci, genome-specific designations are included (e.g., *VRN-H1* and *VRN-A1*, or *FR-H2* and *FR-A2*).

Sequences

Sequences have been deposited with GenBank under accession numbers DQ445232–DQ445253, DQ480160, EU593530–EU593542, and EU650230–EU650231. They are listed in Table SIV.

Results

CBF genomic region overview

To investigate the possibility that allelic differences in the *CBF* genes at *FR-H2* underlie the molecular basis of *FR-H2*, we initiated the systematic isolation and DNA sequence determination of the 5H *CBF* genomic regions through construction and screening of 'Dicktoo', 'Morex', 'Nure', and 'Tremois' bacteriophage λ genomic libraries. Our rationale for using bacteriophage λ as a cloning vehicle over other vectors capable of accommodating larger inserts stemmed from the knowledge that Arabidopsis, tomato, and rice harbored three *CBF* genes linked in a cluster spanning less than 10 kb (Gilmour et al. 1998; Yu et al. 2002; Zhang et al. 2004). Initial screening of the genomic libraries was carried out with a mixed probe containing *CBF2*, *CBF3*, *CBF4*, *CBF6*, *CBF8*, *CBF9*, and *CBF10* (Skinner et al. 2005). Screening 1.25×10^6 primary recombinants of each genotype resulted in 155 positive clones that were then taken through plaque purification. Genomic clones were fingerprinted via DNA blot hybridization using a panel of restriction enzymes and gene-specific probes encompassing the 3′ regions. Three novel *CBF*s not present as part of the original probes, *CBF12*, *CBF13*, and *CBF14*, were isolated in the initial screen. Subsequent screens of the 'Nure' and 'Tremois' λ libraries were carried out for *CBF15* and *CBF16* and of the 'Tremois' library for *CBF4* and *CBF9*.

In total, approximately 415,000 bp of non-contiguous sequence information was determined from 'Nure' (175,000 bp), 'Tremois' (165,000 bp), 'Dicktoo' (123,000 bp), and 'Morex' (100,000 bp). GenBank accession numbers for the bacteriophage λ clone inserts isolated and sequenced are summarized in Table SIV.

The *CBF2* and *CBF4* structural organization is similar for 'Dicktoo' and 'Nure' and differs from 'Morex' and 'Tremois'

CBF2B was isolated from both 'Nure' and 'Dicktoo' (Fig. 1a). *CBF2A* was also isolated from both 'Nure' and 'Dicktoo' (Fig. 1b). *CBF2B* was a lone *CBF* gene on the respective bacteriophage λ genomic clones (Fig. 1a). *CBF2A* was physically linked to *CBF4B*, and was inverted relative to *CBF4B* (Fig. 1b). 'Morex' harbored *CBF2A* and *CBF4B* in the same configuration as 'Nure' and 'Dicktoo' (Fig. 1c). The 'Morex' allele possessed 4,229 bp of a CACTA transposon immediately upstream of *CBF2A*, which extended to the end of the λ clone (Fig. 1c). 'Nure'

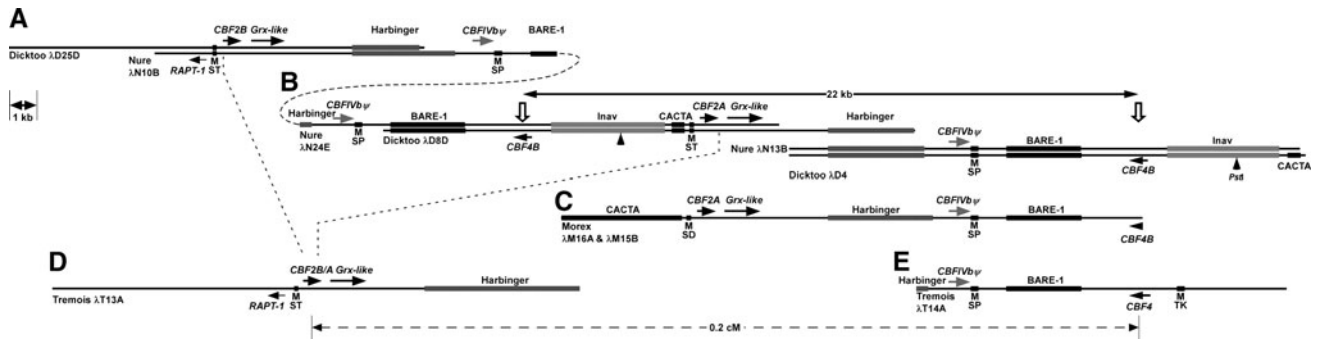

Fig. 1 Physical maps of the 'Dicktoo', 'Morex', 'Nure' and 'Tremois' *CBF2* and *CBF4* genomic regions. Barley genotype and phage clones are identified on the left end of each physical map. **a** 'Dicktoo' and 'Nure' *CBF2B*. **b** 'Dicktoo' and 'Nure' *CBF2A–CBF4B*. The downward-directed *arrows* identify the identical *CBF4B* coding sequences separated by 22 kb. The *horizontal double arrowhead line* encompasses the 22 kb duplicated segment. The two upward-directed *arrowheads* identify the *Pst*I CAPS marker discriminating the Inav retrotransposon on 'Dicktoo' phage clones *λ*D8D and *λ*D4. The *CBF2B* (**a**) and *CBF2A–CBF4B* (**b**) genomic regions are connected by the *dashed line* to indicate linkage order based on 'Nure' × 'Tremois' recombinants (Francia et al. 2007). **c** 'Morex' *CBF2A–CBF4B*. **d** 'Tremois' *CBF2B/A*. The *dotted lines* connect the 'Tremois' *CBF2B/A* genomic region to the physically colinear 'Dicktoo'–'Nure' *CBF2B* and *CBF2A–CBF4B* genomic regions. **e** 'Tremois' *CBF4*. The *dashed line* indicating 0.2 cM between *CBF2B/A* (**d**) and *CBF4B* (**e**) is based on 'Nure' × 'Tremois' recombinants (Francia et al. 2007), however, the physical distances are not known

and 'Dicktoo' possessed less than 500 bp of a CACTA transposon, harboring an Inav retrotransposon immediately beyond the truncated CACTA (Fig. 1b). The 'Morex' *CBF2A* CDSs was identical to 'Dicktoo' *CBF2* (Fig. S1), and the genomic region downstream of 'Morex' *CBF2A* was 99% to those of both 'Nure' and 'Dicktoo' (Fig. 1b, c).

The 'Tremois' *CBF2* and *CBF4* genes each resided as lone *CBF* genes on the respective *λ* genomic clones (Fig. 1d, e). Harbinger DNA transposon elements resided on the respective rightward and leftward termini of the 'Tremois' *CBF2* and *CBF4* genomic clones but their nucleotide sequences differed slightly, indicating that the genomic regions encompassed by these two clones did not overlap. The *CBF2A* and *CBF4B* genes of 'Nure', 'Dicktoo', and 'Morex' were also separated by Harbinger DNA transposon elements but the Harbinger in the 'Tremois' *CBF2* genomic region was comprised of three tandem repeating units whereas the Harbinger DNA transposon in the 'Dicktoo', 'Morex', and 'Nure' *CBF2A-CBF4B* genomic regions consisted of two tandem repeating units.

