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ORIGINAL PAPER

Cbf14 copy number variation in the A, B, and D genomes of diploid and polyploid wheat

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Abstract Freezing tolerance and winter hardiness are complex traits. In the Triticeae, two loci on the group 5 chromosome homoeologs are repeatedly identified as having major effects on these traits. Recently, we found that segments of the genomic region at one of these loci, *Frost resistance-2* (*Fr-2*) is copy number variable in barley. Freezing-tolerant winter-hardy genotypes have greater tandem copy numbers of the genomic region encompassing the *C-repeat binding factor* genes *Cbf2A* and *Cbf4B* at *Fr-H2* than the less freezing-tolerant nonwinter-hardy genotypes. Here we report that in wheat the *Cbf14* gene at *Fr-2* is copy number variable. Using DNA blot hybridizations, we estimated copy numbers of *Cbf14* across the different genomes of diploid and polyploid wheat. Copy numbers of *Cbf14* are lower in the B genome than in the A and D genomes across all ploidy levels. Among hexaploid red wheats, winter genotypes harbor greater *Cbf14* copy numbers than spring genotypes. *Cbf14* copy numbers also vary across the red winter wheats such that hard wheats harbor greater copy numbers than soft wheats. Analysis of hexaploid wheat chromosome 5 substitution lines indicates that *Cbf14* copy numbers in the introgressions are stable in the different backgrounds. Taken together our data suggest that higher copy number states existed in the diploid wild ancestors prior to the polyploidization events and that the loss of *Cbf14* copies occurred in the cultivated germplasm.

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Introduction

Wheat, *Triticum aestivum*, is an allohexaploid comprising three diploid genomes identified as A, B, and D (Gill et al. 2004). Extant diploid relatives of hexaploid wheat include *T. urartu* (AA), a relative of *Ae. speltoides* (BB), and *Ae. tauschii* (DD), all of which are wild species endogenous to either the Fertile Crescent or Central Asia (Giles and Brown 2006; Zohary and Hopf 2000). Approximately 0.5 million years ago, hybridization between *T. urartu* and the *Ae. speltoides* relative occurred, giving rise to tetraploid emmer wheat, *T. turgidum* ssp. *dicoccoides* (AABB) (Luo et al. 2007; Ozkan et al. 2011). Then, approximately 10,000 years ago wild emmer was domesticated by humans giving rise to *T. turgidum* ssp. *dicoccon* (Luo et al. 2007; Matsuoka 2011; Ozkan et al. 2011). Subsequent interspecific hybridization events then occurred between cultivated emmer and *Ae. tauschii*, ultimately producing hexaploid wheat (AABBDD) (Charmet 2011; Matsuoka 2011; Zohary and Hopf 2000).

Today wheat is cultivated throughout the world. It is grown most-successfully in the temperate climate regions at latitudes 30°–60°N and 27°–40°S, but is also grown in more tropical regions (Curtis 2002). In temperate regions, it can be either autumn-sown or spring-sown. Autumn-sown genotypes are usually of a winter growth habit characterized by a requirement for vernalization, an 8–10 week exposure to low temperatures (0–10 °C) that causes the transition from the vegetative to the reproductive growth phase (Distelfeld et al. 2009; Greenup et al. 2009). In comparison, spring genotypes, which are spring-sown, are

inherently reproductively competent and do not require vernalization to flower (Distelfeld et al. 2009; Greenup et al. 2009).

Autumn-sown genotypes must possess a level of freezing tolerance that enables them to survive minimum temperatures in a given region, and also be winter-hardy such that they are able to sustain freezing tolerance for an extended period of time appropriate for a particular region. However, most modern cultivars possess a level of freezing tolerance and winter hardiness that is only marginally greater than that required for the region where they are grown, and as such severe winter kill occurs about once every 10 years (Braun and Săulescu 2002; Fowler and Gusta 1979). Spring genotypes are typically not selected for winter survival and, in general, are much less freezing tolerant than winter genotypes (Fowler and Limin 2004; Wilen et al. 1996).

Hexaploid wheat genotypes usually exhibit much greater freezing tolerance than diploid and tetraploid genotypes (Fowler et al. 1977; Limin and Fowler 1981). Within hexaploid wheat, the hard red winter (HRW) wheats grown in the North American Great Plains tend to be about 6 °C more freezing tolerant, and have a greater endurance for prolonged exposure to freezing temperatures and desiccating conditions than the soft red winter (SRW) wheats typically grown east of the Mississippi (Gusta et al. 1997, 2001).

In diploid and hexaploid wheat, genetic analyses have identified two loci that have a major effect on freezing tolerance and winter hardiness, both of which reside on the long arm of the group 5 homoeologous chromosomes (Båga et al. 2007; Galiba et al. 1995; Roberts 1990; Snape et al. 1997; Sutka and Snape 1989; Tóth et al. 2003; Vágújfalvi et al. 2003). These are identified as *Frost resistance-1* (*Fr-1*), and *Fr-2*. *Fr-1* and *Fr-2* have also been identified in barley at genetically co-linear positions (Francia et al. 2004, 2007; Hayes et al. 1993; Knox et al. 2010; Skinner et al. 2006). *Fr-1* is thought to be due to the product of the *Vernalization-1* (*Vrn-1*) gene acting to concomitantly induce flowering and reduce freezing tolerance, the latter of which occurs in part through the downregulation of genes at *Fr-2* (Dhillon et al. 2010; Stockinger et al. 2007). *Fr-2* consists of a cluster of at least 11 different *C-repeat binding factor* (*Cbf*) gene coding sequences spanning approximately 1 cM genetic distance and 1 Mb physical distance (Knox et al. 2008, 2010; Miller et al. 2006; Skinner et al. 2005). CBFs are DNA-binding transcriptional activator proteins and are a major regulatory hub that affects control over the low temperature transcriptome in plants (Stockinger et al. 1997; Thomashow 2010). In barley, several 11 *Cbf* orthologs are duplicated, existing as identical or nearly identical paralogs in individual genomes (Knox et al. 2010). A single barley genome may harbor both *Cbf2A* and *Cbf2B*, *Cbf10A* and *Cbf10B*, *Cbf12A* and *Cbf12B*, and *Cbf15A* and *Cbf15B* (Knox et al. 2010). Additionally, the winter-hardy winter and facultative genotypes possess multiple copies of a tandemly duplicated 22 kb genomic segment encompassing *Cbf2A* and *Cbf4B*, whereas the spring genotypes possess only a single copy of *Cbf2* and *Cbf4* (Knox et al. 2010). Expression analyses indicate that the higher *Cbf2A*–*Cbf4B* genomic region copy numbers give rise to increased levels of *CBF2* and *CBF4* transcripts (Stockinger et al. 2007). As increasing *CBF* transcript levels increase freezing tolerance (Thomashow 1999), the increase in *Cbf2A*–*Cbf4B* genomic region copy numbers in winter barleys over spring barleys suggests that copy number variation (CNV) plays a role in underlying the effect of *Fr-H2*.

