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ORIGINAL PAPER

Large deletions in the *CBF* gene cluster at the *Fr-B2* locus are associated with reduced frost tolerance in wheat

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Abstract Wheat plants which are exposed to periods of low temperatures (cold acclimation) exhibit increased survival rates when they are subsequently exposed to freezing temperatures. This process is associated with large-scale changes in the transcriptome which are modulated by a set of tandemly duplicated *C-repeat Binding Factor* (*CBF*) transcription factors located at the *Frost Resistance-2* (*Fr-2*) locus. While *Arabidopsis* has three tandemly duplicated *CBF* genes, the *CBF* family in wheat has undergone an expansion and at least 15 *CBF* genes have been identified, 11 of which are present at the *Fr-2* loci on homeologous group 5 chromosomes. We report here the discovery of three large deletions which eliminate 6, 9, and all 11 *CBF* genes from the *Fr-B2* locus in tetraploid and hexaploid wheat. In wild emmer wheat, the *Fr-B2* deletions were found only among the accessions from the southern subpopulations. Among cultivated wheats, the *Fr-B2* deletions were more common among varieties with a spring growth habit than among those with a winter growth habit. Replicated freezing tolerance experiments showed that both the deletion of nine *CBF* genes in tetraploid wheat and the complete *Fr-B2* deletion in hexaploid wheat were associated with significant reductions in survival after exposure to freezing temperatures. Our results suggest that selection for the wild-type *Fr-B2* allele may be beneficial for breeders selecting for varieties with improved frost tolerance.

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Introduction

Wheat is one of the world's most important crops, providing approximately one-fifth of all calories consumed globally. Hexaploid bread wheat (*Triticum aestivum*)—which accounts for 95 % of all wheat grown—has been adapted to grow in environments spanning latitudes from 65°N to 45°S, a range which encompasses large variations in temperature during the growing season (Dubcovsky and Dvorak 2007). Due to their longer growing season, fall-sown wheat varieties (winter wheat) are generally higher yielding than those sown in spring (spring wheat). However, to achieve this greater yield potential winter wheat varieties must be able to withstand freezing temperatures during the winter.

Variation in a plant's freezing tolerance is closely associated with cold acclimation, the process by which extended exposure to gradually decreasing, but non-freezing temperatures, results in improved cold tolerance and survival rates when the plants are subsequently exposed to freezing temperatures (Thomashow 2010). During cold acclimation, plants undergo large-scale changes in their transcriptome, which in turn activates the production of an array of proteins to aid the plant's survival during subsequent freezing stress (Kocsy et al. 2010; Winfield et al. 2010).

Despite these large changes in global gene expression patterns, it appears that a relatively small number of loci account for a large proportion of the observed variance in cold tolerance between wheat varieties (Vágújfalvi et al. 2000). Chromosome substitution experiments using the cold-hardy variety Cheyenne as a donor and the cold-susceptible variety Chinese Spring as a recipient showed that transferring chromosomes 5A, 5D and, to a lesser extent, 5B conferred the greatest improvements in freezing tolerance (Sutka 1981; Veisz and Sutka 1989; Toth et al. 2003). Using these same chromosome substitution lines, it was shown that chromosome 5A substitutions also conferred stronger induction of *COR14b*, a central cold-responsive gene contributing to the protection of chloroplasts during exposure to freezing temperatures (Vágújfalvi et al. 2000). This gene, as well as numerous other cold-responsive genes, harbors a CRT-DRE box within its promoter, a conserved binding domain for the *CBF* (*C-repeat Binding Factor*) family of transcription factors. One such gene, *TmCBF3*, was mapped to the same region as the QTL for differential *COR14b* expression—designated the *Fr-2* (*Frost resistance-2*) locus—suggesting that the strong effect this locus has on cold tolerance can be attributed to the *CBF* genes (Vágújfalvi et al. 2003). Furthermore, several different *CBF* genes which also map to the *Fr-2* locus were shown to exhibit significantly higher expression in cold-hardy plants than cold-sensitive ones (Vágújfalvi et al. 2005).

An in-depth study of the *CBF* gene family in *T. monococcum* identified 15 *CBF* genes, 11 of which were present as a cluster at the *Fr-2* locus on chromosome $5A^m$ (Miller et al. 2006). A subsequent high-density mapping study of this region suggested that the *CBF12*, *CBF14* and *CBF15* genes were most likely to account for the observed differences in cold tolerance within the specific *T. monococcum* mapping population used in this study (Knox et al. 2008). The *CBF* genes are a family of AP2/ERF transcription factors and have been studied in greatest depth in *Arabidopsis*. They play a crucial role in the co-ordination of cold response and their expression is induced within 15 min of exposure to cold temperatures, which is closely followed by the up-regulation of cold-responsive genes around 2 h later (Thomashow 2010). This so-called *CBF* regulon has been estimated to represent a significant proportion of cold-inducible genes; for example, 28 % of all cold-inducible genes were also up-regulated in *Arabidopsis CBF2* over-expression transgenic plants (Vogel et al. 2005). These include genes coding for enzymes that make cryoprotective and other membrane-stabilizing proteins, which help to prevent cellular damage when plants are subjected to the extreme dehydration associated with freezing stress.

In *Arabidopsis*, the transgenic constitutive over-expression of the *CBF* genes results in an increase in freezing tolerance even without prior cold acclimation (Gilmour et al. 2004; Jaglo-Ottosen et al. 1998; Liu et al. 1998), but this also results in a slow-growing dwarf phenotype, as a consequence of interactions between *CBF* genes and the gibberellin biosynthetic pathway (Achard et al. 2008). This suppression of growth illustrates the negative pleiotropic effects associated with expressing a transcriptome designed for cold response in warm environments.

While *Arabidopsis* has three closely related *CBF* genes tandemly arrayed in a cluster on chromosome four (Gilmour et al. 1998; Medina et al. 1999; Stockinger et al. 1997), wheat and other temperate cereals have undergone an expansion in this family. In hexaploid wheat, for example, there are at least 15 *CBF* genes in each of the three wheat genomes, including 11 at each of the three *Fr-2* loci (Badawi et al. 2007). In barley, variation in *CBF* copy number was found at the *Fr-H2* locus and this variation was associated with differences in freezing tolerance (Knox et al. 2010). The presence of natural variation within each of the wheat homeologous *Fr-2* loci is illustrated by the clear differences in frost tolerance conferred by the inter-varietal chromosome substitution lines involving all three homeologous group 5 chromosomes, where the substitution of chromosome 5A confers the largest increases in frost tolerance and chromosome 5B the least (Sutka 1981; Veisz and Sutka 1989).

In the current study, we describe the discovery of deletions of multiple *CBF* genes from the *Fr-B2* locus on chromosome 5B in tetraploid and hexaploid wheat and report their distribution among accessions with different growth habit, as well as their effect on frost tolerance in controlled freezing experiments. In both tetraploid and hexaploid wheat varieties, deletions at the *Fr-B2* locus are more commonly found in lines with a spring rather than winter growth habit and are associated with reduced freezing tolerance. We discuss the potential applications of our results for breeding programs aimed toward increasing wheat freezing tolerance.

Materials and methods

Screening for deletions at the *Fr-B2* locus and growth habit

To determine the presence or absence of each *CBF* gene, homeolog-specific PCR reactions were carried out using

the primers listed in Supplementary Materials, Table S1. All PCR reaction volumes were 20 µl and included 0.2 µM of both forward and reverse primers, 5 % DMSO and 0.75 U of *Taq* polymerase. Reactions were carried out in an ABI9700 thermocycler using a touchdown PCR protocol consisting of an initial incubation at 94 °C for 2 min, followed by 10 cycles of touchdown PCR 94 °C 20 s, 63 °C 30 s (−0.5 °C/cycle), 72 °C 1 min/kb, followed by 25 cycles of standard PCR using an annealing temperature of 58 °C. The expected amplified PCR product size for each reaction is listed in Supplementary Materials, Table S1.