The 'Tremois' *CBF2* paralog was *CBF2B*-like in its 5′ region and *CBF2A*-like in its 3′ region (Fig. 1d). Of eight nucleotide polymorphisms distinguishing the 'Nure' and 'Dicktoo' *CBF2A* paralog from the *CBF2B* paralog the 'Tremois' *CBF2B/A* paralog shared the first two with *CBF2B* and the last six with *CBF2A* (Fig. S1). The identity between 'Tremois' *CBF2B/A* in its 5′ region with the *CBF2B* paralogs continued through the CDS of an AP2 domain-containing protein identified here as *Related to AP2 Triticeae-1* (*RAPT-1*) to the end of the genomic clone (Fig. 1a, d). Similarly the identity between 'Tremois' *CBF2B/A* and the *CBF2A* paralogs continued in the 3′ region through the glutaredoxin-like CDS and into the Harbinger DNA transposon. The overall nucleotide identity between the genomic regions immediately upstream and downstream of 'Tremois' *CBF2B/A*, and the 'Dicktoo'–'Nure' *CBF2B* upstream region, and the *CBF2A* upstream region of 'Dicktoo', 'Nure', and 'Morex' was 98 and 99%, respectively. The 'Morex' and 'Tremois' *CBF4* genomic regions were physically colinear with the 'Dicktoo'–'Nure' *CBF2A–CBF4B* genomic regions from the Harbinger transposon through *CBF4* (Fig. 1b, e). 668 nucleotides upstream of *CBF4* the 'Tremois' genomic region diverged from the other sequences.

To verify the presence and absence of the different *CBF2* forms in the genomes of the four barley cultivars we created PCR primer sets that could discriminate between *CBF2B* and *CBF2A*. We developed one set specific for the 5′ region of *CBF2A* and another that was specific for the 5′ region of *CBF2B*. The *CBF2B*-specific primers amplified products from 'Dicktoo', 'Nure', and 'Tremois' genomic DNA templates, but not from a 'Morex' template (Fig. 2a). The *CBF2A*-specific primers amplified products from 'Dicktoo', 'Morex', and 'Nure', but not from 'Tremois' (Fig. 2a). A second PCR primer-pair amplified across an insertion/deletion that discriminated the 3′ region of *CBF2B* from *CBF2A*. Amplification with the *CBF2* 3′ region primer-pair resulted in the predicted product sizes for *CBF2B* from 'Dicktoo' and 'Nure' and in the predicted product sizes for *CBF2A* from all four genotypes (Fig. 2b). Taken together these data confirmed that 'Dicktoo' and 'Nure' harbored both of these *CBF2* paralogs while 'Morex' and 'Tremois' harbored only a single of these *CBF2* paralogs.

'Nure' and 'Dicktoo' *CBF2A–CBF4B* genomic regions are tandemly duplicated

Sequencing two independent *λ* genomic clones encompassing *CBF4B* from 'Dicktoo', *λ*D8D and *λ*D4, indicated they

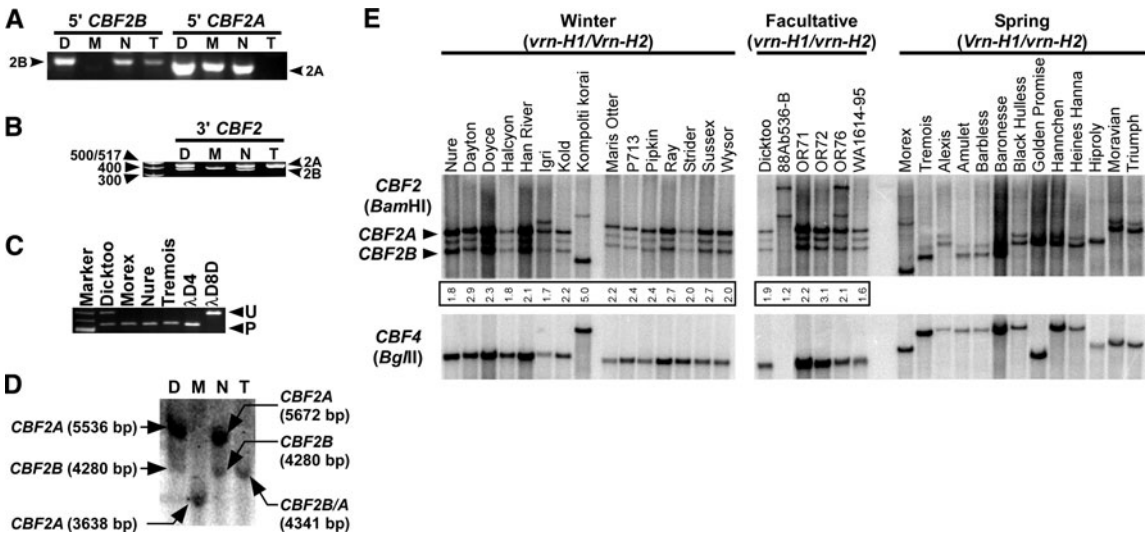

Fig. 2 Structural analyses of the *CBF2* and *CBF4* genomic regions. Amplification products of the *CBF2B* and *CBF2A* upstream (**a**) and downstream (**b**) regions. 'Dicktoo' (*D*), 'Morex' (*M*), 'Nure' (*N*), and 'Tremois' (*T*). **c** CAPS marker assay discriminating the 'Dicktoo' *CBF4B–CBF2A* duplicated regions. *U* undigested PCR products, *P* the larger of two *Pst*I-restricted fragments. **d** *CBF2* gene-specific probe hybridized to 'Dicktoo', 'Morex', 'Nure' and 'Tremois' *Bam*HI digested genomic DNAs. Predicted fragment sizes are indicated. **e** Hybridization of *CBF2* (*upper*) and *CBF4* (*lower*) to genomic DNAs of winter, facultative, and spring genotypes. The relative signal intensity ratios of the upper *CBF2A* to *CBF2B* bands is shown below the *CBF2* hybridization image. For 'Kompolti korai', the lower MW fragment is taken to be *CBF2A*, and for OR76, the ratio reported is for the 'Dicktoo'–'Nure' allele (the ratio of the 88AB536-B allele is 1.6)