A CNV is defined as a DNA segment ranging in size from 1 kb to several megabases (Mb) whose copy numbers are variable between the genomes of two or more individuals within a species (Korbel et al. 2008; Stankiewicz and Lupski 2010). In mammalian genomes where CNV has been more extensively characterized, the frequency of CNVs is two-four orders of magnitude greater than that of single nucleotide polymorphisms (SNPs) (Korbel et al. 2008; Stankiewicz and Lupski 2010). Dosage-sensitive genes within copy number variable regions or genes involved in regulatory roles have pronounced phenotypic consequences when present in variable copy numbers (Korbel et al. 2008; Stankiewicz and Lupski 2010). In plants and mammals, the major classes of genes encompassed by CNV regions seem to be those involved with environmental response (Cook et al. 2012; DeBolt 2010; Díaz et al. 2012; Knox et al. 2010; Korbel et al. 2008; McHale et al. 2012).

The increase in copy numbers of the *Cbf2A*–*Cbf4B* genomic region in winter barley genotypes over their spring genotype counterparts raised the question whether *Cbf* gene CNV and the distinction between winter and spring genotypes were more widespread in the Triticeae. Several independent lines of investigation suggested that *Cbf14* might be copy number variable between winter and spring growth habit types, and between different market classes of winter wheats. Expression analyses of *Cbf2*, *Cbf9*, and *Cbf14* in hexaploid wheat indicated that these genes were expressed to higher levels in winter wheats than in spring wheats and that *Cbf14* is expressed to higher levels in the HRW wheats than in the SRW wheats (Galiba et al. 2013; Stockinger et al. 2007). Additionally, single chromosome recombinants having the *Fr-2* region from the HRW wheat 'Cheyenne' express *Cbf7* (*Cbf14* using revised nomenclature) to fourfold higher levels than recombinants having the *Fr-2* region from *T. spelta* (Vágújfalvi et al. 2005). To test whether *Cbf14* might be copy number variable a small collection of hexaploid spring and winter wheats was surveyed using DNA blot hybridization (Knox et al. 2010). Hybridization of a *Cbf14* gene-specific probe obtained from the *T. monococcum Cbf14* promoter to *Sac*I-digested

wheat DNAs indicated that *Cbf14* was copy number variable; and suggested CNV occurred on all three group 5 chromosome homoeologs (Knox et al. 2010). Here, we further explore the extent of *Cbf14* CNV in wheat. To determine the genome identity (A, B, and D) of the *Cbf14* cross-hybridizing bands, we utilize the set of chromosome 5 nullisomic-tetrasomic hexaploid wheat lines (Sears 1966). Mapping to the individual genomes in combination with normalization of copy numbers through the use of the single copy *Puroindoline b* (*Pinb*) gene (Chantret et al. 2005) allowed us to determine the extent of *Cbf14* CNV across the A, B, and D genomes of hexaploid wheat genotypes, irrespective of their growth habit and market class. We also ask what the *Cbf14* copy number states were in the genomes of the diploid wheat species *T. urartu*, *Ae. speltoides*, and *Ae. tauschii*. In addition, we look at the inter-varietal chromosome substitution lines that have a long and established historical role in furthering our understanding of freezing tolerance and winter hardiness, as these were some of the first genetic tools used that played a key role in the current focus on chromosome 5 (reviewed in Stockinger et al. 2006).

Materials and methods

Plant material

Wheat genotypes, their ploidy level, seed source, growth habit or market class, pedigree, and location where the accession was collected or developed are provided in Table S1. Seedling tissue used for DNA extractions from all hexaploid wheats went through single seed descent. Tetraploid durum wheat seedlings used for DNA extractions utilized seed provided by the breeder. Diploid wheat DNAs were prepared from one to three plants grown from seed obtained from the source.

DNA extractions and hybridizations

For tetraploid and hexaploid wheats, about 50 seeds were sown in 9 cm × 9 cm pots (Kord Products, http://www.kord.ca) filled with Bacto high porosity soil mix, and placed in the greenhouse. At approximately 10 cm height, seedlings were cut about 2 cm above the soil. Leaf tissue was flash frozen in liquid nitrogen, lyophilized, and used for the isolation of high molecular weight (MW) DNAs as described (Stockinger et al. 1996). Diploid wheat plants were grown to larger size prior to tissue harvest.

Approximately, 10 µg DNA was digested with *Sac*I or *Bgl*II restriction endonucleases (NEB, http://www.neb.com), ethanol precipitated, resuspended in 10 mM Tris (pH 8.5), and quantified using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen, http://www.invitrogen.com). Equal quantities of each DNA sample were electrophoresed on 0.8 % TAE agarose gels, transferred to Hybond N membrane (GE Healthcare, http://www.gelifescience.com) and UV-crosslinked using standard procedures (Ausubel et al. 1993). Blots were hybridized with a 189 bp *Cbf14* promoter fragment (subsequently referred to as the *Cbf14* 5′ fragment) and a 201 bp *Puroindoline b* (*Pinb*) coding sequence (CDS) fragment. To prepare hybridization probes, *Cbf14* 5′ fragment 368 bp distal to the *Cbf14* MET initiator codon was PCR amplified from *T. monococcum* accession DV92 using forward primer: 5′-agcgagctgtccttgtcatt-3′ and reverse primer: 5′-gcatcttttgtggcgaaaat-3′. An alignment of the *Cbf14* genomic region from *T. monococcum* genotypes DV92 and G3116 and two different A genome *Cbf14* variants from the HRW wheat cultivar 'Norstar' (Knox et al. 2008; Miller et al. 2006; Ratnayaka et al. 2005) is presented in Figure S1, showing primer sites and the regions used as a probe. The *Pinb* CDS was PCR amplified from the *T. aestivum* N5A/T5D nullisomic-tetrasomic line using forward primer: 5′-tgatggagcgatgtttcaca-3′ and reverse primer: 5′-atacctcacctcgccaaatg-3′. Primers were designed using Primer3 (http://frodo.wi.mit.edu). The Tm-*Cbf14* 5′ and Ta-*Pinb* CDS templates were cloned into pGEM-T Easy (Promega, http://www.promega.com) and sequenced. Cloned *Cbf14* 5′ and *Pinb* CDS fragments were used for generating hybridization probes by PCR amplification using the vector's M13 primer sites. Amplified fragments were then labeled with α-dCTP ^{32}P via random hexamer labeling using the MegaPrime DNA labeling kit (GE Healthcare, http://www.gelifescience.com). Hybridization and washes were carried out as described (Knox et al. 2010). Blots were exposed to phosphorscreens and images were scanned using a Molecular Dynamics Storm840 PhosphorImager (GE Healthcare, http://www.gelifesciences.com). Following hybridization, the probe was stripped from the filter using 0.1 % SDS at 65 °C, and the filters were reused.