For tetraploid lines, growth habit was determined using PCR markers to screen for spring and winter alleles of *VRN-A1* (Fu et al. 2005). In hexaploid lines, growth habit was determined using PCR markers for the genes *VRN-A1*, *VRN-B1*, *VRN-D1* (Fu et al. 2005) and *VRN-B3* (Yan et al. 2006), and information from the National Plant Germplasm System (http://www.ars-grin.gov/npgs/).

Plant materials

To develop sister lines for the nine-gene deletion at the *Fr-B2* locus in tetraploid wheat, we crossed the durum varieties Durelle (no *Fr-B2* deletion, wild-type photoperiod-sensitive allele) and Kronos (*Fr-B2* deletion, *Ppd-A1a* photoperiod-insensitive allele) and then backcrossed the F_1 hybrid to Kronos for three generations. Selection for the photoperiod-sensitive allele at each generation was carried out using the *Ppd* markers described by Wilhelm et al. (2009) and the *Fr-B2* deletion was selected using the PCR markers described above. The three *Fr-A2* genes (*CBF-A12*, *CBF-A14* and *CBF-A15*) analyzed in this study were sequenced using the primers described in Supplementary Materials, Table S1. For all three genes, sequences were identical in both parental lines.

Two different BC_3F_1 plants heterozygous for these two loci were self-pollinated and two sets of homozygous photoperiod-sensitive BC_3F_2 sister lines (~94 % identical) with and without the *Fr-B2* deletion were selected. These plants were self-pollinated to generate sufficient BC_3F_3 seeds, which were used in the freezing tolerance experiments. These lines all carry the dominant *VRN-A1* allele conferring a spring growth habit, so before exposure to freezing temperatures, plants were maintained under short-day conditions (8 h light/day) to prevent *VRN-1* up-regulation, which, through its role in inducing reproductive development, contributes to the down-regulation of the cold-responsive genes (Dhillon et al. 2010).

Frost tolerance tests

Four survival tests at different freezing temperatures were carried out in Martonvásár, Hungary, to determine freezing tolerance of the tetraploid sister lines. These included two preliminary tests at −9 and −8 °C to determine optimum freezing temperatures for this material and two tests at −7 °C using different cold acclimation conditions. Two additional freezing tests, one using the same tetraploid sister lines and another using a set of 103 hexaploid lines, were performed at Washington State University (WSU), Washington, USA at −6 °C using different cold acclimation protocols as described below.

Martonvásár, *Hungary*: Germinated seedlings were potted in wooden boxes using a randomized block design. Two different cold acclimation schemes (A and B) were used that differed mainly in the duration of their initial growth and pre-hardening phases. In scheme B, this period was shortened and a short-day photoperiod was maintained throughout the acclimation process to slow reproductive development before the freezing test. The two cold acclimation schemes are described in detail below.

Scheme A: Plants were grown for 20 days in a PGR-15 growth chamber (Conviron, Manitoba, Canada) at 20/15 °C (day/night), 75 % relative humidity under short-day (SD) conditions (8 h light and 16 h dark) using a light intensity of 260 µmol m^{-2} s^{-1}. Temperatures were reduced to 15/10 °C for 22 days for pre-hardening, maintaining all other growth parameters. Temperatures were then decreased by 2 °C per day to 4/4 °C and the length of the light period was increased to 16 h (long days, LD). This cold-hardening period was maintained for 18 days and was followed by a second period of cold hardening of −2 °C for 6 h, +2 °C for 7 h, then −2 °C for a further 17 h and −4 °C for 22 h before temperatures were reduced to the target freezing temperature (−9 °C) in the growth chamber (C812, Conviron). The freezing temperatures were applied for 24 h and in darkness. After freezing, the temperature was gradually increased by 2 °C per hour to 16 °C. At this point, leaves were removed and the plants were left to undergo a 2-week recovery period at 17/16 °C. The evaluation of survival rates was carried out as described previously (Knox et al. 2008).

Scheme B: The initial growth phase (20/15 °C) was shortened from 20 to 7 days and the pre-hardening phase (15/10 °C) from 22 to 14 days. As in scheme A, temperatures were reduced by 2 °C per day to 4/4 °C for cold hardening and maintained for 12 days, but in this scheme plants were kept under SD conditions until the final 7 days of cold hardening, when lighting was adjusted to LD conditions. The second cold-hardening phase was as described in scheme A, which was applied before plants were exposed to the target temperature (−8 °C). Recovery and scoring were also as described in scheme A.

Two −7 °C freezing tests were subsequently carried out to discriminate survival rates between genotypes, one using scheme A and the other using scheme B.

WSU, *USA*: Two additional freezing tests (one in tetraploid and the other one in hexaploid wheat) were performed in the frost tolerance facility at WSU using their standard protocols. Since this protocol resulted in low survival rates in a preliminary experiment at −7 °C, the freezing temperature was changed to −6 °C. The cold acclimation procedure used at WSU was more similar to scheme B described above, in that the plants were maintained under SD during the cold acclimation period. Plants were germinated in soil-less potting mix (Sunshine Mix #1/LC1, Sun-Gro Horticulture, Seba Beach, CA) in plastic cell–packs, six cells per pack and with 20 seedlings of a single genotype per cell, under a SD regime (8 h light, 16 h dark) at 22/15 °C for 7 days in a PGR-15 growth chamber. During cold acclimation, plants were exposed to a constant temperature of 4 °C for 5 weeks and maintained under short-day conditions. For the freezing test, plants were moved into an LU113 programmable temperature cabinet (ESPEC NA, Hudsonville MI). Emergence of the 20 seedlings was recorded and plants were cut to a height of 1″. The planting mix was drenched with ice water containing 10 mg/L Snowmax® (a commercial product that results in uniform ice nucleation at about −3 °C) and a layer of crushed ice was placed on the soil surface. The temperature of the programmable chamber was lowered to −3 °C for 16 h to allow the heat produced during ice formation to dissipate. The temperature was decreased to −6 °C at a rate of 4 °C per hour. Soil temperatures were traced with sensors buried within each pot. After the 16 h at −3 °C, temperature changes in the air and the soil were very similar, and in 45 min both reached the target temperature of −6 °C. The target temperature was maintained for one additional hour and then increased to 4 °C at a rate of 4 °C per hour. The tray was then returned to a growth chamber set to an SD regime. The chamber was set to a constant temperature of 4 °C for the first 24 h, before temperatures were increased to 22/15 °C thereafter. Survival was scored 3 weeks after the date of freezing.

The survival rate of hexaploid wheat lines was determined using the same conditions as described above with the exception that the cold acclimation period was conducted under a LD (18 h light/6 h dark) regime, instead of SD. For these tests, survival was calculated as the average of four biological replicates for each variety, with each replicate consisting of 20 seedlings.

Statistical analysis

Since less than 3 % of the plants survived the freezing tests at −8 °C (3 survived out of 216 tested) and −9 °C (2 survived out of 85 tested), results from these experiments were excluded from the statistical analyses. Survival data for the other three tests were analyzed using a factorial ANOVA. The model included genotype (two sets of sister lines with and without the nine-gene *Fr-B2* deletion), experiment (two −7 °C experiments and one −6 °C experiment), genotype × experiment interaction, and blocks nested within experiments. The differences between the lines with and without the nine-gene *Fr-B2* deletion were tested using a simple statistical contrast. ANOVA assumptions for this analysis were validated using Levene's test for homogeneity of variances ($P > 0.05$) and the Shapiro–Wilk test for normality of residuals ($P > 0.05$).