differed in 50 positions over a 10,350 bp nucleotide region (99% identity). Based on gene order and other landmarks the genomic regions on these two clones appeared structurally the same (Fig. 1b). Additionally, a 4,652 bp segment on the rightward end of λD8D was 100% identical to the leftward end of λD4 (Fig. 1b). These data suggested the 4,652 bp segment might be a region of overlap between λD8D and λD4 and that the two clones might represent different segments of tandem duplications through the *CBF2A–CBF4B* genomic region. Similarly two independent λ genomic clones encompassing *CBF4B* from 'Nure' were 99% identical over a region appearing structurally the same (Fig. 1b). Conveniently on the 'Dicktoo' clone λD8D there was an Inav retroelement that resided between *CBF4B* and *CBF2A* that differed from the Inav retroelement upstream of *CBF4B* on 'Dicktoo' clone λD4 by two single nucleotide polymorphisms (SNPs) and a single bp insertion/deletion, which together resulted in a *Pst*I cleaved amplified polymorphic site (CAPS) that should discriminate the two regions. Using this CAPS marker with λD4 and λD8D templates indicated that indeed λD4 was restricted by *Pst*I and that λD8D was not (Fig. 2c). Amplification from 'Dicktoo' genomic DNA confirmed that both the *Pst*I-restricted and the *Pst*I non-restricted Inav retroelement forms existed in the 'Dicktoo' genome (Fig. 2c). As a further test of "genetic purity" of the 'Dicktoo' source tissue, and to also determine whether these two genomic regions were stably inherited, the CAPS assay was carried out on six of the 'Dicktoo' × 'Morex' doubled haploid lines having the 'Dicktoo' *CBF2* allele (Hayes et al. 1997). All six lines had both the *Pst*I-restricted and the *Pst*I non-restricted Inav retroelement forms (not shown). The nucleotide changes that resulted in the loss of the *Pst*I site were unique to 'Dicktoo', however, because none of the other three barley genotypes possessed the non-*Pst*I-restrictable form (Fig. 2c). Instead a *Mwo*I SNP was found to discriminate the repeating units of 'Nure' (not shown). Additionally, the rightward ends of the two 'Nure' clones, λN13B and 'Nure' λN24E completely differed from one another just downstream of the Inav retroelement. 'Nure' λN24E possessed the CACTA element, whereas λN13B did not (Fig. 1b). This suggested that λN13B might exit the iterated region and anchor to a single copy region. Taken together these data indicated that the genomic region encompassing *CBF2A–CBF4B* was tandemly duplicated in both 'Dicktoo' and 'Nure' and that the duplicated segments were not an artifact of heterogeneous tissue or DNA used for library construction. Moreover, the two distinct units were stably inherited.

DNA blot hybridizations were also carried out with 'Nure', 'Dicktoo', 'Morex', and 'Tremois'. *CBF2* and *CBF4* gene-specific probes were hybridized to DNAs restricted with *Bam*HI, and *Bgl*II, respectively. DNA sequence analyses indicated *Bam*HI should produce an RFLP discriminating *CBF2A* and *CBF2B* in 'Dicktoo' and 'Nure'. (No convenient RFLP was identified that discriminated the individual genomic units encompassing *CBF4B*.) Gene-specific probes that cross-hybridized to regions immediately upstream of the CDS (*CBF4*) or immediately downstream of the CDS (*CBF2*) were used to avoid cross-

hybridization with closely related sequences and pseudogenes. Hybridizations with the *CBF2* probe produced single fragments of the predicted sizes in both 'Morex' and 'Tremois', and two fragments of predicted sizes corresponding to *CBF2B* and *CBF2A* in 'Dicktoo' and 'Nure' (Fig. 2d). Hybridizations with the *CBF4* probe produced single fragments of the predicted sizes in all four genotypes (Fig. 2e). Quantification of the signal intensity corresponding to the *CBF2A* and *CBF2B* fragments indicated a *CBF2A*:*CBF2B* ratio of approximately 2:1 (Fig. 2e).

CBF2A–CBF4B tandem segmental duplications occur in cultivated barleys possessing *vrn-H1* winter alleles

As the *vrn-H1* genotypes 'Dicktoo' and 'Nure' harbored both the segmental duplications through the *CBF2A–CBF4B* genomic region and the two *CBF2* paralogs whereas the *Vrn-H1* spring genotypes 'Morex' and 'Tremois' lacked the segmental duplications and harbored only a single *CBF2* paralog, one question raised was whether these genotypes typified other barley genotypes carrying *vrn-H1* winter and *Vrn-H1* spring alleles. To address this question we carried out DNA blot hybridizations using the *CBF2* and *CBF4* gene-specific probes on additional winter, facultative, and spring barley genotypes having diverse origins and different end product uses (listed in Tables SI, SII). Some of the cross-hybridization banding patterns were shared with the four reference genotypes while some were new and distinctly different (Fig. 2e). In most of the winter and facultative genotypes hybridization with the *CBF2* probe produced the characteristic two-band pattern typical of 'Nure' and 'Dicktoo' along with a less-intense third cross-hybridizing fragment migrating intermediate to *CBF2A* and *CBF2B* (Fig. 2e). In most of these genotypes, the *CBF2A* fragment produced about twofold stronger signal intensity than the *CBF2B* fragment (Fig. 2e).

However, two additional and distinct banding patterns were also present in the winter and facultative genotypes. One occurred in 'Kompolti korai', and the second occurred in 88AB536-B and OR76 (Fig. 2e). OR76 exhibited both the 'Dicktoo'–'Nure' banding pattern and the 88AB536-B banding pattern (Fig. 2e). Subsequent genotyping of individual progeny from OR76 using a CAPS that occurred within the *CBF4* coding sequence (Skinner et al. 2006) indicated that plants were heterozygous and this banding pattern was not due to a recombination even within the *CBF* gene cluster. The 88AB536-B *CBF2* allelic form was subsequently traced to a winter growth habit Korean landrace Mumie Pori through genotyping lines in the 88AB536-B pedigree (Fig. S2). 'Kompolti korai', a Hungarian cultivar, was traced by pedigree analysis to French, Belgian, and Hungarian landraces (Karsai et al. 2005). In 'Kompolti korai' the MW of the cross-hybridizing fragments were higher and lower than the other *vrn-H1* genotypes and the upper fragment was clearly the less intense (Fig. 2e). In 88AB536-B there were two higher MW fragments (Fig. 2e). In contrast to the winter and facultative genotypes hybridization of the *CBF2* probe to the spring genotypes produced an array of different MW fragments across accessions but there was only a single strongly cross-hybridizing fragment in each accession (Fig. 2e). These data suggest the presence of two *CBF2* paralogs in the genomes of *vrn-H1* genotypes 'Kompolti korai' (KK) and Mumie Pori (MP), and a single *CBF2* paralog in the genomes of all *Vrn-H1* genotypes surveyed.

We also hybridized a *CBF4* gene-specific probe to *Bgl*II restricted DNAs of these same genotypes. Most winter and facultative genotypes exhibited a single fragment at the same approximate MW as 'Nure' and 'Dicktoo' (Fig. 2e). Two exceptions were again 'Kompolti korai' and 88Ab536-B (Figs. 2e, S2). 'Kompolti korai' produced a single higher MW cross-hybridizing fragment whereas 88Ab536-B and the genotypes tracing to the Korean landrace Mumie Pori failed to cross-hybridize with both a *CBF4* 3′ gene-specific probe (Fig. 2e) and a *CBF4* 5′ gene-specific probe (Fig. S2). (Although there was very weak signal in the Mumie Pori lane this appears to be due to contamination by the heavily overloaded adjacent lane since none of the other genotypes having the Mumie Pori allele cross-hybridized at this position.) 88Ab536-B does harbor *CBF4*, however (Skinner et al. 2006), which was confirmed by both PCR amplification of the genomic DNA used for the RFLP analysis and hybridization of a *CBF4* CDS probe to genomic DNA, which produced an array of different MW fragments (not shown). These data suggest the Mumie Pori (MP) allelic form of *CBF4* exists in a different contextual genomic environment from that the 'Dicktoo'–'Nure' (DN) and 'Kompolti korai' (KK) *CBF4* allelic forms.

