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#### Authors

Zhu, Jie  
Pearce, Stephen  
Burke, Adrienne  
et al.

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# Copy number and haplotype variation at the *VRN-A1* and central *FR-A2* loci are associated with frost tolerance in hexaploid wheat

Jie Zhu · Stephen Pearce · Adrienne Burke ·  
Deven Robert See · Daniel Z. Skinner ·  
Jorge Dubcovsky · Kimberly Garland-Campbell

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## Abstract

**Key message** The interaction between *VRN-A1* and *FR-A2* largely affect the frost tolerance of hexaploid wheat.

**Abstract** Frost tolerance is critical for wheat survival during cold winters. Natural variation for this trait is mainly associated with allelic differences at the *VERNALIZATION 1* (*VRN1*) and *FROST RESISTANCE 2* (*FR2*) loci. *VRN1* regulates the transition between vegetative and reproductive stages and *FR2*, a locus including several tandemly duplicated *C-REPEAT BINDING FACTOR* (*CBF*) transcription factors, regulates the expression of *Cold-regulated* genes. We identified sequence and copy number variation at these two loci among winter and spring wheat varieties and characterized their association with frost tolerance.

We identified two *FR-A2* haplotypes—‘FR-A2-S’ and ‘FR-A2-T’—distinguished by two insertion/deletions and ten single nucleotide polymorphisms within the *CBF-A12* and *CBF-A15* genes. Increased copy number of *CBF-A14* was frequently associated with the *FR-A2-T* haplotype and with higher *CBF14* transcript levels in response to cold. Factorial ANOVAs revealed significant interactions between *VRN1* and *FR-A2* for frost tolerance in both winter and spring panels suggesting a crosstalk between vernalization and cold acclimation pathways. The model including these two loci and their interaction explained 32.0 and 20.7 % of the variation in frost tolerance in the winter and spring panels, respectively. The interaction was validated in a winter wheat  $F_{4,5}$  population segregating for both genes. Increased *VRN-A1* copy number was associated with improved frost tolerance among varieties carrying the *FR-A2-T* allele but not among those carrying the *FR-A2-S* allele. These results suggest that selection of varieties carrying the *FR-A2-T* allele and three copies of the recessive *vrn-A1* allele would be a good strategy to improve frost tolerance in wheat.

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J. Zhu and S. Pearce contributed equally to this work.

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J. Zhu · D. Z. Skinner · K. Garland-Campbell  
Crop and Soil Sciences, Washington State University,  
Pullman, WA 99164, USA

S. Pearce · J. Dubcovsky (✉)  
Department of Plant Sciences, University of California, Davis,  
CA 95616, USA  
e-mail: jdubcovsky@ucdavis.edu

A. Burke · D. R. See · D. Z. Skinner · K. Garland-Campbell (✉)  
USDA-ARS Wheat Genetics, Quality, Physiology and Disease  
Research Unit, United States Department of Agriculture,  
Pullman, WA 99164-6420, USA  
e-mail: kgcamp@wsu.edu

D. R. See  
Department of Plant Pathology, Washington State University,  
Pullman, WA 99164, USA

J. Dubcovsky  
Howard Hughes Medical Institute and Gordon and Betty Moore  
Foundation Investigator, Chevy Chase, MD 28015-6789, USA

## Introduction

Temperate cereals such as wheat (*Triticum* spp.) and barley (*Hordeum vulgare* L.) can be classified into spring or winter types based on their growth habit. Winter varieties are sown in autumn and must be able to withstand freezing temperatures for their reproductive success. These varieties require long exposures to low non-freezing temperatures (vernalization) to accelerate flowering whereas spring varieties, generally sown after the risk of exposure to freezing temperatures has passed, do not have this requirement. Cold acclimation is the process by which gradual exposure to low, non-freezing temperatures leads to an increase in frost tolerance. Two loci, *FROST RESISTANCE 1* (*FR1*) and *FROST RESISTANCE 2* (*FR2*), both located on the long arm of homoeologous group 5 chromosomes, are key components of the cold acclimation regulatory network in wheat and barley (Vágújfalvi et al. 2003; Francia et al. 2004; reviewed in Galiba et al. 2009). An additional smaller QTL for frost tolerance, distinct from *FR2*, has recently been reported on chromosome 5B (Zhao et al. 2013). This QTL, identified in wheat germplasm adapted to central Europe, was not part of this study.