The differences in survival in the hexaploid lines were tested using the non-parametric Wilcoxon two-sample test because the Shapiro–Wilk test for normality of residuals was not met for this data, even after transformation. Comparisons were made between winter and spring lines carrying the wild-type *Fr-B2* allele and between lines with and without the *Fr-B2* deletion allele. This last comparison was performed only for the hexaploid spring lines because we identified only a single winter line carrying the deletion at the *Fr-B2* locus among the lines tested for frost tolerance. All statistical tests were performed using SAS 9.3 (SAS Institute, Cary, NC).

Results

A deletion of nine genes at the *Fr-B2* locus on chromosome 5B in tetraploid wheat

A previous high-density mapping study identified *CBF12*, *CBF14* and *CBF15*—three adjacent genes in the center of the *Fr-B2* locus (Fig. 1)—as being critical in accounting for the differences in frost tolerance in *T. monococcum* (Knox et al. 2008). To characterize the natural allelic variation in these genes in tetraploid wheat, we developed homeolog-specific primers (Supplementary materials, Table S1) to sequence the A and B genomic copies of these three genes. We were able to amplify and sequence both genomic copies of these three genes from the winter durum wheat variety Durelle, demonstrating that all six primer pairs were functional and genome specific. However, when using Kronos genomic DNA as a template, we were able to amplify only the A genome copies of all three genes. We designed a second B genome-specific primer pair for each of these genes (Supplementary materials, Table S1), but as with the previous primer pairs, while we were able to amplify the B genome copies of *CBF12*, *CBF14* and *CBF15* from Durelle, we were unable to do so from Kronos. The lack of amplification of the B genome copies of these genes using multiple primer pairs, different sources of Kronos genomic DNA and positive and negative control PCR reactions led us to the conclusion that the *CBF12*, *CBF14* and *CBF15* genes were deleted from chromosome 5B of Kronos.

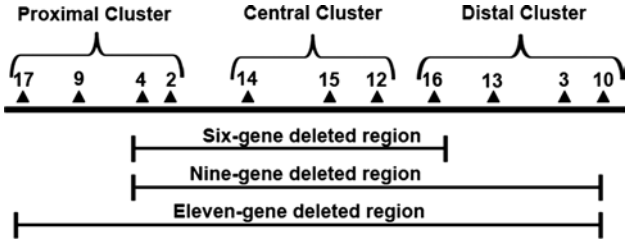

Fig. 1 The *CBF* gene cluster on chromosome 5B of wheat, showing the gene order in the proximal cluster as proposed by Miller et al. (2006) (see "Discussion" for further details on alternative gene orders at this locus). Also shown are the regions of the *CBF* cluster included in the 6-, 9- and 11-gene deletions identified in this study

The deletion of three adjacent genes was suggestive of a larger deletion within this region, so we attempted to amplify the other genes at the *Fr-B2* locus to determine if they too were included in this deleted region. Taking a similar approach, B genome-specific primer pairs were designed to amplify the remaining eight genes of this cluster (Supplementary materials, Table S1), and while all eight *CBF* genes from the B genome were amplified in Durelle, in Kronos, we were able to amplify only *CBF-B9* and *CBF-B17*.

To provide further evidence for the *Fr-B2* deletion, we made use of a transcriptome recently assembled from 488.9 M 100 bp paired-end reads generated from Kronos tissue samples (Krasileva et al. 2013). The A and B homeologs of all 11 *CBF* genes at the *Fr-2* loci were used as queries in a BLAST analysis against the assembled transcriptome contigs. Although the tissues used to construct the RNAseq libraries were not exposed to cold, 5,973 paired-end reads showed identity to 7 of the 11 *CBF* genes indicating an adequate basal transcription level to study genome representation (Table 1). For the *CBF* genes located outside the *Fr-B2* deletion (*CBF9* and *CBF17*), we detected transcripts from both the A and B genomes (>99.3 % identity, Table 1). In contrast, all other 953 paired-end reads were assembled into contigs that were >99.5 % identical to the *Fr-A2* genes (Table 1). Visual examination of the read pile-up confirmed that none of the assembled reads included B genome haplotypes (Table 1).

Taken together, the PCR amplification patterns of the 11 *CBF* genes from genomic DNA and the RNAseq results indicate that Kronos carries a deletion of 9 of the 11 genes in the *CBF* cluster at the *Fr-B2* locus (Fig. 1).

Distribution of *Fr-B2* deletions among tetraploid wheat populations

To screen for this deletion among populations of domesticated and wild wheats, we used a subset of the B genome-specific PCR markers targeted to the different regions of the *Fr-B2* locus—one from the proximal cluster (*CBF-B4*), one from the central cluster (*CBF-B12*) and one from the distal cluster (*CBF-B10*). Amplification of *CBF-B9* was included as a positive control, since it lay outside of the identified deleted region.

Wild emmer (*T. turgidum* ssp. *dicoccoides*): The progenitor of domesticated durum wheat, wild emmer, can be divided into two distinct sub-populations within the Fertile Crescent; a northern sub-population arising from the area surrounding Iraq, Iran and Turkey and a southern sub-population centered on Syria, Lebanon and Israel (Luo et al. 2007). We screened 30 wild emmer varieties and identified 6 which carried the deletion of nine *CBF* genes at the *Fr-B2* locus, while 14 lines carried the wild-type allele without the deletion (Table 2). However, there were

Table 1 BLAST analysis using *CBF* genes to query the Kronos transcriptome (Krasileva et al. 2013)

Gene	A Genome (*Fr-A2*)			B Genome (*Fr-B2*)		
	Contig	% Id.	Reads	Contig	% Id	Reads
CBF-17	UCW_Tt_k51_contig_5419	100	636	UCW_Tt_k31_contig_34427	99.6	64
CBF-9	UCW_Tt_k61_contig_1703	99.4	3,112	UCW_Tt_k55_contig_19370	100	1,204
CBF-4	UCW_Tt_k45_contig_47704	99.8	166	–	–	–
CBF-2	–	–		–	–	–
CBF-14	UCW_Tt_k45_contig_73235	100	310	–	–	–
CBF-15	–	–	–	–	–	–
CBF-12	–	–	–	–	–	–
CBF-16	–	–	–	–	–	–
CBF-13	UCW_Tt_k31_contig_66792	100	55	–	–	–
CBF-3	UCW_Tt_k61_contig_45414	100	221	–	–	–
CBF-10	UCW_Tt_k45_contig_13157	99.6	205	–	–	–

Reads indicate the number of 100 bp paired-end reads stringently mapped to each contig. "–" represents no hit found with >99 % identity to any transcriptome contig

Table 2 Distribution of the nine-gene and six-gene *Fr-B2* deletions in *T. turgidum* ssp. *dicoccoides* (wild emmer) and *T. turgidum* ssp. *dicoccum* (domesticated emmer)

Wheat	*Fr-B2* locus	*N*	Varieties[a]
T. turgidum ssp. *dicoccoides*	Nine-gene deletion	6	UH 20, UH 23, UH 27, UH 29, UH 32, UH 33
	Six-gene deletion	10	UH 5, UH 7, UH 8, UH 9, UH 11, UH 17, UH 19, UH 24, UH 28, UH 40
	Wild type	14	UH 1, UH 41, UH 42, UH 43, UH 44, PI 428017, PI 428020, PI 428028, PI 428036, PI 428041, PI 428047, PI 428055, PI 428058, PI 428061
T. turgidum ssp. *dicoccum*	Deletion	0	
	Wild type	57	PI 355498, Cltr 17675, PI 94640, PI 254180, PI 254158, PI347230, Cltr 17676, PI182743, PI 319868, PI 319869, PI 352329, PI 470737, PI 470739, PI 606325, PI 94626, PI 94627, PI 352352, PI 355454, PI 352347, PI 352357, PI 352367, PI 355496, PI 113961, PI 174108, PI 591868, IDG 8634, IDG 8649, IDG 8727, MG 3428, MG 3429, MG 3430, MG 4376, MG 4382, MG 5269, MG 5270, MG 5273, MG 5274, MG 5275, MG 5276, MG 5282, MG 5306, MG 5307, MG 5312, MG 5314, MG 5315, MG 5340, MG 5366, MG 5389, MG 5390, MG 5398, MG 5399/3, MG 5463, MG 5465, MG 5466, MG 5567, MG 28056, MG 28057