Estimation of *Cbf14* copy numbers

Copy numbers of *Cbf14* in hexaploid and diploid wheats were estimated using the grain hardness gene *Pinb* as a reference standard. Signal intensities of the *Cbf14* 5′ and *Pinb* CDS cross-hybridizing probes were quantified using the ImageQuant 5.0 software (Molecular Dynamics). *Cbf14*/*Pinb* ratios were then used to estimate haploid copy numbers of *Cbf14*. *Pinb* is a 447 bp open reading frame that is present in single copy on the D genome in hexaploid wheat, is deleted in tetraploid wheats, and is present in single copy in diploid wheats (Chantret et al. 2005; Gautier et al. 2000). In all of the hexaploid wheats, the *Pinb* cross-hybridizing pattern detected consisted of a single MW fragment. Absence of *Pinb* from the tetraploid

wheat genomes was consistent with our hybridization data in which there was no *Pinb* cross-hybridizing fragment in any of the *T. turgidum* accessions characterized. In some of the diploid wheats, however, more than one *Pinb* cross-hybridizing band was produced. In all instances, the cumulative signal intensities of the *Pinb* cross-hybridizing bands were used to estimate *Cbf14*/*Pinb* ratios. For hexaploid wheat, *CBF14* copy number differences between the A and D genomes of an individual genotype were also estimated by normalizing the signal intensities of the A genome cross-hybridizing fragment to that of the D genome cross-hybridizing fragment. For the tetraploid *T. turgidum* wheat accessions, only the *Cbf14*-A/*Cbf14*-B ratios were determined.

Nomenclature and gene symbols

Reference to loci and genes follows the Recommended Rules for Gene Symbolization in Wheat (http://wheat.pw.usda.gov/GG2/Triticum/wgc/2008/Catalogue2008.pdf). As this convention uses an all uppercase non-italic format for specifying protein, an all uppercase italic was also used to specify transcripts; e.g., *CBF* and *VRN-1*, when referring specifically to the mRNAs from these genes.

Results

Genome identity of the *Cbf14* cross hybridizing fragments

In an earlier study, it was suggested that the *Cbf14* gene might be variable in copy number across hexaploid wheat (Knox et al. 2010). Hybridization of a unique fragment representing the *T. monococcum Cbf14* promoter (*Cbf14* 5′) region to hexaploid wheat DNAs restriction-digested with *Sac*I produced four cross-hybridizing bands (Knox et al. 2010). We hypothesized the different bands represented the three homoeologous *Cbf14* genes from the A, B, and D genomes. To test whether this was the case, the *Cbf14* 5′ fragment was used as a hybridization probe against six wheat lines nullisomic-tetrasomic for the group 5 homoeologous chromosomes (Sears 1966). In these lines, deletion of a chromosome pair is compensated by the addition of its homoeologous pair. For example, the line N5A/T5B is nullisomic for chromosome 5A and tetrasomic for chromosome 5B; i.e. it lacks the chromosome 5A pair, and carries an extra copy of the 5B pair.

Hybridization of the *Cbf14* 5′ fragment to this panel of nullisomic-tetrasomic lines produced two or three cross-hybridizing bands (Fig. 1). Using a *Sac*I digest, lines nullisomic for 5A (N5A/T5B and N5A/T5D) and 5D (N5D/T5A and N5D/T5B) produced three cross-hybridizing bands, while lines nullisomic for 5B (N5B/T5A and N5B/T5D) produced two cross-hybridizing bands (Fig. 1a). Using a *Bgl*II digest, three cross-hybridizing bands were produced for the 5B and 5D nullisomic lines, and two cross-hybridizing bands were produced for the 5A nullisomics (Fig. 1b). With both digests, the absent band(s) corresponded to the homoeolog for which the lines were nullisomic (Fig. 1a, b). In this way, the chromosome 5 nullisomic-tetrasomic lines revealed the genome identity of the cross-hybridizing bands. Additionally, the banding pattern indicates the presence of a *Sac*I restriction site internal to the cross hybridizing region of the B genome *Cbf14* (Fig. 1a) and a *Bgl*II site in that of the A genome *Cbf14* (Fig. 1b).

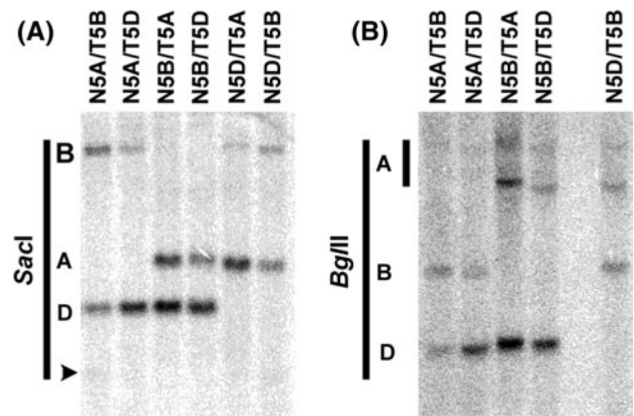

Fig. 1 *Cbf14* hybridization patterns in wheat lines nullisomic/tetrasomic for group 5 chromosome homoeologs. *Sac*I (**a**) and *Bgl*II (**b**) digested DNAs were hybridized with a probe generated from the *T. monococcum Cbf14* promoter. The *A*, *B*, and *D*, *Cbf14* cross-hybridizing fragments, MWs 2.3, 4.6, and 1.65 kb, respectively, are identified and labeled according to their respective homoeolog. *Sac*I restricts the *B Cbf14* homoeolog into two cross-hybridizing bands; the lower MW of the two is indicated using an arrowhead. *Bgl*II restricts the *A Cbf14* homoeolog into two cross-hybridizing bands. Insufficient quantity of DNA precluded the N5D/T5A line from the *Bgl*II digest

The A and D genomes of hexaploid wheat have greater *Cbf14* copy numbers than the B genome

Data indicating variation in *Cbf14* copy numbers across different accessions of hexaploid wheat were obtained by surveying a small collection of genotypes (Knox et al. 2010). To explore the extent of *Cbf14* CNV in wheat, we surveyed a panel of 50 hexaploid wheats that included 47 *T. aestivum* subsp. *aestivum* accessions and three *T. aestivum subsp. sphaerococcum* accessions. Of the 47 subsp. *aestivum* accessions, 35 are assigned to one of the five market classes—white spring (WhS), hard red spring (HRS), white winter (WhW), SRW, and HRW (Cox 1991; Zeven and van Hintum 1992); NPGS http://www.ars-grin.gov/npgs/index.html), whereas the remaining 12 *T. aestivum* accessions are unclassified.