'Dicktoo' and 'Nure' *CBF13* is an intact CDS but 'Tremois' *CBF13* is a pseudogene

Sequencing the genomic region encompassing *CBF3* and *CBF13* from 'Nure', 'Dicktoo' and 'Tremois' indicated that the 'Tremois' *CBF13* CDS harbored multiple non-sense codons in all three reading frames while the 'Nure' and 'Dicktoo' *CBF13* CDSs were intact open reading frames 759 nucleotides in length. In all genotypes *CBF3* was an intact open reading frame. The 'Dicktoo' and 'Nure' *CBF13* CDS was 12.5 kb downstream of *CBF3* (Fig. 3a). In 'Tremois' the distance was 6.4 kb (Fig. 3b). The 'Morex' *CBF13* genomic region was of interest for comparative purposes; however, our 'Morex' clone terminated approximately 700 bp beyond *CBF3* (Fig. 3c). Within this region of physical overlap the 'Morex' and 'Tremois' *CBF3* downstream regions were 98% identical (Fig. 3b, c). DNA

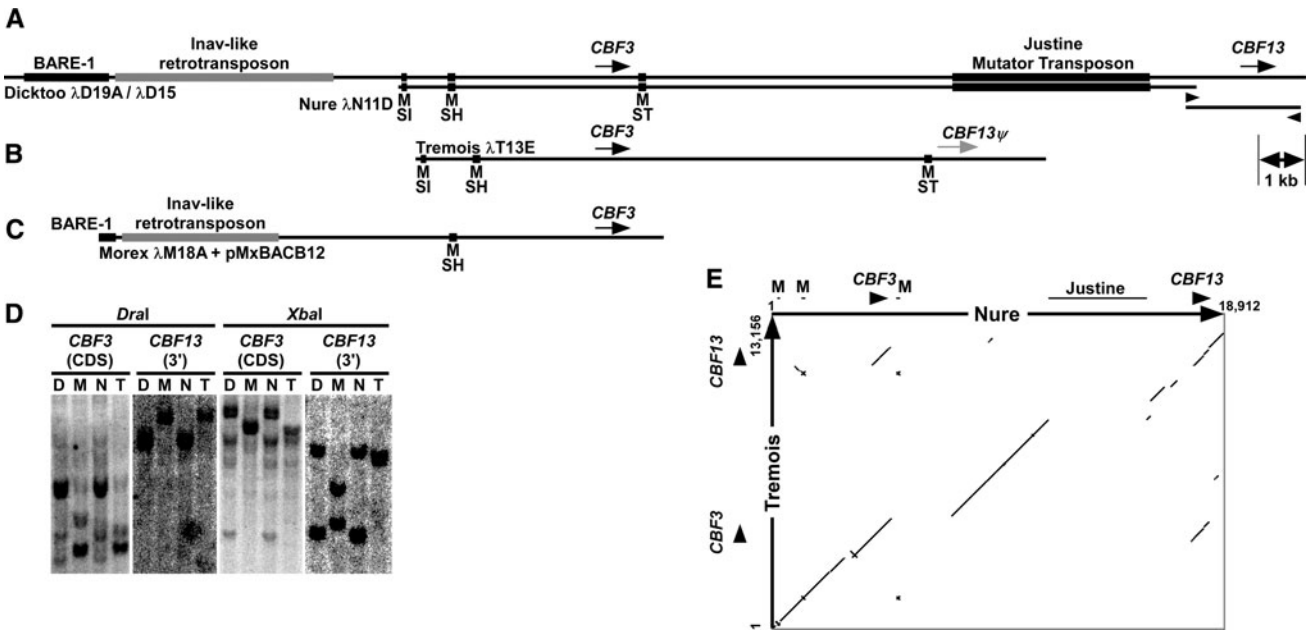

Fig. 3 Physical maps of the *CBF3–CBF13* genomic regions. **a** 'Dicktoo' and 'Nure'. The genomic region encompassing *CBF13* PCR amplified from 'Nure' genomic DNA is shown below the genomic clone. **b** 'Tremois'. **c** 'Morex'. **d** DNA blot hybridizations using the *CBF3* CDS and the 'Dicktoo'–'Nure' *CBF13* 3′ region. Hybridizations are to the same filter, and images are aligned to the same MW position. An *Xba*I site bisects the region encompassed by the *CBF13* hybridization probe in 'Dicktoo', 'Nure', and 'Tremois'; this results in two cross-hybridizing fragments. The 'Tremois' fragment appearing as single band is likely a doublet. **e** Dot plot comparing the 'Nure' (*X* axis) and 'Tremois' (*Y* axis) *CBF3 – CBF13* genomic regions. The region immediately downstream of 'Nure' *CBF3* and the Justine mutator transposon are absent from 'Tremois'

blot hybridization using a probe encompassing the 'Dicktoo'–'Nure' *CBF13* downstream region cross-hybridized to all four genotypes suggesting *CBF13* was present in 'Morex' but that there were likely to be structural rearrangements that made it distinct from 'Dicktoo'–'Nure' and 'Tremois' (Fig. 3d). Primers directed to either the 'Tremois' *CBF13* CDS or the 'Dicktoo'–'Nure' *CBF13* CDS also failed to amplify a product from 'Morex'. A dot plot comparison of the 'Dicktoo'–'Nure' and 'Tremois' *CBF3–CBF13* intergenic regions indicated that a 2.5 kb segment immediately downstream of *CBF3* and a 4.1 kb Justine mutator transposon upstream of 'Dicktoo'–'Nure' *CBF13* were absent from 'Tremois' (Fig. 3e). In sum these data indicate 'Tremois' *CBF13* is a pseudogene and that there have been multiple rearrangements in the *CBF3–CBF13* intergenic region.

Duplications of the genomic regions encompassing the *CBF*s is a recurring theme

CBF9 genomic clones were sequenced from 'Morex' and 'Tremois' (Fig. 4a, b). Immediately upstream and downstream of the CDSs the two clones were nearly identical. However, 2 kb downstream of the CDSs the two genomic regions were completely dissimilar except for a short inverted segment (Fig. 4a, b). Sequencing portions of additional *CBF9*-positive 'Tremois' λ genomic clones confirmed 'Tremois' also harbored the 'Morex' *CBF9* form (not shown). Hybridization with a *CBF9* promoter fragment and digestion with an enzyme that did not bisect the probe produced two distinct cross-hybridizing fragments in 'Dicktoo', 'Morex', and 'Tremois' (Fig. 5a) indicating that these genomes likely harbored two *CBF9* paralogs.