[a] PI and Cltr germplasms correspond to Germplasm Resources Information Network (*GRIN*) numbers. Other numbers correspond to 'Location–Genotype' identification numbers from the University of Haifa (*UH*) wheat germplasm collection (Nevo and Beiles 1989; Peleg et al. 2005) and to varieties from the University of Bologna *T. dicoccum* collection (MG and IDG), seeds of which were kindly provided by Dr. Marco Maccaferri (University of Bologna, Italy)

also an additional set of ten lines, from which we were able to amplify the *CBF-B10* gene, indicating this was not included in the deleted region in these lines. Further PCRs were carried out using the B genome-specific primers for the other genes in this cluster, which revealed that all these ten lines carried a deletion of six *CBF* genes, rather than the nine originally identified (Fig. 1).

The distribution of these lines revealed that all those carrying deletions of either six or nine *CBF* genes originate from the southern sub-population, in regions close to the Mediterranean Sea. The only accession from this region that did not show the *Fr-B2* deletion was the one collected from Mt. Hermon (University of Haifa code 1 (UH1)). However, this accession was collected at 1,300 m altitude from an area on the northern border of the southern sub-population where the average growing season temperature is 11 °C, much cooler than the surrounding regions (Fig. 2, Nevo and Beiles 1989). In all other cases, those lines carrying the *Fr-B2* wild-type allele originated from the northern sub-population of wild emmer, including varieties from Turkey and Iraq.

Domesticated emmer (*T. turgidum* ssp. *dicoccum*): Current evidence suggests that the most likely site for the initial domestication of emmer wheat was the northern sub-population of wild emmer, which was followed by its transfer to southern sub-populations (Luo et al. 2007). We screened 57 varieties of domesticated emmer from sources including Georgia, Iran, Turkey, Ethiopia and India, but found no lines carrying any deletion within the *Fr-B2* locus (Table 2).

Cultivated durum (*T. turgidum* ssp. *durum*): We screened a total of 91 durum varieties from different parts of the world and found that the nine-gene *Fr-B2* deletion was present in approximately half of those lines with a spring growth habit (37 out of 73, 50.7 %, Table 3). In durum wheat varieties with a winter growth habit, the nine-gene *CBF* deletion was less common, present in 7 of the 18 lines we screened (38.9 %, Table 3). We found no domesticated durum line carrying the six-gene *CBF* deletion.

Synthetic wheats: To expand the diversity of the D genome in the common wheat germplasm, several breeding programs undertook the creation of a large number of synthetic wheat-hexaploid varieties derived from crossing modern tetraploid (AABB) durum varieties with different accessions of *Aegilops tauschii*, the wild diploid donor of the D genome (Warburton et al. 2006). We screened a small collection of ten synthetic wheat lines to determine the extent to which the nine-gene *Fr-B2* deletion had been incorporated into these wheats via the tetraploid parent. Of the ten synthetic wheats we screened, we identified the nine-gene *Fr-B2* deletion in one accession, W7984 (Table 4). The source of the AB genomes for this line was the durum variety "Altar 84", which also carries this deletion (Table 3).

A deletion of 11 *CBF* genes on chromosome 5B in hexaploid wheat

To determine if deletions at the *Fr-B2* locus were also present in hexaploid wheat (*T. aestivum*), we screened a

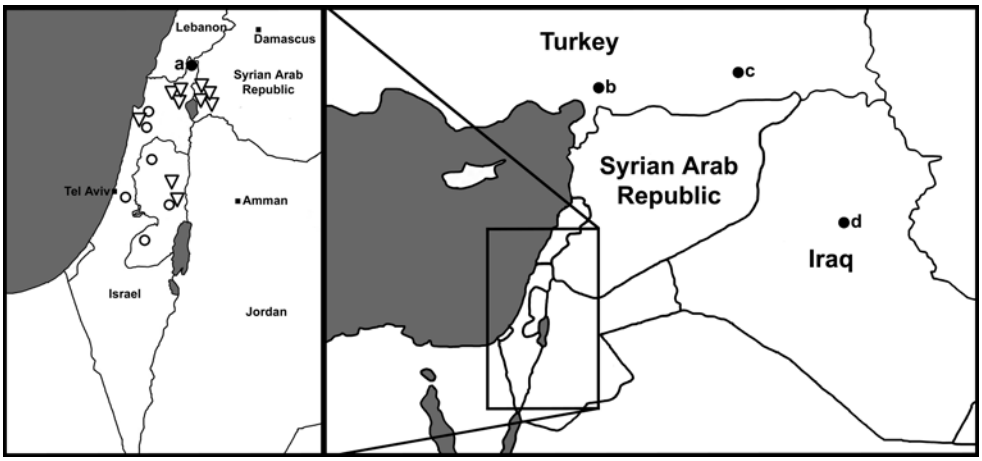

Fig. 2 Geographic location of *T. dicoccoides* accessions carrying deletions of six (*empty triangles*) or nine (*empty circles*) *CBF* genes from the *Fr-B2* locus and wild-type *Fr-B2* allele without the deletion (*filled circles*). *a* Mt. Hermon (see main text for discussion of this accession). *b* One accession from Maras, Turkey. *c* Eight accessions from Diyarbakir, Turkey. *d* Four accessions from Iraq. All accessions from Turkey and Iraq have the wild-type *Fr-B2* allele with no deletion of any *CBF* genes

Table 3 Distribution of the nine-gene *Fr-B2* deletion among spring and winter cultivated durum varieties (*T. turgidum* ssp. *durum*)

Growth habit	*Fr-B2* allele	*N*	Location	Varieties
Spring	Deletion	37	Italy	Ofanto, Messapia, L35, Colosseo, Cirillo, Capelli, Appulo, Appio, Adamello, Valforte, Valnova, Varano, Russello SG7
			Mexico	Aconchi 89, Altar 84, Mexicali 75, Anhinga, Croc 1, Flamingo:Dr, Jori 69, Scoter, Tehuacan 60
			Tunisia	Khiar, Inrat 69, Karim
			USA	WB881, Kronos, Colorado, Rugby, Waskana, Tacna
			France	Neodur, Exeldur, Durfort
			Canada	AC Navigator, AC Pathfinder
			Austria	Grandur
	Wild type	36	Italy	Latino, Duilio, Ciccio, Valbelice, San Carlo, Trinakria, Vitron[a], Zenit, Iumillo
			USA	Ben, Belzer, Plaza, Lloyd, Maier, Monroe, Munich, Renville, Lakota, Langdon, Edmore, Vic, Mindum, Leeds, Wells
			Canada	AC Avonlea, AC Melita, AC Morse, Hercules, Kyle, Medora, Plenty, Sceptre, Wakooma
			France	Nefer
			Austria	Helidur
			Israel	Gaza
Winter	Deletion	7	USA	VA05WD-12[b], VA05WD-10, VA05WD-16, XVAD99147-1, Produra
			Italy	Karel, Saragolla
	Wild type	11	USA	VA05WD-42, VA05WD-40, VA05WD-31, XVAD99068-14
			Austria	Astrodur, Extradur, Goldur, Frankodur, Semperdur, Topdur
			France	Durelle