The *FR1* locus was isolated during a study to identify loci contributing to differential cold acclimation in winter vs spring wheat (Galiba et al. 1995; Sutka et al. 1999). *FR1* is most likely a pleiotropic effect of the *VERNALIZATION1* (*VRN1*) gene (Limin and Fowler 2006; Stockinger et al. 2007; Dhillon et al. 2010) and, therefore, will be referred to hereafter as *VRN1* (with an additional letter indicating the specific wheat genome e.g. *VRN-A1*, *VRN-B1*, and *VRN-D1*). *VRN1* transcript levels increase during exposure to low temperatures and trigger the transition from the vegetative to reproductive growth stage. This transition is associated with suppressed induction of *C-REPEAT BINDING FACTOR* (*CBF*) genes in response to cold, resulting in reduced frost tolerance (Kobayashi et al. 2005; Limin and Fowler 2006; Stockinger et al. 2007; Dhillon et al. 2010).

The presence of dominant *Vrn-A1a* or *Vrn-A1b* alleles eliminates the need for vernalization prior to flowering, resulting in a spring growth habit. Plants carrying dominant *Vrn-B1* and *Vrn-D1* alleles also exhibit a spring growth habit, although these alleles are weaker than the *Vrn-A1*

alleles and plants maintain some residual response to vernalization (Santra et al. 2009). Spring wheat varieties carrying the dominant *Vrn-A1a* or *Vrn-A1b* alleles are more sensitive to frost damage than those carrying the *Vrn-B1* or *Vrn-D1* alleles and both are more sensitive than varieties with a winter growth habit (Koemel et al. 2004; Reddy et al. 2006). Different recessive winter *vrn-A1* alleles have been identified using various nomenclature; including ‘*V*’ and ‘*W*’ which are distinguished by a C/T single nucleotide polymorphism (SNP) in the fourth exon of this gene (Table 1). These alleles have been shown to be associated with differences in vegetative growth patterns (Chen et al. 2009, 2010) and it has been reported that they may also be associated with differences in frost tolerance (Eagles et al. 2011). Wheat genotypes carrying the ‘*V*’ allele had a lower *vrn-A1* copy number and reduced vernalization requirement to induce flowering, than those carrying the ‘*W*’ allele, which were characterized by higher *vrn-A1* copy number and a greater vernalization requirement (Table 1) (Díaz et al. 2012).

The second frost tolerance locus, *FR2*, was mapped ~30 cM proximal to *VRN1* on homoeologous group 5 chromosomes of both wheat and barley and is associated with frost tolerance and expression of the *COLD REGULATED* (*COR*) gene, *Cor14b* (Tóth et al. 2003; Vágújfalvi et al. 2003; McIntosh et al. 2013; Francia et al. 2004; Båga et al. 2007; Motomura et al. 2013). In hexaploid wheat, the differential *CBF* expression detected between frost-sensitive and frost-tolerant varieties was tightly associated with the *FR-A2* locus (Vágújfalvi et al. 2005). QTL for frost tolerance have been identified in several independent studies at this locus (Båga et al. 2007; Motomura et al. 2013). The *FR-A<sup>m</sup>2* locus from diploid wheat *Triticum monococcum* (*A<sup>m</sup>* genome related to the *A* genome of polyploid wheat) was later found to comprise 11 tandemly duplicated *CBF* genes clustered closely together on chromosome 5A<sup>m</sup> (Miller et al. 2006).