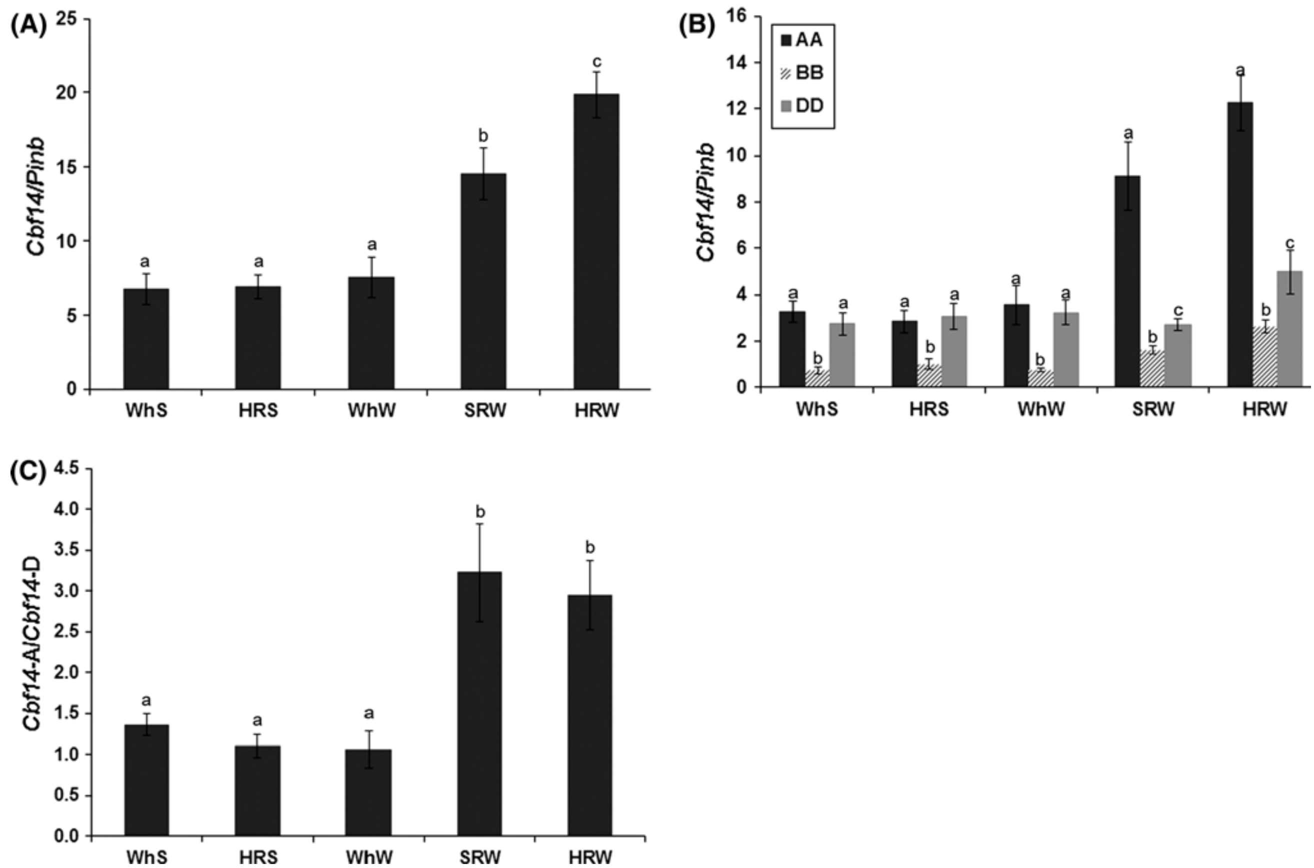

Fig. 2 *Cbf14* copy number estimates in hexaploid wheats, *T. aestivum* subsp. *aestivum*. Genotypes are grouped into five market classes—white spring (WhS), hard red spring (HRS), white winter (WhW), soft red winter (SRW), and hard red winter (HRW). **a** *Cbf14* copy numbers relative to *Pinb* in the hexaploid genome; **b** comparisons of *Cbf14/Pinb* ratios across the three genomes within each class for each genome; **c** *Cbf14* copy numbers in the A genome relative to those in the D genome (*Cbf14*-A/*Cbf14*-D). WhS, HRS, WhW, SRW, and HRW classes include 10, 6, 6, 5, and 8 genotypes, respectively (identified in Fig. S2 and Table S2). *Error bars* represent standard error of mean (SEM). Statistically significant differences are indicated by *different letters* above the *error bars* ($P < 0.05$)

In 43 of the 50 accessions, the *Cbf14* cross-hybridizing bands migrated to the same MW positions as those of the nullisomic-tetrasomic lines (Fig. S2A and S3A; Fig. 1). Of the remaining seven accessions, three including 'Blueboy', 'Mayview', and 'Odessa' did not produce the typical D genome fragment and instead cross-hybridized to a fragment of higher MW, just below that of the B genome fragment (Fig. S3A). These three genotypes have both the A and B cross-hybridizing fragments. Loss of the fragment cross-hybridizing at the D genome MW and appearance of the novel MW support it to be from the D genome and a Restriction Length Polymorphism, but it is also possible that this is a disomic addition from another genome and the D genome compliment is absent. Four accessions, including 'Sonora' and 'Mandel Gehun' (Fig. S2A), and 'PI 70711', and 'PI 40941' (Fig. S3A), did not produce a *Cbf14* cross-hybridizing B genome fragment.

To estimate *Cbf14* copy numbers, we hybridized the same filters to a coding sequence probe of the *Puroindoline b* (*Pinb*) gene and quantified *Cbf14/Pinb* signal intensity ratios (Fig. S2 and S3; Table S2). *Cbf14/Pinb* ratios were determined for the A, B, and D genomes (Fig. 2, Table S2). These ratios were then used to estimate total copy numbers of *Cbf14* in the hexaploid genome. Comparison of total *Cbf14* copy numbers revealed the order HRW > SRW > HRS = WhW = WhS (Fig. 2a). The differences in *Cbf14* copy numbers between HRW, SRW, and HRS wheats were significant (Fig. 2a). In all genotypes, regardless of their market class and growth habit, *Cbf14* copy numbers were significantly higher in the A and D genomes compared to the B genome (Fig. 2b; Table S2). In SRW and HRW wheats, *Cbf14* copy numbers in the A genome were significantly higher than those in the D genome (Fig. 2b). Additionally, *Cbf14* copy numbers in the A and B genomes of HRW and SRW wheats were significantly higher than those of the other three classes (Table S2).

To assay relative genome copies of *Cbf14* within a genotype, the signal intensity of the A genome *Cbf14*

cross-hybridizing fragment was normalized to that of the D genome fragment. The ratios of *Cbf14*-A/*Cbf14*-D were approximately 1:1 for WhS, WhW, and HRS wheats (Fig. 2c; Table S2). One exception was HRS wheat 'Cadet', which exhibited about threefold greater copy numbers in the D genome compared to A genome (Table S2). In comparison, the *CBF14*-A/*CBF14*-D ratios for SRW and HRW wheats were about 3:1 (Fig. 2c; Table S2).

Taken together, these data indicate that most HRW wheats have greater copy numbers of *Cbf14* than the wheats of other market classes. Greater *Cbf14* copy numbers of HRW wheats over the SRW wheats are significant for the B genome and also appear to occur for the A genome, however, differences between HRW and SRW in the A genome copies were not significant. Among red wheats, *Cbf14* copy numbers are much higher in winter genotypes (hard and soft) compared to spring genotypes primarily due to increased copy numbers in the A genome. Differences in *Cbf14* copy numbers between winter and spring white wheats were not detected in any genome however. Regardless of the market class and growth habit, the B genome harbored the fewest copies of *Cbf14*.