[a] Vitron origin is Italy–Spain

[b] Winter durum varieties with VA or XVA identification numbers were kindly provided by Dr. Carl Griffey (Virginia Tech University, USA)

collection of 45 varieties previously used as parental lines of RIL populations by the Wheat Coordinated Agricultural Project (WheatCAP, Table 5). In 3 of these 45 lines (IDO556, RSI5 and PI 610751), we failed to amplify not only *CBF-B4*, *CBF-B12* and *CBF-B10*, but also *CBF-B9*, suggestive of a larger deletion also including the *CBF-B9* gene. An additional B genome-specific primer pair was designed to the *CBF-B9* gene (Supplementary materials, Table S1), which successfully amplified this gene in all other hexaploid varieties, but again failed to amplify a product in these three lines. Using the other B genome-specific primers listed in Table S1, we tested for the presence of all 11 genes at the *Fr-B2* locus. For each gene, primers were confirmed to be functional in the other hexaploid varieties. We found that, in addition to the nine *CBF* genes deleted in tetraploid wheat varieties, both *CBF-B9* and *CBF-B17* were also deleted in these three lines. These observations led us to the conclusion that these three hexaploid varieties carry a complete deletion of the *Fr-B2* locus encompassing all 11 *CBF* genes on chromosome 5B. All

Table 4 Distribution of the nine-gene *Fr-B2* deletion among synthetic wheats

Name	Source of AB genomes	*Fr-B2* allele
RL5402[a]	TetraCantach	Wild type
RL5403[a]	TetraCantach	Wild type
RL5405[a]	TetraCantach	Wild type
RL5406[a]	TetraCantach	Wild type
62052_4[a]	*T. durum* "Croc-1"	Wild type
62056_4[a]	*T. durum* "Croc-1"	Wild type
161725_0[a]	*T. durum* "Ceta"	Wild type
Sear's synthetic[a]	Unknown	Wild type
W7984[b]	Altar84	Deleted
PI610750[c]	*T. durum* "Croc-1"	Wild type

[a] Akhunov et al. (2010)

[b] Sorrells et al. (2011)

[c] Mujeeb-Kazi et al. (2000)

three of these varieties have a spring growth habit, while none of the 29 varieties with a winter growth habit in this collection carried any deletion at the *Fr-B2* locus (Table 5).

Pedigrees of the three lines carrying the complete *Fr-B2* deletion (IDO556, RSI5 and PI 610751) were traced back to their founding landraces. Where possible, ancestors of these lines were obtained from the US National Small Grains Collection or from the wheat germplasm bank at CIMMYT. Analyses to determine the presence of the *CBF* genes within these lines by PCR allowed us to trace the deletion of 11 *CBF* genes to the early founder lines Federation, Sonora 64 and Yaqui 50, which have been widely used in wheat breeding in the USA.

We also screened for this deletion within a set of 103 hexaploid wheat varieties, which were previously characterized for frost tolerance tests (Table 6, see "Materials and methods"). Combining these results with those from the WheatCAP population (Table 5), the current study describes the screening of 148 common wheat varieties for the 11-gene *Fr-B2* deletion. We found this deletion to be present in 22 % (22 out of 99) of the tested common wheat varieties with a spring growth habit, but in only 2 % (1 out of 49) of the tested varieties with a winter growth habit. These results suggest that the 11-gene *Fr-B2* deletion is approximately tenfold more abundant in spring than in winter varieties of common wheat.

Deletions at the *Fr-B2* locus are associated with reduced freezing tolerance in wheat

To test the effect of the nine-gene deletion in the *Fr-B2* locus of tetraploid wheat on freezing tolerance, we selected two sets of BC_3F_3 sister lines homozygous for the presence and absence of the *Fr-B2* deletion in a photoperiod-sensitive background (see "Materials and methods"). Sequencing revealed no polymorphisms between the parental plants used to generate these lines in the three genes analyzed at the *Fr-A2* locus in this study (*CBF-A12*, *CBF-A14* and *CBF-A15*). These lines carried the dominant *VRN-A1* allele conferring a spring growth habit. Since it is known that *VRN-1* expression and the subsequent transition to the reproductive stage can negatively affect the induction of the *CBF* and cold-induced genes (Dhillon et al. 2010), plants were maintained under short-day conditions (8 h light/16 h dark) before exposure to the target freezing temperature to preclude the induction of *VRN-1* and to minimize the effects of the transition to the reproductive growth phase. Apical meristems from a small sample of representative plants were harvested and observed 1 day before freezing to confirm that the plant apices remained at the vegetative stage of development (data not shown).

We carried out two initial freezing tolerance experiments at −8 and −9 °C using two different cold acclimation schemes to optimize the best temperature to differentiate the wild-type and *Fr-B2* nine-gene deletion genotypes (see "Materials and methods"). After exposure to these temperatures, survival rates were very low and the only plants to survive this treatment were those carrying the wild-type *Fr-B2* allele. At −8 °C, 3 out of 138 plants with all *CBF*

Table 5 Growth habit and distribution of the 11-gene *Fr-B2* locus deletion among hexaploid wheats from the Wheat CAP collection (http://maswheat.ucdavis.edu/Mapping/index.htm)

Growth habit	*Fr-B2* allele	*N*	Varieties
Spring	Deletion	3	IDO556, RSI5, PI610751
	Wild type	13	UC1110, Zak, McNeal, Thatcher, PI 658244, Louise, Penawawa, GRN*5/ND614-A, Reeder/Bw-277 "R", Redder/Bw-277 "S", Weebill 1, Jupateco 73S, Express
Winter	Deletion	0	
	Wild type	29	Rio Blanco, IDO444, OS9 (Stephens), Finch, Eltan, NY18/CC 40-1, Platte, CO940610, TAM 105, Jagger, Heyne, KS01HW163-4, Harry, Wesley, 2174, SS550, Pioneer 26R46, P91193, P92201, Cayuga, Caledonia, Pioneer 25R26, Foster, USG3209, Jaypee, McCormick, Pioneer 26R61, Kanqueen, Clark's Cream

Table 6 Growth habit and distribution of the 11-gene *Fr-B2* deletion among the hexaploid wheats used in frost tolerance tests