*CBF* proteins are AP2/ERF transcription factors which contain five conserved amino acid motifs (AP2, CMIII-1, CMIII-2, CMIII-3 and CMIII-4) that distinguish them from other members of the ERF family (Nakano et al. 2006). Among these five motifs, the AP2 domain is a critical functional domain required for the *CBF* proteins to bind to the

**Table 1** Variable nomenclature and characteristics of two *vrn-A1* alleles in different studies

<i>vrn-A1</i> allele	Chen et al. 2009	Eagles et al. 2011	Exon 4 SNP, nucleotide 349 <sup>a</sup>	Amino acid 117 <sup>a</sup>	Vernalization requirement	Copy number in the winter panel
<i>V</i>	<i>vrn-A1a</i>	Jagger (J)	C	L	Shorter	Seven out of eight varieties <3 copies
<i>W</i>	<i>vrn-A1b</i>	Wichita (K)	C and T	L and F	Longer	53 out of 58 varieties = 3 copies

<sup>a</sup> Nucleotide and amino acid residue numbers are relative to ‘Jagger’ *VRN-A1* coding sequence and *VRN-A1* protein

C-repeat/dehydration-responsive elements (CRT/DRE) located in the promoter regions of their target *COR* genes (Stockinger et al. 1997; Liu et al. 1998). The *CBF* genes have been best characterized in *Arabidopsis*, where three tandemly duplicated *CBF* genes are present in a cluster on chromosome 4 (Stockinger et al. 1997; Gilmour et al. 1998; Medina et al. 1999). The *CBF* gene family in the temperate grasses has undergone an expansion dated to the Eocene–Oligocene transition during a period of global cooling ~33 MYA (Sandve and Fjellheim 2010). Interestingly, deletions and duplications of several *CBF* genes have been detected at the *FR2* locus in wheat and barley (Skinner et al. 2005; Francia et al. 2007; Fricano et al. 2009; Knox et al. 2010; Pearce et al. 2013), potentially allowing for a more flexible response to cold stress and suggesting that this gene cluster is subject to dynamic expansions and contractions.

Three adjacent genes, *CBF12*, *14* and *15*, located at the center of the eleven-gene cluster at the *Fr-A<sup>m</sup>2* locus were previously shown to be completely linked to differences in frost survival and accumulation of *Cor14b* and *DEHYDRIN 5 (DHN5)* transcripts at 12–15 °C in diploid wheat (Knox et al. 2008). Their critical role in frost tolerance was also supported by several expression studies. In hexaploid wheat recombinant substitution lines, transcript levels of *CBF14* and *15* were more than fourfold higher when the *FR-A2* allele from the frost-tolerant wheat cultivar ‘Cheyenne’ was present as compared to the frost-sensitive allele from *T. spelta* (*CBF12* was not included in this study) (Vágújfalvi et al. 2005). Expression of *CBF12* and *CBF14* after long-term cold acclimation differed between frost-tolerant and frost-sensitive mutant lines derived from the wheat cultivar ‘Winoka’ (Sutton et al. 2009). In barley, *HvCBF12* and *14* were more highly expressed in the frost-tolerant cultivar ‘Nure’ than in the frost-sensitive cultivar ‘Tremois’ (Stockinger et al. 2007). Studies of *CBF* nucleotide sequence variation, insertion/deletions (indels), and copy number variation (CNV) also support an important role of these genes in frost tolerance (Knox et al. 2008, 2010). In barley, allelic variation at *HvCBF14* was associated with differences in frost tolerance (Fricano et al. 2009). In wheat, the copy number of *CBF14* was found to be higher in winter wheat than spring wheat (Knox et al. 2010; Dhillon and Stockinger 2013). More recently, a large multi-gene deletion at the *Fr-B2* locus, which included *CBF-B12*, *CBF-B14* and *CBF-B15*, was shown to be associated with reduced frost tolerance in both tetraploid durum (*Triticum durum* L.) and hexaploid bread wheat (Pearce et al. 2013). Taken together, the expression experiments and the genetic data suggest that *CBF12*, *CBF14* and *CBF15* play a critical role in frost tolerance in the temperate cereals.