Two distinct genomic clones encompassing *CBF15* orthologs, λT16 and λT25, were recovered from 'Tremois' (Fig. 4c, d). The *CBF15* CDS on 'Tremois' λT16 was deemed *CBF15A*, and the *CBF15* CDS on λT25 was deemed *CBF15B*. Downstream of λT16 *CBF15A* was a Vincent CACTA transposon (Fig. 4c), whereas downstream of λT25 *CBF15B* was a BARE-1 retrotransposon (Fig. 4d). *CBF15B* consisted of two additional codons at the NH_3-terminus as a result of a T to G transversion four nucleotides upstream of the relative *CBF15A* MET codon. 'Tremois' λT16 was physically colinear with the 'Nure' clone λN3B (Fig. 4c). The *CBF15A* CDS on 'Tremois' λT16 and 'Nure' λN3B were more similar to each other and differed from *CBF15B* at two positions conserved between 'Nure' and 'Tremois' *CBF15A*. Hybridization with a *CBF15* promoter fragment produced two distinct cross-hybridizing fragments in a subset of genotypes suggesting two distinct *CBF15* paralogs also occurred in these genotypes (Fig. 5b).

Three distinct genomic regions encompassing *CBF12* were isolated from the four cultivars. The 'Nure' and

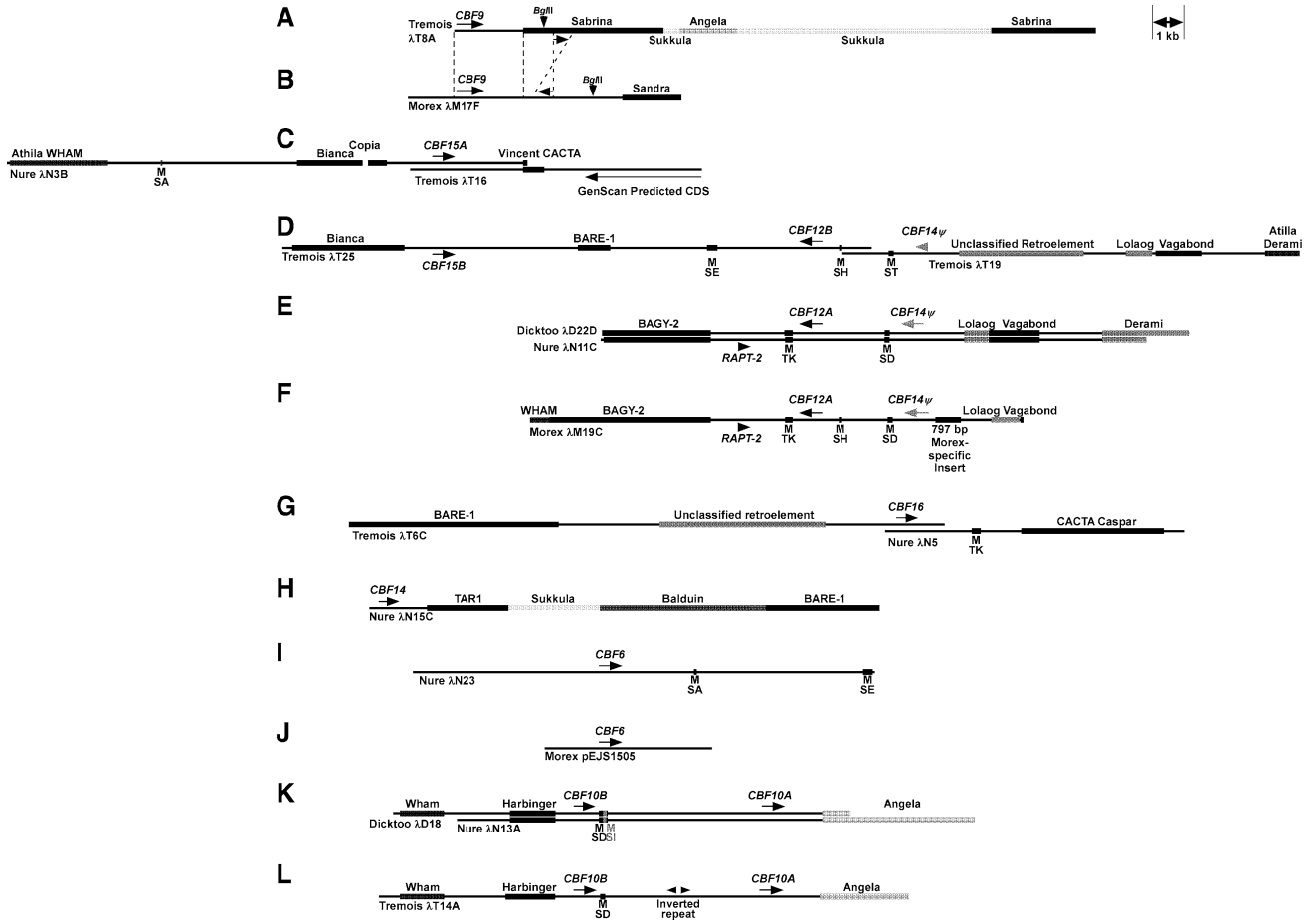

Fig. 4 Physical maps of additional *FR-H2 CBF* gene orthologs. **a** *CBF9* 'Tremois'. **b** *CBF9* 'Morex'. The *dashed lines* identifies the regions conserved between 'Tremois' and 'Morex' *CBF9* genomic regions. **c** *CBF15A* 'Nure and 'Tremois'. **d** *CBF15B–CBF12B* 'Tremois', **e** *CBF12A* 'Dicktoo' and 'Nure', **f** *CBF12A* 'Morex', **g** *CBF16* 'Tremois' and 'Nure, **h** *CBF14* 'Nure', ***i*** *CBF6* 'Nure', **j** *CBF6* 'Morex', **k** *CBF10B–CBF10A* 'Dicktoo' and 'Nure', **l** *CBF10B–CBF10A* 'Tremois'

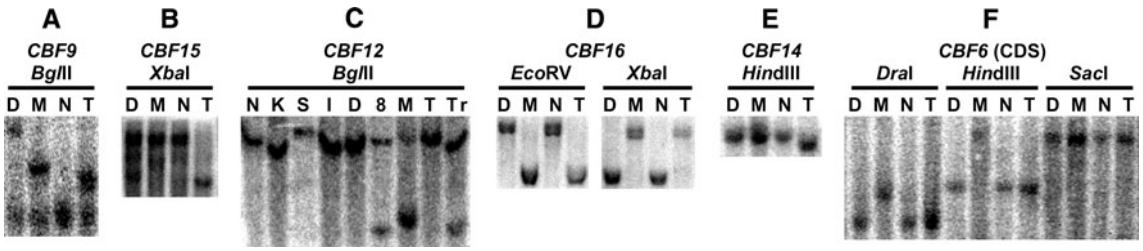

Fig. 5 DNA blot hybridization with additional *FR-H2 CBF*s. **a** *CBF9*, **b** *CBF15*, **c** *CBF12*, **d** *CBF16*, **e** *CBF14*, **f** *CBF6*. All probes except *CBF6* recognize sequences outside the CDS. 'Dicktoo' (*D*), 'Morex' (*M*), 'Nure' (*N*), 'Tremois' (*T*), 'Kompolti korai' (*K*), 'Strider' (*S*), 'Igri' (*I*), '88Ab536-B' (*8*), 'Triumph' (*Tr*)