Growth habit	*Fr-B2* allele	*N*	Origin	Varieties	
				Fr-A2 Haplotype-S[b]	*Fr-A2* Haplotype-T
Spring	Deletion	19	Australia	Bobin (PI 106120), Dundee (PI 89424), Gular (PI 113489), Insignia (PI 210975), Thew (PI 41087)	Federation (PI 41080), Gallipoli (PI 55857), Hard Federation (PI 41079), Ranee (PI 67875)
			Mexico	Borlaug M95[a], Cajeme 71 (PI 412955), Sonora 64 (Cltr 13930), Yaqui 50 (PI 210890), Yaqui 54 (Cltr 13218)	Lerma Rojo (Cltr 13651)
			USA	Prospur (Cltr 17408), Probrand 775 (PI 601334)	
			India	NP876 (PI 322271)	Indian F (PI 93986)
	Wild type	64	Australia	Baart (PI 5078), Cleveland (PI 89191), Clubhead (PI 116224), Eden (PI 224658), Falcon (PI 292578), Florence (PI 38349), Gabo (PI 155431), Gluclub (PI 67326), Onas (PI 46796), Steinwedel (PI 41081), Yandilla King (PI 42120)	Currawa (PI 42105), AUS 90168 (PI 422410)
			Mexico	Bluebird 'S' (PI 519318), Gabo 55 (PI 583713), Inia 66 (PI 412973), Kentana 48 (Cltr 12980)	Lerma 52 (PI 210887), Lerma Rojo 64 (Cltr 13929), Lerma Rojo 64A (PI 342642), Siete Cerros 62 (PI 338921), Sonora 64A (PI 320109), Yaktana 54A (PI 351913)
			USA	Fielder (Cltr 17268), Hope (Cltr 8178), Springfield (Cltr 14589), Sterling (Cltr 17859), Tadinia (PI 494096), Thatcher (PI 168659), Timstein (Cltr 12347), Yecora Rojo (Cltr 17414)	Little Club (Cltr 4066), Turkey (Cltr 5757, this particular Turkey accession carries the spring allele *VRN-B1*)
			Argentina	Barleta (Cltr 8398), Chino (Cltr 12601), Klein 157 (PI 161825), Klein Lucero (Cltr 14047), Tezanos Pintos Precoz (PI 345731)	
			Canada	Marquis (Cltr 3641), Prelude (Cltr 4323)	Fife (PI 283820), Red Fife (PI 348919), White Fife (Cltr 4412)
			Kenya	Kenya 324 (PI 283840), Kenya 58 (Cltr 12471), Kenta C 9906 (PI 351682), Kenya (PI 192099)	
			Germany	Peragis (PI 184582), Derenburger Silber (PI 422410)	Heines Kolben (Cltr 11772)
			Russia	Ladoga (Cltr 4795)	Lutescens 62 (PI 74489)
			Japan	Aka-Daruma (PI 325843)	Aka-komugi (PI 45234)
			Other countries	Napo 63, Colombia (PI 337711), Gehun, India (PI 116066), Americano 44D, Uruguay (PI 191937), Sol, Sweden (Cltr 6009)	Chile 1B, Chile (PI 320098), Frontana, Brazil (Cltr 12470), Mentana, Italy (Cltr 12448), Noe, France (Cltr 5015), Red Egyptian, South Africa (Cltr 12345), Squarehead, UK (PI 51694)
Winter	Deletion	1	India	C591 (PI 180965)	
	Wild type	19	USA	Oro (Cltr 8220), Goldcoin (Cltr 4156)	Fultz (PI 5493), Kanred (Cltr 5146), Hussar (Cltr 4843), Mediterranean (Cltr 3332), Turkey Red (PI 565343)
			Germany	Merlin (PI 351584)	Heines IV (PI 180583), Heines VII (PI 201195), Tadorna (PI 338011)
			Sweden	Kronen (PI 278526), Pansar III (PI 52322)	
			Argentina	Klein Atlas (PI 344459), Klein Rendidor (PI 351622)	
			Other countries	Golden drop, Australia (PI 92399); Wilhelmina, Netherlands (Cltr 11389)	Norin 10, Japan (PI 156641); Blaue Dame Russia (PI 278451)

[a] Borlaug M95 from CIMMYT

[b] *Fr-A2* haplotype data based on CBF genes *CBF-A12*, *CBF-A14* and *CBF-A15* (10 SNP + 2 indels) from Zhu et al. (2013)

Table 7 Survival rates of BC_3F_3 sister lines carrying the wild-type *Fr-B2* allele (Wild type) and the nine-gene deletion at the *Fr-B2* locus (Deletion) in response to freezing temperatures

	−6 °C		−7 °C (Scheme A)		−7 °C (Scheme B)		Overall	
	Deletion	Wild type	Deletion	Wild type	Deletion	Wild type	Deletion	Wild type
n	92	105	43	44	51	74	186	222
Survival rate (%)	3.3 %	18.4 %	74.0 %	87.5 %	39.1 %	44.8 %	44.3 %	54.7 %

Experimental conditions are described in "Materials and methods"

genes present survived, whereas all 78 plants with the *CBF* deletion were killed. Similarly, only 2 out of 44 plants with all *CBF* genes present survived freezing at −9 °C, while all 41 plants with the *CBF* deletion were killed. Since no plant carrying the *Fr-B2* deletion survived exposures to freezing temperatures of −8 or −9 °C, subsequent experiments were performed at −7 °C (two tests) and −6 °C (two tests).

Analysis of the survival data using a factorial ANOVA including experiments and genotypes as factors showed significant differences in survival between experiment ($P < 0.0001$) and between genotypes ($P = 0.0026$). The frost tolerance results by individual line are presented in Supplementary Materials Table S2. The lack of significant interaction between experiments and genotypes ($P = 0.10$) indicated that the results among the three experiments were consistent and that, therefore, they could be combined in a single statistical analysis (main effects). The complete ANOVA model explained 91 % of the variation in survival, indicating that the selected model provided an adequate description of the major factors affecting survival in these experiments.

The planned contrast between the *Fr-B2* alleles for survival to freezing temperatures was highly significant ($P = 0.008$). The average survival across experiments was 54.7 % for lines with the wild-type *Fr-B2* allele and 44.3 % for the lines carrying the nine-gene deletion at the *Fr-B2* locus (Table 7). Thus, the deletion of these nine *CBF* genes was associated with an average reduction in survival of 10.4 %.

To test the effect of the larger 11-gene *Fr-B2* deletion in hexaploid wheat, we took advantage of a set of 103 hexaploid wheat accessions (Table 6 and Supplementary Materials Table S3.) previously characterized for frost tolerance at WSU (see "Materials and methods"). Among all lines with the wild-type *Fr-B2* allele, those with a winter growth habit showed a fivefold higher survival rate than the lines with a spring growth habit (51.5 ± 5.9 vs. 9.5 ± 1.3 %, Wilcoxon two-sample test $P < 0.0001$, Fig. 3). This is consistent with previous studies which have demonstrated that wheat varieties with a spring growth habit exhibit reduced frost tolerance than those with a winter growth habit (Dhillon et al. 2010; Fowler and Limin 2004).

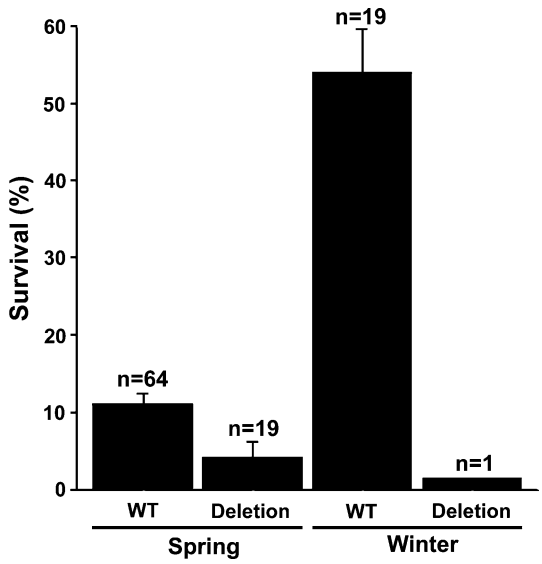

Fig. 3 Survival rates of hexaploid lines with a spring and winter growth habit following exposure to −6 °C in artificial freezing experiments. Lines are separated into those carrying the wild-type *Fr-B2* allele (*WT*) and those carrying the 11-gene deletion at this locus (*deletion*)

Within the 83 spring lines, those carrying the *Fr-B2* deletion (19 accessions, 4.1 ± 2.1 % survival) showed a significant reduction in frost tolerance compared to those with the wild-type *Fr-B2* allele (64 accessions, 11.1 ± 1.4 % survival, Wilcoxon two-sample test $P = 0.0023$, Fig. 3). Among the 20 winter hexaploid lines tested for frost tolerance, only 1 carried the *Fr-B2* deletion and, therefore, we were unable to perform any statistical tests within the winter class. However, it is worth noting that the survival rate of this single line carrying the *Fr-B2* deletion was more than two standard deviations below the average survival of the winter lines without the deletion (within the lower 10 % percentile, Fig. 3).