In the current study, we analyze the sequences of *CBF12*, *CBF14* and *CBF15* homoeologs from 146 hexaploid wheat varieties and identify SNP and indel allelic

variation in *VRN-A1* and the *CBF* genes located at the central cluster of *FR-A2*. We determine frost survival for these varieties and for a bi-parental population segregating for the allelic variation at the *VRN-A1* and *FR-A2* loci. We also identify CNV at *VRN-A1* and *FR-A2* and determine that interactions between these two loci are associated with differential frost survival in both winter and spring wheat.

## Materials and methods

### Plant materials

Two wheat diversity panels and one bi-parental *F*<sub>4:5</sub> segregating population were analyzed during the current study:

#### Winter panel

A diversity panel of 65 hexaploid winter wheat varieties was selected to represent varieties grown in diverse regions of the world, including accessions originating from the USA (36), Russia (8), Romania (6), Canada (4), Ukraine (2), Finland (2), Denmark (1), France (1), Germany (1), Serbia (1), Switzerland (1) and Poland (1) and one accession of undetermined geographic origin (Supplemental Material Table S1).

#### Spring panel

A second diversity panel of 81 hexaploid spring wheat varieties from North America (34), Australia (22), South America (8), Europe (8), Africa (5) and Asia (4) was selected to represent spring wheat varieties from different regions of the world (Supplemental Material Table S2).

The growth habit of varieties in both panels was determined by genotyping the vernalization loci *VRN-A1*, *VRN-B1* and *VRN-D1* (see “Molecular markers” below) and confirmed with information provided by the Germplasm Resources Information Network (GRIN) database of the National Plant Germplasm System (<http://www.ars-grin.gov/npgs/>).

#### Bi-parental *F*<sub>4:5</sub> segregating population (Eltan/ORFW)

The unreleased, soft white wheat genotype ‘Oregon Feed Wheat No. 5’ (ORFW) carries a major genetic factor conditioning freezing sensitivity (Skinner and Campbell 2008). A heterozygous *F*<sub>2:4</sub> family derived from a cross between the frost-tolerant winter wheat cultivar ‘Eltan’ (PI 536994) (Peterson et al. 1991) and ORFW was self-pollinated and the progeny were genotyped for sequence variation at the *FR-A2* and *VRN-A1* loci. Seventy individuals homozygous for each of the two *FR-A2* haplotypes (S and T) and each

of the two *vrn-A1* alleles segregating in this population ( $V = 2$  copies and  $W = 3$  copies) were selected to generate a segregating  $F_{4;5}$  population. No recombination event was detected between *CBF-A12*, *CBF-A14* and *CBF-A15*, so the effects of the three central *CBF* genes at the *FR-A2* locus could not be separated in this population.

In all frost tolerance assays for winter wheat accessions, the soft white wheat cultivars ‘Eltan’ and ‘Stephens’ (PI 658243) (Kronstad et al. 1978) developed in the Pacific Northwest (PNW) of the US were used as frost-tolerant and frost susceptible controls, respectively. In the frost-tolerance assay for the 81 spring wheat cultivars, the cultivars ‘Alpowa’ (PI 566596) and ‘Louise’ (PI 634865) developed in the PNW (Kidwell et al. 2006) were included as frost tolerant and sensitive controls, respectively.

To screen for diversity within ancestors of modern wheat, we selected 25 *Triticum turgidum* spp. *dicoccoides* accessions from the University of Haifa germplasm collection (Supplemental Material Table S5, Nevo and Beiles 1989; Peleg et al. 2005) and two accessions of *Triticum urartu* (‘ICWT 500612’ and ‘G1812’).

#### DNA extraction and gene sequencing

Leaf tissue was harvested from 3-week-old seedlings and genomic DNA extracted using the BioSprint 96 DNA Plant Kit (Qiagen, Valencia, CA, USA).

Genome-specific primers were developed for each of the three homoeologous copies of *CBF12*, *CBF14* and *CBF15* (Supplemental Material Table S3). All PCRs were carried out in 20  $\mu$ l volumes containing 5 % DMSO, 10 mM Tris–HCl, 50 mM KCl, 3 mM  $MgCl_2$ , 0.2 mM dNTPs, 0.375  $\mu$ M of each primer, 50–100 ng genomic DNA and 0.75 U *Taq* DNA polymerase. Amplifications were performed using a standard touchdown PCR protocol with the appropriate annealing temperature (Supplemental Material Table S3).