'Dicktoo' clones λN11C and λD22D were nearly identical (Fig. 4e). The 'Morex' *CBF12* genomic region shared most features with 'Nure' and 'Dicktoo' and had several additional sequence elements (Fig. 4f). The 'Tremois' *CBF12* genomic region was distinctly different. As such its *CBF12* CDS was identified as *CBF12B*. 'Tremois' *CBF12B* was physically linked to *CBF15B* (Fig. 4d). Upstream of *CBF12B* was an unclassified repeat element that was not present in the *CBF12A* upstream region (Fig. 4d–f). The 'Tremois' *CBF12B* downstream region also lacked the CDS encoding a 127 amino acid AP2 domain-containing polypeptide, *RAPT-2*, that was downstream of *CBF12A* (Fig. 4d–f). To determine whether *RAPT-2* expression differed between genotypes expression analyses were carried out using RNAs from low temperature time course experiments. These experiments indicated that *RAPT-2* was constitutively expressed in all four barley genotypes (not

shown). This expression data suggested 'Tremois' harbored *RAPT-2*. Hybridization with a *CBF12* promoter fragment produced two distinct cross-hybridizing fragments in 'Morex' and 'Tremois' as well as several additional genotypes (Fig. 5c). Taken together these experiments suggested two distinct *CBF12* paralogs existed in individual genomes.

Genomic clones encompassing *CBF16* were recovered and sequenced from each 'Nure' and 'Tremois' (Fig. 4g). The two sequences were 99% identical over a 1.6 kb overlapping region suggesting this 1.6 kb region was physically colinear. DNA blot hybridizations using the *CBF16* promoter fragment produced a doublet with an *Eco*RV digest in 'Dicktoo' and 'Nure', and a doublet with an *Xba*I digest in 'Morex' and 'Tremois' (Fig. 5d). This data suggested there might also be two *CBF16* paralogs because neither enzyme restricted within the region used as the hybridization probe.

In sum, duplicate forms of *CBF9* and *CBF15* were found in individual genomes, and indirect evidence suggests duplicate forms of *CBF12* occur in individual genomes. Differences in these genes between 'Nure' and 'Tremois' that were clear cut and might be considered as factors underlying *FR-H2* were not identified.

CBF6, *CBF10*, and *CBF14*

Sequencing of genomic clones alongside DNA blots indicated *CBF14* and *CBF6* existed as single genes and that *CBF10B* and *CBF10A* existed as a tandemly duplicated gene pair (Figs. 4h–l, 5e, f). The CDSs of all these genes were intact open reading frames in all four genotypes. The physical map of the single *CBF14* genomic clone sequenced (from 'Nure') is shown in Fig. 4h. DNA blot hybridizations with the *CBF14* promoter fragment produced only a single fragment in each of the four primary genotypes (Fig. 5e). *CBF6* genomic sequences were determined from 'Nure' and 'Morex' (Fig. 4i, j). Their genomic regions exhibited nearly 100% identity from 2 kb upstream through the CDS, and diverged about 0.6 kb downstream (not shown). The DNA blot hybridization patterns of 'Morex' were also distinct from 'Dicktoo', 'Nure', and 'Tremois' (Fig. 5f). The genomic sequences encompassing *CBF10B* and *CBF10A* of 'Dicktoo' and 'Nure' were nearly identical (Fig. 4k) and differed from 'Tremois' (Fig. 4l). The *CBF10B* CDS of 'Dicktoo' and 'Nure' also harbored an in-frame, 39 bp deletion (3′ to the AP2 domain) relative to 'Tremois' *CBF10B*. Collectively these data indicated the *CBF* genes in the *FR-H2* genomic region were nearly identical between 'Dicktoo' and 'Nure' and differed from 'Morex' and 'Tremois', which also differed from each other.

Greater *CBF14* copy numbers occur in winter wheats than spring wheats

An obvious question raised is whether *CBF*s also exist in variable copy numbers in other Triticeae cereals. To begin to address this question DNA blot hybridization was carried out with wheat *CBF14*. *CBF14* was selected because it shows significant variability in expression levels across wheat genotypes that differ in their freezing tolerance (Stockinger et al. 2007; Vágújfalvi et al. 2005).

A *T. monococcum CBF14* promoter fragment was hybridized to a panel of three *Vrn-1* spring, and nine *vrn-1* winter hexaploid wheat genotypes. DNAs were restriction digested using four enzymes; *Dra*I, *Hin*dIII, *Sac*I, and *Xba*I. No polymorphism was detected across the 12 genotypes with these four enzymes (Fig. 6, and data not shown). For all genotypes, hybridization to *Dra*I, *Hin*dIII, and *Xba*I restricted DNA produced three distinct cross-hybridizing bands, and hybridization to *Sac*I restricted DNA produced four distinct cross-hybridizing bands (Fig. 6, and data not shown). The three bands were attributed to cross-hybridization to the three homoeoalleles (AA, BB, and DD), and in the instance of the *Sac*I digestion, the four bands were attributed to the enzyme restricting within the region recognized by the probe for one homoeoallele. Several of these cross-hybridizing bands exhibited substantially reduced signal intensity in the three *Vrn-1* spring genotypes relative to the *vrn-1* winter genotypes (Fig. 6). Comparisons across the *vrn-1* winter wheats indicated that there were also differences within the winter wheat germplasm. The soft

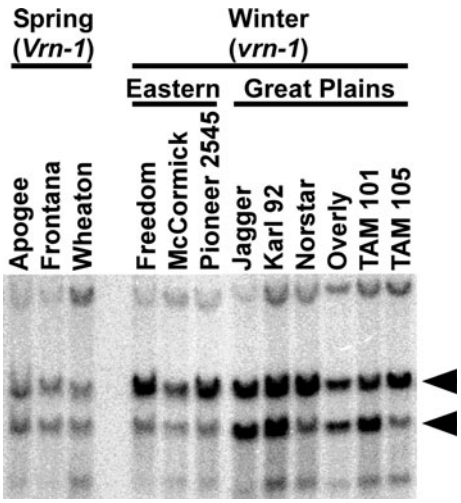

Fig. 6 Hybridization of a *T. monococcum CBF14* promoter fragment to hexaploid wheat genomic DNAs restriction digested with *Sac*I. *Upper arrow* identifies the MW fragment showing greater signal intensity in the Eastern and Great Plains winter wheat groups than in the spring wheats, *lower arrow* identifies the MW fragment showing increased signal intensity in most Great Plains wheats but not in Eastern wheats

red winter wheats grown in the Eastern US exhibited reduced signal intensity for one of the cross-hybridizing bands (i.e., on one of the homoeologs) relative to the hard red winter wheats grown on the Great Plains (Fig. 6). These data indicate *CBF* gene copy number variation also occurs in wheat.