Since the *Fr-A2* locus is known to have a large effect on frost tolerance, we performed an additional statistical test to determine if the observed differences in frost tolerance between the two *Fr-B2* alleles were affected by an unbalanced distribution of *Fr-A2* alleles between the two *Fr-B2* classes. We obtained haplotype data for the *Fr-A2*

locus from a related study characterizing variation at the *Fr-A2* locus (Zhu et al. 2013, submitted) and performed independent ANOVAs for *Fr-B2* and growth habit within each of the two *Fr-A2* haplotype classes. Significant differences between the presence and absence of the *Fr-B2* deletion were detected both among the 38 accessions carrying the tolerant *Fr-A2* "T" haplotype ($P = 0.036$) and among the 65 accessions carrying the *Fr-A2* "S" haplotype ($P = 0.011$, Table 6). This result confirmed that the observed differences in frost survival detected at the *Fr-B2* locus were not an artifact generated by an unbalanced distribution of *Fr-A2* alleles within the *Fr-B2* classes.

Discussion

Whereas *Arabidopsis* has three *CBF* genes present as a tandem cluster, wheat and other members of the *Pooideae* family have undergone a large expansion of this gene family, with 11 genes at the *Fr-2* locus, 8 of which are *Pooideae* specific (Badawi et al. 2007; Miller et al. 2006; Skinner et al. 2005). This expansion has been dated to coincide with a period of global cooling during the Eocene–Oligocene transition approximately 33 MYA (Sandve and Fjellheim 2010), and it has been proposed that the increase in the number of *CBF* genes and diversity at this locus may have occurred as a result of selection pressure for improved frost tolerance by this period of lower temperatures. This hypothesis is supported by the finding that copy number variation exists among the *CBF* genes in both wheat and barley and that this is associated with differences in freezing tolerance (Knox et al. 2010).

In the current study, we describe the characterization and distribution of large deletions within the *Fr-B2* locus on chromosome 5B in both hexaploid and tetraploid wheat. In hexaploid wheat, we identified some varieties which carried a deletion of all 11 genes from the *Fr-B2* locus (Fig. 1, Tables 5, 6), while in tetraploid wheat two *Fr-B2* deletions were identified, one including 9 of the 11 *CBF* genes and the other one restricted to *T. dicoccoides*, including only 6 of these genes (Fig. 1). The exclusion of *CBF-B9* and *CBF-B17* from both deleted regions in tetraploid wheat provides indirect evidence supporting one of the two alternative orders previously proposed for the four proximal *CBF* genes at the *Fr-2* locus. During the original mapping of this locus, the *Fr-A^{m}2 CBF* region in *T. monococcum* was divided by recombination into three clusters (Fig. 1), but the orientation of the four linked *CBF* genes in the proximal cluster (which were all present in a single BAC clone) could not be established due to the lack of recombination among these genes. A *CBF-17–CBF-9–CBF-4–CBF-2* order was initially proposed to maximize the physical proximity of the more similar paralogs (Miller et al. 2006), but the alternative order (*CBF-2–CBF-4–CBF-9–CBF-17*) was later favored by Knox et al. (2008) to be consistent with the *CBF* gene order found in barley (Francia et al. 2007). If we assume that these large deletions within the *Fr-B2* cluster arise from distinct single deletion events, the most likely order of the proximal cluster is *CBF17–CBF9–CBF4–CBF2* as initially suggested by Miller et al. (2006) and as presented in Fig. 1. However, with the available information we cannot rule out the possibility of two independent deletions in this region and a gene order similar to that observed in barley (Francia et al. 2007).

It is interesting to note that all three deletions identified in this study span the entire central *CBF* cluster, which consists of the genes *CBF12*, *CBF14* and *CBF15*. A high-resolution QTL mapping study in diploid wheat *T. monococcum* mapped the differences in frost tolerance to a region of the *Fr-A^{m}2* locus including these three genes (Knox et al. 2008). With the available information, it is not possible to determine whether these three deletions arose independently, or as a result of additional deletions occurring in a line already carrying a smaller deletion. The occurrence of deletions in a region including multiple copies of related genes is not unexpected. In addition, the large wheat genome is subject to very high rates of deletions, whose deleterious effects are buffered by polyploidy (Dubcovsky and Dvorak 2007). Sequencing the distal and proximal borders of each of these deletions may reveal whether these are common and thus provide information to clarify the origin of these different deletions.

Associations between segregation for deletions at the *Fr-B2* locus and freezing tolerance

The importance of genes present on chromosome 5 in improving wheat frost tolerance was first demonstrated using chromosome substitution lines, where the transfer of chromosomes 5A, 5B and 5D from the cold-hardy variety Cheyenne to the cold-susceptible Chinese Spring resulted in the greatest increases in cold tolerance among the different homeologous groups (Sutka 1981; Veisz and Sutka 1989). Within homeologous group 5, the substitution of chromosome 5A had the largest effect on frost tolerance, while the substitution of chromosome 5B had the smallest effect (Sutka 1981; Veisz and Sutka 1989). However, neither Cheyenne nor Chinese Spring, the donor and recipient genotypes used in the chromosome substitution experiments described above, carries any of the *Fr-B2* deletions described in the current study. Therefore, it is not possible to compare the relative effect of the *Fr-B2* deletion with the effect of allelic differences at the *Fr-A2* and *Fr-D2* loci.

The impact of the chromosome substitutions on frost tolerance was later found to be associated with the presence of the vernalization gene *VRN-1* and the frost tolerance

locus *Fr-2*, which were mapped ~30 cM apart on the long arms of homeologous group 5 chromosomes (Vágújfalvi et al. 2000, 2003, 2005). It was initially thought that an additional frost tolerance locus existed which was closely linked to *VRN-1* (*Fr-1*), but it is now generally accepted that this is a pleiotropic effect of the earlier initiation of flowering caused by the dominant *VRN-1* alleles (Dhillon et al. 2010). Differences in frost tolerance in the 5A and 5D chromosomes in hexaploid wheat were also associated with differences at their respective *Fr-2* loci (Båga et al. 2007; Snape et al. 1997). Although the frost tolerance locus on chromosome 5B was originally published as *Fr-B1* (Toth et al. 2003), the name was later corrected to *Fr-B2* in the 2004 supplement of the Catalogue of Gene Symbols for Wheat (McIntosh et al. 2004).

We show here that the deletions at the *Fr-B2* locus on chromosome 5B are associated with highly significant reductions in survival rates following exposure to freezing temperatures both in tetraploid (Table 7) and hexaploid wheat (Fig. 3). In tetraploid wheat, plants carrying the deletion exhibited an average reduction in survival of 10.4 % in comparison to wild-type lines, following exposure to temperatures of −6 and −7 °C. The effect on freezing tolerance associated with this large deletion would likely be larger were it not for the redundancy provided by the homeologous *CBF* genes on chromosome 5A in tetraploid wheat. In hexaploid wheat, the negative effect of the *Fr-B2* deletion on frost tolerance was also observed in a survey of 103 hexaploid wheat lines. Among the lines with a spring growth habit, those carrying the deletion of all 11 *CBF* genes from the *Fr-B2* locus showed a significant reduction in frost tolerance ($P = 0.0023$, Fig. 3). The difference between *Fr-B2* alleles was even larger among winter lines (Fig. 3), but we were unable to confirm this statistically since this frost tolerance test included a single winter line carrying the *Fr-B2* deletion.