Cbf14 copy numbers vary between the A, B, and D genomes of diploid wheats

Differences in *Cbf14* copy numbers across the A, B, and D genomes of hexaploid wheats raised the question as to what was the state of *Cbf14* copy numbers in the ancestral diploid wheats. To address this question, we surveyed accessions of the diploid ancestors, *T. urartu* (AA), *Aegilops speltoides* (BB), and *Ae. tauschii* (DD). We also examined several accessions of einkorn wheat, *T. monococcum* (A^mA^m), a cultivated relative of *T. urartu*.

Using a *Sac*I digest and the same *Cbf14* promoter fragment as a probe, most of the A, B, and D genome diploid wheats produced bands of similar MWs as those of hexaploid wheats (Fig. S4A). Nonetheless, there was some variability. The MWs of the cross-hybridizing bands in *T. urartu* accessions, PI 428183 and PI 428316 ran lower than the A genome band of hexaploid wheats (Fig. S4A). *T. urartu* accession G1812 did not cross-hybridize to the *Cbf14* 5′ probe (Fig. S4A). Testing a *Cbf2* gene-specific probe on the same filter resulted in a cross-hybridizing band with G1812 (not shown), indicating other *Fr-2 Cbf*s were still present in this genotype. The *Ae. speltoides* accessions exhibited several different hybridization patterns (Fig. S4A). The *Ae. tauschii* accessions exhibited two different banding patterns both of which consisted of one strong, and one weak cross-hybridizing band (Fig. S4A). As *T. urartu* and *Ae. tauschii* are self-pollinating species, the two different banding patterns in each suggest at least two allelic forms in each species, and that there is a *Sac*I site unevenly bisecting the region cross-hybridizing to the probe in *Ae. tauschii*. In comparison, *Ae. speltoides* is an outcrosser, and as such two bands of relative equal intensity suggest that the DNA may be heterogeneous and comprising two allelic forms.

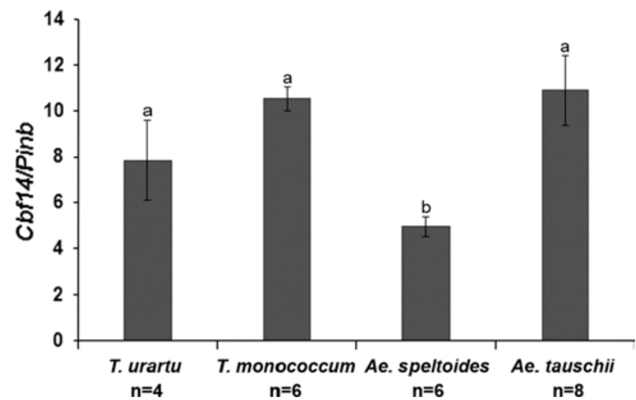

Fig. 3 Mean *Cbf14/Pinb* ratios in diploid wild (*T. urartu* (AA), *Ae. speltoides* (BB), and *Ae. tauschii* (DD) and cultivated (*T. monococcum* A^mA^m) wheats. Number of genotypes within each species (*n*) is listed on the *X* axis. *Error bars* represent SEM. Statistically significant differences are indicated by *different letters* above the *error bars* ($P < 0.05$)

To estimate copy numbers of *Cbf14,* the same blots were subsequently hybridized with *Pinb* and the *Cbf14/Pinb* ratios were determined (Fig. S4B). The resulting *Cbf14* copy number estimates ranged 3–11 in *T. urartu* (excluding G1812), 8–12 in *T. monococcum*, 3–7 in *Ae. speltoides*, and 5–20 in *Ae. tauschii* (Table S3). Average *Cbf14* copy numbers were significantly lower in *Ae. speltoides* (BB) compared to the other diploid wheats (Fig. 3). No significant differences were detected in the average *Cbf14* copy numbers between the *T. urartu*, *T. monococcum*, and *Ae. tauschii* accessions (Fig. 3). Additionally, no association was detected between growth habit and *Cbf14* copy numbers in the *T. monococcum* accessions (Fig. S4A; Table S3).

Overall, *Cbf14* copy numbers in diploid wheats were lower in the B genome compared to the A and D genomes, a result which paralleled the findings of the hexaploid wheats.

Cbf14 CNV between the A and B genomes of tetraploid wheats

Cbf14 copy numbers were also assayed in a population of tetraploid (AABB), *T. turgidum* subsp. *durum* wheats resulting from a breeding program directed at developing winter durum wheats (Hall et al. 2011; Schilling et al. 2003). Among this group, the majority of the accessions produced three *Cbf14* cross-hybridizing bands (Fig. S5), the MWs of which were similar to those of the A and B

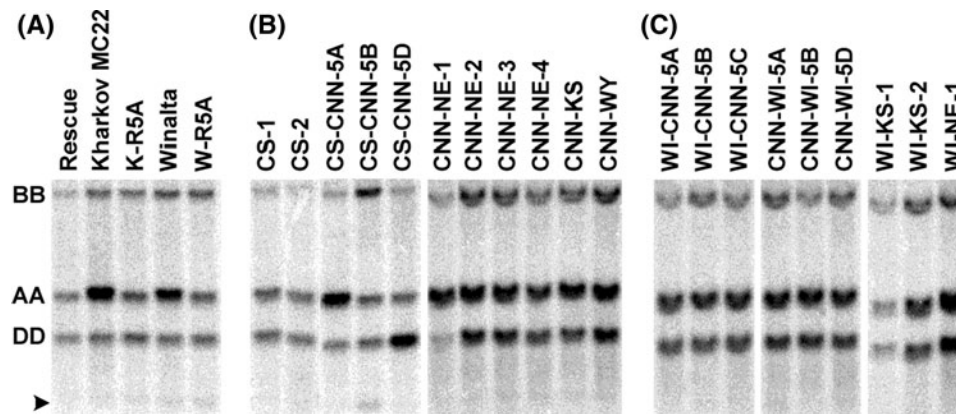

Fig. 4 *Cbf14* hybridization patterns in the inter-varietal chromosome 5 substitution lines of hexaploid wheat. **a** Substitution lines K-R5A and W-R5A have the 5A homoeolog of 'Rescue' in the 'Kharkov MC22' and 'Winalta' backgrounds, respectively, **b** CS-CNN substitution lines in which the 'Cheyenne' 5A, 5B, and 5D homoeologs replace the corresponding 'Chinese Spring' homeolog in the 'Chinese Spring' background, and **c** reciprocal 5A, 5B, and 5D substitutions between 'Cheyenne' and 'Wichita'. DNA for each substitution line was isolated from seedlings produced from a single individual plant whose heads were bagged to prevent cross pollination. The MW of the A, B, and D, cross-hybridizing fragments are 2.3, 4.6, and 1.65 kb, respectively. Note: Signal intensity differences between the 'Wichita' accessions, WI-KS-1, WI-KS-2, and WI-NE-1 are attributed to DNA quantity loading differences. *CS* Chinese Spring, *CNN* Cheyenne, *WI* Wichita. The *numbers* and *letters* added to CS, CNN, and WI indicate different sources of accessions, which are identified in Table S1