Discussion

A primary objective of this study was to explore whether there were structural differences in the *CBF* genes between 'Nure' and 'Tremois' that might account for the phenotypic effect of *FR-H2*. We found that there were at least two major allelic differences. One was copy number differences in which the 'Nure' allele possessed a greater number of *CBF2* and *CBF4* gene copies than did 'Tremois'. 'Tremois' possessed a single *CBF2* gene in the form of a *CBF2B/A* paralog while 'Nure' possessed both *CBF2B* and *CBF2A*. The data reveal 'Nure' also harbored at least two *CBF4B* gene copies as a result of a tandem segmental duplication through the genomic region encompassing *CBF4B*. These tandem segmental duplications and the *CBF2A* to *CBF2B* signal ratios (determined from the DNA blot hybridizations) also indicate at least two *CBF2A* genes. The second difference occurred in *CBF13* in which 'Nure' *CBF13* was an intact coding sequence, whereas the 'Tremois' *CBF13* was a pseudogene. While we cannot as yet rule out the possibility that other differences contribute to *FR-H2*, the single-copy *CBF2* and *CBF4* genes in 'Tremois' compared to the multiple gene copies in 'Nure' is compelling. *CBF2* and *CBF4* are expressed in response to low temperatures, and greater *CBF2* and *CBF4* expression levels cosegregate with the 'Nure' allele at *FR-H2* (Stockinger et al. 2007). *CBF13* expression in contrast has eluded detection (Badawi et al. 2007; Stockinger et al. 2007).

Different markers have been developed along the *FR-H2* cluster using this sequence information (Francia et al. 2007) and can be used to select one or more *CBF*s, or the entire cluster in a breeding program. It may also be possible to track cultivar-specific *CBF* alleles among germplasm groups. For example, the SNP in the Inav retrotransposon element separating *CBF2A* and *CBF4B* can be used to track inheritance of the 'Dicktoo' *CBF2A–CBF4B* allele in a segregating population in which 'Dicktoo' is in the pedigree. Similarly the *Mwo*I SNP could be used to track the 'Nure' *CBF2–CBF4* genomic region. At present it is unknown as to how genotype-specific these signatures are or to what extent they occur in the germplasm base. PCR amplification and sequencing of the *CBF2A–CBF4B* intergenic regions from individual genotypes may be a means to generate genotype-specific signatures were this warranted. Screening additional barley genotypes suggests there are genotypes with *CBF2A–CBF4B* genomic region copy numbers that exceeds those of 'Nure' and 'Dicktoo' (TD and EJS, unpublished data). Having genotype-specific signatures would be a very valuable tool to select for these alleles, especially in winter × winter crosses in which variability in the gene coding sequences is likely to be non-existent.

The barley genotypes assayed here also revealed the variability of the *FR-H2* locus and the limitation of some of the tools. Use of the 5′ and 3′ sequences flanking the *CBF* coding sequences, while avoiding cross-hybridization of the closely related gene sequences and pseudogenes failed to cross-hybridize with the Mumie Pori *CBF4* allelic form despite the presence of *CBF4* in the genome. Similarly, surveying European cultivars, landraces, and *H. spontaneum* accessions for variation in *CBF3*, *CBF6*, *CBF9*, and *CBF14* using a PCR-based approach did not amplify *CBF3* and *CBF9* from a significant number of the lines (Fricano et al. 2009). Rather than being absent from the genome, these *CBF* CDS may exist in different contextual environments in which the sequences flanking the CDS differ from the reference.

Why two *CBF2* paralogs and why the *CBF2A–CBF4B* genomic region amplification in the *vrn-H1* winter genotypes 'Dicktoo' and 'Nure'? Perhaps *CBF2* and *CBF4* play more critical roles due to expression in tissues important for winter survival. In *Arabidopsis thaliana*, At-*CBF2* is expressed in different tissues than At-*CBF1* and At-*CBF3* (Novillo et al. 2007). This implies that CBF-targeted genes are also differentially regulated in these same tissues. Similar spatial regulatory patterns may occur in barley and the other Triticeae cereals. Indeed candidate downstream target genes of the CBFs in barley do exhibit spatial restriction in their expression patterns (Pearce et al. 1998). The activity of CBF2 and CBF4 are also stimulated by low temperature (Skinner et al. 2005; Xue 2003) and so these CBFs may be "better equipped" to activate target genes under conditions of low temperature stress. Alternatively the *CBF2A–CBF4B* genomic environment may be more favorable for amplification.

Multiple identical or nearly identical *CBF9*, *CBF12*, and *CBF15* coding sequences also appear to be present in the genomes of most of these barley genotypes. Some may be defective in the 'Tremois' genome although we found no evidence to indicate that this was the case. Clear cut copy number differences were also not apparent for these other *CBF* genes. However, it is important to point out that the amplified copies of *CBF4B* in 'Nure' and 'Dicktoo' were indistinguishable from one another and were only recognizable as duplicate copies of the same coding sequence by virtue of polymorphisms present within the flanking repetitive DNA—the *CBF4B* coding sequences themselves were invariant. The immediate flanking sequences also lacked convenient polymorphisms that would allow separation of

these duplicated *CBF4* genes using standard RFLP technology. As such, if there are copy number differences in other *CBF* genes, they may be masked.

Hybridization of *CBF14* to the hexaploid wheat genotypes indicates the phenomenon of *CBF* genomic region amplification is more widespread in the Triticeae and that there is a prevalence of increased copy numbers in the *vrn-1* winter wheat genotypes. Moreover, differences in *CBF14* copy numbers in wheat occurred on at least two of the three homoeologs, and these differences appeared to define the different wheat germplasm groups. Eastern US wheats are descendant from Western and Northern European landraces whereas Great Plains wheats are descendant from Crimean landraces (Quisenberry and Reitz 1974; Zeven and van Hintum 1992). Winter wheats descendant from Crimean landraces exhibited increased *CBF14* copy numbers on more of the homoeologs than wheats from Western and Northern Europe. This essentially parallels data showing that wheats having a Crimean pedigree are generally more freezing tolerant and more winter hardy that those from Western and Northern Europe (Fowler and Gusta 1979; Gusta et al. 2001). Crimean wheats made possible the cultivation of winter wheat on the Great Plains where previous attempts at cultivation of Western and Northern Europe genotypes met with failure (Quisenberry and Reitz 1974; Zeven and van Hintum 1992). In essence it is possible that all three homoeologs carry increased copy numbers of multiple *CBF* gene orthologs, giving rise to a highly complex homoeologous allelic series in which variation occurs at the individual gene ortholog level and in copy numbers of a given gene ortholog. Ultimately freezing tolerance levels of individual genotypes may be an additive effect of all *CBF* genes at *FR-2*.