The presence of large deletions in the tetraploid and hexaploid wheat varieties has likely been favored by the redundancy provided by polyploidy. So far, no such deletions have been reported in diploid *T. monococcum* or barley, suggesting that deletions of multiple *CBF* genes may have a detrimental effect on the plant's ability to adapt to environmental changes. Instead, observed differences in frost tolerance between varieties of diploid species could be accounted for by mutations within individual *CBF* genes (Knox et al. 2008) or by copy number variation (Knox et al. 2010).

It is important to point out that the tight linkage among *CBF* genes present in the *Fr2* loci makes it very difficult to establish causal relationships between observed changes in some *CBF* genes and frost tolerance. Therefore, SNPs, deletions or copy number variants of specific *CBF* genes should be considered markers of a particular haplotype for the complete *Fr2* locus, rather than as evidence of a causal relationship between an observed polymorphisms and the frost tolerance phenotype.

Distribution of the *Fr-B2* deletions

The distribution of the *Fr-B2* alleles in tetraploid and hexaploid wheat shows some correlation with both geographic distribution and growth habit. Among the wild emmer wheat accessions screened in this study (*T. turgidum* ssp. *dicoccoides*), the distribution of the *Fr-B2* alleles closely matches the distribution of the two sub-populations, originally characterized using AFLP markers (Ozkan et al. 2002). The northern sub-population is found in the region including Turkey, Iraq and Iran, and is characterized by higher altitude and colder average temperatures than the southern sub-population, which is centered on Israel, Lebanon and Syria and lies closer to the Mediterranean Sea (Ozkan et al. 2002). Our analysis of a small sample of wild emmer populations shows that none of the varieties originating from the northern sub-population carry any deletion at the *Fr-B2* locus. In contrast, all accessions from the southern sub-population, with the exception of the one located at the northern border of the distribution, carry either the six-gene or nine-gene *Fr-B2* deletion (Fig. 2). This exception is an accession from Mt. Hermon, collected at 1,300 m of elevation and with a much lower average growing temperature than the surrounding region (Fig. 2). Strong evidence exists to suggest that domesticated emmer (*T. turgidum* ssp. *dicoccum*) originated from the northern sub-population of wild emmer (Luo et al. 2007). Therefore, it is not surprising that all 57 accessions of domesticated emmer screened in this study carry the *Fr-B2* wild-type allele without any *CBF* deletion (Table 2). These results do not rule out the possibility that this deletion may be present in a small proportion of domesticated emmer.

In cultivated durum wheat (*T. turgidum* ssp. *durum*), we found the nine-gene *Fr-B2* deletion to be widespread among varieties with a spring growth habit from diverse parts of the world (Table 3), where the deletion was present in 50.7 % of the varieties we screened. Among the smaller number of winter durum wheats that we analyzed, the deletion was less common and present in just 38.9 % of the varieties. Winter wheats are sown in the fall and are generally exposed to freezing temperatures during the winter and thus require improved frost tolerance. In contrast, spring-sown varieties are planted later in the growing season to avoid exposure to these freezing temperatures. Although in some Mediterranean regions spring wheats are sometimes sown in the fall to take advantage of winter precipitation, these regions usually have milder winters than the regions where true winter wheat varieties are grown. Therefore, it is reasonable to assume that winter wheat varieties are

subject to a stronger selection pressure for freezing tolerance than spring varieties. This selection pressure is distributed between the genes present at both *Fr-A2* and *Fr-B2* loci in tetraploid wheat and also at *Fr-D2* in hexaploid wheat (plus other loci affecting frost tolerance) and, therefore, the correlation between the *Fr-B2* deletion and growth habit is not expected to be perfect.

A higher frequency of the *Fr-B2* deletion among accessions with a spring growth habit was also observed in the 148 bread wheat varieties (*T. aestivum*) analyzed in this study (Tables 5, 6). The complete deletion of all 11 *CBF* genes from the *Fr-B2* locus was identified in 22 % of spring varieties, but in only 2 % of winter varieties. We traced the origins of this deletion in three modern common wheat varieties and found that the deletion of all 11 *CBF* genes at the *Fr-B2* locus was already present in some of the founders of the modern US wheat breeding programs. It is tempting to speculate that the very low frequency of the *Fr-B2* deletion observed among cultivated winter varieties of common and durum wheat might reflect the selection for the wild-type *Fr-B2* allele by winter wheat breeders when targeting improved frost tolerance.

We also analyzed ten synthetic wheat varieties to determine whether the nine-gene deletion at the *Fr-B2* locus had been incorporated into hexaploid wheat via the tetraploid donor (Table 4). We identified one synthetic variety carrying this deletion, the tetraploid parent of which, Altar84, also carries this deletion (Table 3). This result demonstrates an additional possible means by which deletions at the *Fr-B2* locus could be incorporated into modern common wheat varieties. Winter wheat breeding programs interested in utilizing synthetic wheat and improving freezing tolerance will benefit from a preliminary screening of synthetic accessions for those that do not carry deletions at the *Fr-B2* locus.

The lower frequency of deletions at the *Fr-B2* locus in *T. dicoccoides* varieties from the northern sub-population or from high altitude, as well as in modern wheat varieties with a winter growth habit relative to those with a spring growth habit, suggests that the wild-type *Fr-B2* allele has been favored by selection for increased frost tolerance. This hypothesis is further supported by the demonstration in both tetraploid and hexaploid wheat that lines carrying deletions at the *Fr-B2* locus exhibit reduced frost tolerance in comparison to those lines carrying the wild-type *Fr-B2* allele (Table 7; Fig. 3). Although the results from experiments carried out in hexaploid wheat will require validation using segregating populations or sister lines, the analysis of freezing tolerance in these 83 spring lines, in combination with the results from replicated freezing experiments using tetraploid sister lines, suggest that deletions at the *Fr-B2* locus have a negative impact on frost tolerance in wheat.

It is also possible that deletions in the *Fr-B2* locus may confer some advantage to accessions grown in warmer conditions, although this hypothesis is more difficult to test. It is known that the activation of the cold-induced *CBF* regulon comprises the induction of hundreds of genes (Fowler and Thomashow 2002) and the synthesis of many different proteins which are required to protect the cell from freezing injury. Therefore, it is not unreasonable to assume that there is an energy cost to a plant in maintaining the activation of the *CBF* regulon. This idea is supported by the slow-growing, dwarf phenotype of *Arabidopsis* transgenic lines over-expressing *CBF-2* when grown under warm conditions (Achard et al. 2008). It would be interesting to study isogenic lines of spring wheat with and without the *Fr-B2* deletion, to determine whether these deletions confer an increase in fitness in warmer regions where the plants are not exposed to freezing temperatures.

Conclusions and practical applications

In summary, this study describes the discovery of large deletions spanning the *CBF* gene cluster at the *Fr-B2* locus and shows that that *Fr-B2* deletions are more common among wild emmer accessions from the southern sub-population in the Fertile Crescent than among those from the northern sub-populations, and that, in cultivated durum and bread wheats, deletions are more common among spring varieties rather than those with a winter growth habit. Since the ancestral expansion of the *CBF* gene family in the *Triticeae* has been linked to an improved ability to respond to low temperatures, we hypothesize that the deletions described in the current work might be associated with reduced fitness in regions where the plants are exposed to freezing temperatures.

This study also shows that *Fr-B2* deletions in tetraploid and hexaploid wheat are associated with decreased survival rates after exposure to freezing temperatures. These increases in frost tolerance are likely sufficient to justify the selection of the *Fr-B2* wild-type allele in wheat breeding programs interested in improving frost tolerance. Although direct selection for frost tolerance has likely been sufficient to maintain a low frequency of the *Fr-B2* deletion among modern commercial winter wheat varieties, the information provided in this study will allow breeders to use molecular markers to select for the wild-type *Fr-B2* alleles in the parental lines used in crosses and during the early stages of their breeding programs.

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