genomic fragments in hexaploid wheats. Six accessions did not produce B genome *Cbf14* cross-hybridizing bands including Odessa#66, VA05WD-1, VA05WD-12, VA05WD-16, XVAD99067-24, and XVAD99147-1 (Fig. S5). Because *Pinb* is absent from the tetraploid wheats (Chantret et al. 2005; Gautier et al. 2000), copy numbers of *Cbf14* were estimated in the A genome relative to the B genome. The *Cbf14*-A/*Cbf14*-B ratios were in the range of 4–26 (Table S4). These ratios were among the highest in XVAD99069-18 and VA05WD-39, and lowest in XVAD99068-14 (Table S4). These *Cbf14*-A/*Cbf14*-B ratios are at best only a rough estimate of the *Cbf14* copy numbers in the A genome relative to the B genome. Nonetheless, the hybridization patterns together with the *Cbf14-A/Cbf14-B* ratios indicate lower copy numbers in the B genome compared to the A genome of tetraploid wheats.

Cbf14 copy number differences in the inter-varietal wheat chromosome substitution lines

Chromosome substitution lines have played an important role in the genetic analyses of freezing tolerance and winter hardiness in wheat and have been instrumental in the identification of *Fr-2*. We hypothesized that *Cbf14* copy numbers may be different between the donor and recipient genomes of these substitution lines. To test this hypothesis, we examined three independent sets of disomic chromosome 5 substitution lines.

One set included the Kharkov-Rescue 5A (K-R5A) and Winalta-Rescue 5A (W-R5A) lines in which the 5A chromosome pair from the spring wheat 'Rescue' replaces the 5A of winter wheats 'Kharkov MC22' and 'Winalta', respectively (MacDonald 1987; Roberts and MacDonald 1988). Hybridization of the *Cbf14* 5′ probe to this set of lines indicated that the signal intensity of the A genome *Cbf14* cross-hybridizing fragment was much greater in the two winter wheats 'Kharkov MC22' and 'Winalta' compared to that of the spring wheat 'Rescue' (Fig. 4a). Signal intensities of the 5A bands in the K-R5A and W-R5A substitution lines were comparable to that of 'Rescue' (Fig. 4a).

Two additional sets of examined substitution lines had the winter wheat 'Cheyenne' as either the donor or recipient of chromosome 5 homoeologs. One of these was the set in which the 5A, 5B, and 5D homoeologs of 'Cheyenne' (CNN) replace those of the spring wheat 'Chinese Spring' (CS), and are identified as CS-CNN-5A, CS-CNN-5B, and CS-CNN-5D, respectively (Morris et al. 1966). The other was the set in which the 5A, 5B, and 5D homoeologs of the two winter wheats 'Cheyenne' and 'Wichita' are reciprocally substituted in the background of the other (Zemetra and Morris 1988; Zemetra et al. 1986). In addition, parental lines 'Chinese Spring', 'Cheyenne', and 'Wichita' obtained from multiple sources were also examined (Table S1).

The *Cbf14* hybridization patterns detected with the substitution lines having the 'Cheyenne' 5A, 5B, and 5D homoeologs in the 'Chinese Spring' background indicated that in each case there was greater signal intensity of the substituted chromosome from the 'Cheyenne' donor over that of the corresponding homoeolog of 'Chinese Spring'

(Fig. 4b). Comparison of the CS-CNN substitution lines to a panel of 'Cheyenne' accessions obtained from different sources indicated that there was variability across the different 'Cheyenne' accessions (Fig. 4b). The most apparent difference occurred with 'Cheyenne' accession CNN-NE-1. Whereas the other five 'Cheyenne' accessions exhibited increased signal intensities of the A, B, and D genome cross-hybridizing fragments over 'Chinese Spring', CNN-NE-1 exhibited greater signal intensity only in the A genome cross-hybridizing fragment (Fig. 4b).

Analysis of the 'Cheyenne' and 'Wichita' parental lines, and the set of reciprocal substitution lines indicated that the 5B homoeolog from 'Cheyenne' had greater signal intensity of the *Cbf14* cross-hybridizing band than that of 'Wichita' 5B (compare WI-CNN-5B and CNN-WI-5B in Fig. 4c). To independently assay differences in B genome copies, we also used a *Bgl*II digest, which produces a lower MW B genome fragment. Using the *Bgl*II digest, the increase in *Cbf14* hybridization signal intensity of the 'Cheyenne' 5B homoeolog over the 'Wichita' 5B homoeolog was much more striking (Fig. S6). This observation was supported by *Cbf14-B/Cbf14-D* ratios of the 'Cheyenne' and 'Wichita' reciprocal substitution lines (Table S5).

Taken together these data indicate that *Cbf14* copy numbers differ among genotypes used to develop substitution lines. These substitution lines were developed 30–50 years ago (MacDonald 1987; Morris et al. 1966; Zemetra et al. 1986). Despite having gone through numerous cycles of selfing since their development (PS Baenziger, UNL, personal communication), the similar signal intensities of the chromosome 5 homoeologs between the substitution lines and the parental donor lines indicate that *Cbf14* copy numbers are stable over generations.

Discussion

The work presented here reveals that *Cbf14* is copy number variable across wheat. Using DNA blot hybridization, *Cbf14* CNV could be robustly scored with a single probe. The *Sac*I digest in combination with the *Cbf14* promoter probe produced a simple banding pattern across a wide array of diploid, tetraploid, and hexaploid wheat germplasm. The promoter fragment provided a gene-specific probe that avoided cross-hybridization to closely related *Cbf* gene family members, which occurs using coding sequence (CDS) probes. This strategy also avoided differential amplification of PCR products which will occur when primer sites differ in the different genomes, and the use of less than optimal primer sites for use in quantitative PCR to obtain genome-specific estimates of *Cbf14* copy numbers.

In the diploid ancestors of hexaploid wheat, a higher *Cbf14* copy number state exists, and in hexaploid wheat significantly greater *Cbf14* copy numbers occur in the red winter wheats over the red spring wheats, and these differences occur on all three group 5 homoeologous chromosomes. These data imply that higher *Cbf14* copy numbers pre-existed in the diploid ancestors, that reduction in *Cbf14* copy numbers probably occurred following polyploidization, and that the ancestral state was retained in some populations and lost in others. *Cbf14* copy number reductions in the hexaploid wheat D genome most likely occurred following amphiploidisation between *T. turgidum* and *Ae. tauschii*. In the case of the hexaploid wheat A and B genomes it is less clear. The B genome fragment was variable among the group of durums assayed, indicating reductions in the B genome may have already existed in tetraploid wheat prior to amphiploidisation with *Ae. tauschii*. As a group the hard red wheats also had greater B genome *Cbf14* copy numbers than the soft red wheats; one scenario that can explain this difference is that these two winter wheat classes are derived from a common hexaploid ancestor and reductions in B genome *Cbf14* copy numbers occurred in a subset of derivative populations. However, as evidence indicates two spatially and temporally separated amphiploidisation events occurred between *T. turgidum* and *Ae. tauschii* (Giles and Brown 2006), another possible scenario is that these two winter wheat classes are derived from separate amphiploidisation events between *Ae. tauschii* and different *T. turgidum* genomes that already differed in their B genome *Cbf14* copy numbers.