CBF14 was examined because it is expressed at significantly higher levels in more freezing tolerant wheat genotypes relative to less freezing tolerant genotypes (Vágújfalvi et al. 2005). Chromosome substitution lines in the 'Chinese Spring' background containing the 'Cheyenne' 5A chromosome also exhibit significantly higher expression levels of *CBF14* (relative to other *FR-A2 CBF*s) than substitution lines containing *T. spelta* 5A chromosome or the non-substituted 'Chinese Spring' background (Vágújfalvi et al. 2005). 'Cheyenne' is derived from a Crimean landrace (Clark 1931). Thus, one explanation for the higher expression levels detected in the 'Chinese Spring' 'Cheyenne' 5A substitution lines may be that it is due to increased *CBF14* copy numbers. 'Cheyenne' is also the source of the 5B and 5D chromosomes generating the substitution lines used for mapping freezing tolerance on 5B and 5D (Snape et al. 1997; Tóth et al. 2003). Thus it is also conceivable that the underlying molecular basis of this increased freezing tolerance is due to increased *CBF* gene copy numbers present on 'Cheyenne' 5B and 5D chromosomes relative to copy numbers present on the 'Chinese Spring' chromosomes.

Upon initial mapping of barley *CBF3* Close and colleagues hypothesized *CBF3* or another tightly linked *CBF* might be a determinant of freezing tolerance in certain genetic backgrounds (Choi et al. 2002). This is indeed the case (Båga et al. 2007; Francia et al. 2004, 2007; Vágújfalvi et al. 2003, 2005). The locus is now known to consist of at least 11 *CBF* orthologs dispersed over a nearly 1 cM interval (Francia et al. 2007; Miller et al. 2006; Skinner et al. 2006) that makes possible a highly complex allelic series. In different genetic backgrounds, different genes along the cluster are implicated in conferring freezing tolerance differences between parental lines (Knox et al. 2008; Vágújfalvi et al. 2005). Variations in copy numbers of individual *CBF* orthologs would add yet an additional level of complexity.

Non-allelic homologous recombination, a mechanism that generates gene copy number changes (Hastings et al. 2009), is likely to be a major factor in generating copy number differences of the genomic regions encompassing the *CBF* genes. Most of the barley *CBF*s resided as isolated genes surrounded by large tracts of Triticeae repeat sequences. These repeat sequences provide the sequence identity necessary for unequal crossing over (Hastings et al. 2009). Homologous recombination also likely played a role in the generation of the *CBF2B/A* paralog. While one scenario is that the *CBF2B/A* paralog is an ancestral gene that was duplicated and those duplicated copies gave rise to the *CBF2B* and *CBF2A* paralogs, another scenario is that the *CBF2B/A* paralog resulted from a homologous recombination between preexisting *CBF2B* and *CBF2A* paralogs. The latter scenario seems more likely as there is only one mismatched nucleotide between 'Tremois' *CBF2B/A* paralog and the corresponding region of the *CBF2B* paralogs, and there is 100% nucleotide identity with the corresponding region of the *CBF2A* paralogs. A similar scenario occurs in the *CBF* genes of Solanum. The *CBF* gene clusters of multiple Solanum species have a basic structural organization of three tandemly linked genes. However, the *S. pimpinellifolium* LA1589 accession harbors an in-frame fusion between the second and third genes of the cluster resulting in the elimination of one *CBF* coding sequence (Pennycooke et al. 2008). Taken together this suggests homologous recombination is continually occurring at the *CBF* gene clusters generating sequence diversity. An intriguing question that arises with these Triticeae cereals is whether the changes in copy number increases or decreases, are due to human-handed selection in combination with cultivation practices.

Purifying selection pressures likely play a role in maintaining the integrity of the *CBF* gene orthologs and the duplicated paralogs in *vrn-H1* genotypes, while relaxed

selection pressure, or perhaps even negative selection pressure exists upon the *CBF* genes in *Vrn-H1* genotypes. Variation in copy number changes occur de novo in both somatic and germ line mammalian cells at a frequency that is several orders of magnitude higher than that at which point mutations occur (Hastings et al. 2009). Increasing *CBF* gene copy numbers is expected to increase *CBF* transcripts, and in turn increase the expression of downstream target genes which together effect increases in freezing tolerance (Jaglo-Ottosen et al. 1998; Jaglo et al. 2001; Liu et al. 1998). Thus, amplification of the genomic regions encompassing the *CBF*s would be a means to increase *CBF* expression levels and provide a selective advantage during winter. The presence of the intact *CBF13* CDS in 'Dicktoo' and 'Nure' and the *CBF13* pseudogene in 'Tremois' suggests purifying selection pressures are also acting on *CBF13*. 'Morex' lacks *CBF10B* (Skinner et al. 2006), and recent studies indicate that 'Morex' *CBF13* is also a pseudogene, albeit with mutations different from those of 'Tremois' *CBF13* (E. Francia, personal communication). Thus, despite the absence of detectable expression of both *CBF10* and *CBF13* these data suggest purifying selection is also acting on these *CBF*s in *vrn-H1* winter genotypes, consistent with their having functional biological roles. On the other hand, there may be a selective disadvantage to having additional copies of *CBF* genes in *Vrn-H1* spring genotypes. High level expression of the *CBF* genes in Arabidopsis results in smaller plants, delayed flowering and other growth abnormalities (Gilmour et al. 2000; Liu et al. 1998; Zhang et al. 2004). Selection for robust plants, high yield, early flowering, or any combination of these traits in *Vrn-H1* spring genotypes would likely favor the selection of genotypes possessing fewer functional *CBF* genes. Similar pressures may also be occurring in *T. monococcum*. Although the *CBF12* coding sequence from the lesser freezing tolerant genotype DV92 harbors a deletion preventing CBF12 protein from binding to its target sequence (while the more freezing tolerance parent G3116 harbors the intact DNA-binding motif), genetic analyses indicate that *CBF* genes distal to *CBF12* also have effect upon freezing tolerance (Knox et al. 2008). As the full complement of *CBF* genes appear to be maintained in winter growth habit forms, and as the winter growth habit form is the ancestral form, it may make sense to establish a complete physical map of the *FR-H2* region using a winter form, perhaps from wild barley, *H. spontaneum*. This physical map would provide a framework in which the allelic structures of modern cultivars, both *vrn-H1* winter genotypes and *Vrn-H1* spring genotypes, could be superimposed. This would allow visualization and direct comparison of the structural changes that occurred in each of these two germplasm groups.

Although *VRN-H1* and *FR-H2* are separated by about 30 cM (Francia et al. 2004; Skinner et al. 2006), the preferential co-association of the allelic state at these two loci may be due simply to the fact that having the winter allele at both loci confers greater low temperature tolerance than that which is conferred by a single locus, a feature that would have been selected for by plant breeders who would have carried out repeated selections and variety trials in multiple environments over multiple years. Preferential co-association may also occur as a result of breeding schemes because barley crosses are primarily limited to winter × winter and spring × spring due to different end product uses of winter (feed for livestock) and spring (malt for brewing) barley types (Fischbeck 2003). Alternatively, the preferential co-association may occur due to more complex interactions between alleles at the two loci.

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