Market classifications and growth habit categorizations are a traditional classification system of wheats grown in North America (Cox 1991; Zeven and van Hintum 1992), which is also used by the National Plant Germplasm System for classifications. Accessions that form the foundation of North American germplasm under these groupings were introduced as landraces and have known Old World origins. Many of the soft red winter wheats were introduced from Northern and Western Europe, the hard red wheats were introduced from the South-West region of the former Soviet Union, and the HRS wheats trace to the Red Fife accession from Poland (Cox 1991; Zeven and van Hintum 1992). In comparison, white wheats were introduced into North America from Old World and New World locations, which suggest that they were first taken by European immigrants to these locations prior to their North American introduction (Cox 1991; Zeven and van Hintum 1992). Most were highly genetically variable at the time they were introduced (Cox 1991; Zeven and van Hintum 1992). While knowing the site of origin of a landrace might provide clues to adaptability based on assumption of a landrace having undergone long-term selection, drawing definitive conclusions about the evolution of a landrace due to its geographic origin is complicated by other factors. For example as the hard red wheats were introduced from

the South-West of the former USSR, a logical assumption might be that these accessions were adapted to that region. While this may be the case, loss of the original landrace and introduction of different landraces from outside regions have occurred through human migration and agricultural practices and thus this may blur our current view (Zeven 1986). Nonetheless introduction of the hard red wheats made possible the cultivation of winter wheat on the Great Plains where previous attempts with the other winter wheats failed due to their inability to survive the more arid and colder winters (Quisenberry and Reitz 1974; Zeven and van Hintum 1992). Whereas our data indicate that *Cbf14* copy numbers of winter and spring white wheats of these early landrace types are more similar to each other and to the red spring wheats than to either class of the red winter wheats, through plant breeding hard white winter wheats are now grown on the Great Plains (Carver et al. 2003; Haley et al. 2003; Ibrahim et al. 2008; Martin et al. 2001; Pike and MacRitchie 2004). We did not assay these modern cultivars but it would be worth knowing whether they possess introgressions of the higher copy number state alleles on all three of the group 5 homoeologs.

It is also noteworthy that the B genome homoeolog has the fewest *Cbf14* copy numbers relative to the A and D genome homoeologs. This trend occurs in hexaploid, tetraploid, and diploid wheats. This is again consistent with the higher *Cbf14* copy number states pre-existing in the diploid ancestors. That the increased copy numbers occur in all of the diploid genomes suggests the amplifications that generated the multiple copies probably occurred in a common ancestor that predates the divergence of the different diploid wheat species. This may have occurred simultaneously with the projected expansion of the *Cbf* gene family in the core Pooids (Sandve and Fjellheim 2010). At a subsequent point in time, and prior to the amphiploidisation between *T. urartu* and the *Ae. speltoides* relative, reductions in *Cbf14* copy numbers occurred in the genome of the B genome diploid wheats. Further reductions in *Cbf14* copy numbers then also continued in the B genome following this amphiploidisation.

In a number of accessions, *Cbf14* was completely absent from a genome. There was no B genome *Cbf14* 5′ cross-hybridizing fragment in four of the spring growth habit hexaploid wheats including ‘Sonora’, ‘Mandel Gehun’, ‘PI 70711’, and ‘PI 40941’. There was also no B genome fragment in six of the tetraploid *T. turgidum* subsp. *durum* wheat accessions including Odessa#66, four lines derived from Odessa#66 (VA05WD-1, VA05WD-12, VA05WD-16, and XVAD99067-24), and XVAD99147-1. The A genome diploid G1812 also did not cross-hybridize to the *Cbf14* 5′ probe. Absence of a *Cbf14* 5′ cross-hybridizing fragment in these accessions may be due to a different *Cbf14* promoter sequence or the complete deletion of *Cbf14* from their genomes. We also screened the G1812 BAC library (Akhunov et al. 2005) using a *Cbf14* CDS probe and did not recover clones (TD and EJS, unpublished data), consistent with the absence of *Cbf14* from this genotype.

Differences across the multiple ‘Cheyenne’ accessions in cross-hybridizing signal intensities of 5B and 5D homoeologs indicate that some of these accessions are different. ‘Cheyenne’ was released as a pure-line selection from a Crimean landrace (Clark 1931). It is possible that ‘Cheyenne’ was originally a heterogeneous population from which subsequent selection and single seed descent resulted in different homogeneous individuals. Pure-line selection as practiced in the early part of the 20th century often involved selecting individuals for similar phenotypes from a phenotypically polymorphic landrace, and did not necessarily involve selection from one single homozygous individual. Most likely a ‘Cheyenne’ accession other than CNN-NE-1 was used to develop the CS-CNN substitution lines.

While *Cbf14* gene CNV occurs on all three homoeologs, the A and D homoeologs exhibit the largest differences across wheat accessions. It is possible that the larger differences in *Cbf14* copy numbers of the 5A and 5D homoeologs are an underlying genetic factor leading to the more frequent identification of 5A and 5D as affecting differences in freezing tolerances (Cahalan and Law 1979; Roberts 1986, 1990; Roberts and MacDonald 1988). Nonetheless 5B is also associated with differences in freezing tolerance, and substitution of the ‘Cheyenne’ 5B into ‘Chinese Spring’ and ‘Wichita’ results in improved freezing tolerance (Tóth et al. 2003; Veisz and Sutka 1989). It must be stressed that *Cbf14* is only one gene in the cluster of at least 11 different *Cbf* gene CDSs at *Fr-2*. Data obtained using CDSs as probes against a panel of nine hexaploid wheat lines suggest that *Cbf2* and *Cbf9* are also copy number variable and that the trend appears to be similar to that of *Cbf14* (Fig. S7). Thus, multiple *Fr-2 Cbf* gene orthologs may be copy number variable, their numbers may act additively, and together they contribute in a quantitative manner to the pathways that they affect regulatory control over.

While contributions of the *Cbf* genes may act in a quantitative manner, mutations in single *Cbf* genes at the *Fr-2* cluster are also associated with differences in freezing tolerance. In *T. monococcum*, genetic analyses identified *Cbf12*, *Cbf14*, and *Cbf15* as candidate genes explaining a large portion of the differences in freezing tolerance between DV92 and G3116 (Knox et al. 2008). *Cbf14* and *Cbf15* sequences of DV92 do not notably differ from those of G3116 (Knox et al. 2008). In the instance of *Cbf12,* there is a 15 bp in-frame deletion in the DNA-binding domain of the DV92 protein that eliminates binding to its cognate binding site whereas the G3116 protein is able to bind (Knox et al. 2008). A smaller QTL effect also

resolves just distal to the *Cbf12–Cbf14–Cbf15* cluster, and includes *Cbf16*, *Cbf13*, *Cbf3*, and *Cbf10* (Knox et al. 2008). Because transcripts for *Cbf12*, *Cbf15*, and *Cbf16* accumulate to higher levels at warmer temperatures in G3116 than in DV92, this differential regulation affect could also not be ruled out as contributing toward differences in freezing tolerance (Knox et al. 2008). It is nonetheless possible that mutations in a single *Cbf12* gene or in other members of this clade (e.g., *Cbf15* or *Cbf16*) have more pronounced phenotypic consequences than copy number differences in *Cbf14* or in other members of this clade (e.g., *Cbf2*, *Cbf4*, or *Cbf9*) as there are a number of differences that discriminate the CBF proteins in these two clades (Skinner et al. 2005; Xue 2003).

DV92 and G3116 both appear to have 10 copies of *Cbf14* based on *Cbf14* to *Pinb* ratios of 9.5 and 10.5, respectively—data consistent with *Cbf14* not being a contributing factor in freezing tolerance differences between these two genotypes (Knox et al. 2008). Although the specific activity of the probes may affect hybridization signal intensities, and thus ratios, both probes were about the same length, 189 bp (*Cbf14*) and 201 bp (*Pinb*). And while ten copies might seem high, it is not unprecedented in plants. At the *rhg1* locus, which confers resistance and susceptibility to soybean cyst nematode, resistance is conferred by an allele having ten copies of a 31 kb region encompassing six genes, three of which contribute toward resistance, whereas susceptibility occurs by the allele having a single copy of this 31 kb region (Cook et al. 2012). At *Fr-2*, it is presently unclear how the physical arrangement of multiple *Cbf14* genes occurs. The *Cbf14* sequence of *T. monococcum* DV92 is derived from a 3.3 kb subclone from BAC clone 119P22 (Miller et al. 2006). Additional identical *Cbf14* paralogs may exist on that same BAC clone, each of which would run at the same MW position on an agarose gel. This is the situation in barley in which independent bacteriophage lambda genomic clones harboring *Cbf2A* and *Cbf4B* recovered from screening non-amplified primary libraries produced identical fingerprints with the restriction enzymes used for fingerprinting (Knox et al. 2010). While the *Cbf2A* and *Cbf4B* CDSs on independent phage clones were identical to the *Cbf2A* and *Cbf4B* CDSs on different phage clones, sequencing those clones revealed polymorphisms in the intergenic regions making it technically feasible to discern that the identical CDSs are embedded in different genomic regions (Knox et al. 2010). In the instance of the wheat *Cbf14* gene, another possibility is that there is only one *Cbf14* CDS on the 119P22 BAC clone but there are additional *Cbf14* gene paralogs residing in similar, but distinct and genetically linked genomic regions. Figure S1 shows an alignment of the genomic regions encompassing *Cbf14* from *T. monococcum* accessions DV92 and G3116, and from two BAC clones, JF758494.1 and JF758498.1, from *T. aestivum* cv. 'Norstar'. These two Norstar BAC clones are derived from the A genome yet they do not encompass identical regions (Fig. S1). These data are consistent with multiple *Cbf14* genes in the A genome, and a single *Cbf14* per BAC insert.

Multiple copies of *Cbf14* across the different wild diploid ancestors and the reductions in copy numbers in the different cultivated tetraploid and hexaploid wheats suggest both functional importance to the multiple copy numbers in these wild ancestors and that copy number reductions likely occurred as a result of changes in selection pressures, possibly in the form of cultivation practices. Diploid wheats do not exhibit greater freezing tolerance than hexaploid wheats. On the contrary, hexaploid wheats exhibit greater freezing tolerance than the diploid and tetraploid wheats (Fowler et al. 1977; Limin and Fowler 1981, 1982). However, the winter growth habit is the ancestral form in the Triticeae (Flood and Halloran 1986; Matus and Hayes 2002; von Bothmer et al. 2003; Yan et al. 2003; Zohary 1969), and thus developmental programming controlling this habit is likely critical for these cereals in their natural habitat. In barley, cultivated genotypes carrying mutations in *Vrn-1* that result in the spring growth habit exhibit reduced copy numbers of the *Cbf2A–Cbf4B* genomic region relative to cultivated winter barleys (Knox et al. 2010). These associations between reduced copy numbers and the spring growth habit in cultivated forms suggest reductions occurred in response to selection for the spring growth habit and possibly earlier flowering, or both. While this relationship did not hold in the *T. monococcum* accessions examined here, three of the four spring accessions (PI 538722, PI 427927, and DV92) retain a winter *vrn-1* allele at *Vrn-1* and are of spring growth habit due to mutations in *Vrn-2* (Yan et al. 2004). (The fourth accession (PI 355549) was not genotyped for *Vrn-2* in that work and we do not know whether it too carries the same mutation.) With a winter *vrn-1* allele, *VRN-1* transcripts do not accumulate until cued by endogenous and exogenous signals (Distelfeld et al. 2009; Trevaskis 2010). In the absence of *VRN-1* accumulation when plants are in the vegetative phase, *CBF* mRNAs accumulate to much higher levels than when *VRN-1* transcripts accumulate and plants are in the reproductive phase (Dhillon et al. 2010; Stockinger et al. 2007). It is not clear what mechanisms control this process, but this indicates that the plant has a mechanism in place to reduce *CBF* transcript levels during the reproductive transition. Selection for the spring growth habit via mutations in the *Vrn-1* gene which results in the constitutive expression of *VRN-1* may have rendered higher *Cbf* copy numbers functionless, and thus they were lost. This hypothesis could be more rigorously tested through determination of *Vrn-1* allelic states, *Cbf14* copy numbers, and growth habit form in a more expansive survey of *T. turgidum* that includes

the earliest cultivated *T. turgidum* ssp. *dicoccon* alongside wild *T. turgidum* ssp. *dicoccoides* exhibiting known kinship relationships to the cultivated forms (Luo et al. 2007). However, CBFs also delay flowering (Gilmour et al. 1998; Liu et al. 1998), and as such selection for earlier flowering should not be excluded as a factor actively driving reductions in *Cbf* gene copy number. In essence, these relationships indicate that the primary functional role of the *Cbf*s probably occurs during the vegetative growth phase and not during the reproductive phase. Thus, copy numbers may be acting additively and the high copy number states in the wild diploid species may be one integral component in a mechanism to maintain and possibly even promote developmental processes associated with vegetative growth for these plants during the winter and spring seasons in their natural habitat. Retention of the ancestral higher copy number state in landrace populations giving rise to the HRW wheats may be due to constancy in the selection pressures for the winter growth habit and higher yield through a more extended vegetative growth phase. A plant that is actively maintaining the vegetative growth phase is also likely to be manifest in its ability to regrow shoots and roots from a meristematic region following winter kill. Whereas the diploid wheats have a single cadre of *Cbf* genes, hexaploid wheats have three cadres, which may simply act additively in contributing toward this phenotype.

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