All PCR products were purified using ExoSAP-IT<sup>®</sup> (Affymetrix, Santa Clara, CA, USA) and sequencing templates were prepared with BigDye<sup>®</sup> Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All samples were sequenced at the USDA-ARS Western Regional Small Grains Genotyping Laboratory, Pullman WA, USA.

#### Molecular markers

Previously developed PCR markers were employed to genotype the *Vrn-A1* (*Vrn-A1a*, *Vrn-A1b* alleles, Yan et al. 2004), *Vrn-B1* and *Vrn-D1* alleles (Fu et al. 2005). The two allelic variants of the recessive *vrn-A1* allele in the winter wheat varieties were classified into ‘V’ and ‘W’ alleles based on the markers described previously [forward:

5′-CAACTTGTTTGGGACTAAAGGC-3′; reverse: 5′-CTG CAACTCCTTGAGATTCAAAG-3′ (Chen et al. 2009)] and confirmed by analyzing the sequencing traces of the 375-bp amplified product.

Two cleaved amplified polymorphic sequences (CAPS) markers were developed to distinguish the ‘FR-A2-S’ and ‘FR-A2-T’ haplotypes, based on the SNPs that were discovered through sequencing *CBF-A12* and *CBF-A15* (details in “Results” below). The PCR primers for *CBF-A12* and *CBF-A15* were those used for sequencing and are listed in Supplemental Material Table S3. For the *CAPS-A12* marker, *ZraI* was used to digest the *CBF-A12* PCR product into fragments of 706/476 bp (FR-A2-S) or 400/304/476 bp (FR-A2-T). For the *CAPS-A15* marker, *SalI* was used to digest the *CBF-A15* PCR product into fragments of 403/605 bp (FR-A2-S) or an undigested band of 1,017 bp (FR-A2-T) (Supplemental Material Fig. S1).

Taqman<sup>®</sup> assays to determine VRN-A1 and CBF copy number

*VRN-A1* copy number was determined using the Taqman<sup>®</sup> assay described by Díaz et al. (2012) with the exception that FAM-BHQ1 and CY5-BHQ3 were used as the 5′ fluorophore and 3′ quencher in target (*VRN-A1*) and control *CONSTANS2* (*CO2*) probes, respectively (Supplemental Material Table S4). Homoeologue-specific Taqman<sup>®</sup> assays were developed for *CBF-A12*, *CBF-A14* and *CBF-A15* genes. For each assay, primers to amplify the template were redundant for all homoeologs, while probes were designed to be homoeolog-specific (Supplemental Material Table S4). Probe specificity was confirmed by testing each assay for amplification in *T. aestivum* nullisomic–tetrasomic lines missing each of the homoeologous group 5 chromosomes. For example, primers were considered A-genome specific, when no fluorescence from the Taqman<sup>®</sup> assay was detected in the line missing chromosome 5A. Each 20  $\mu$ l reaction consisted of 0.2  $\mu$ M of both forward and reverse primers of target and control genes, 0.1  $\mu$ M of both probes, 1 $\times$  Taqman<sup>®</sup> Fast Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and 20 ng of sample DNA. Samples were run as multiplex reactions, including the target gene and control assays in each well. In all cases, the single-copy wheat gene *CONSTANS 2* (*CO2*) was used as the control. Reactions were run in an ABI 7500 Fast qRT-PCR cycler (Applied Biosystems, Foster City, CA, USA). Copy number of each target gene was estimated from the average fold-change ratio between target genes relative to *CO2* based on four biological replicates. Comparisons between control and target gene assays are relative because they use different fluorophores (FAM and CY5), so the ratio between a single copy target and a single copy control could be different from one.

### Quantitative real-time PCR (qRT-PCR)

Six biological replicates of individuals from the Eltan/ORFW  $F_{4,5}$  segregating population were grown under greenhouse conditions for 4 weeks and leaf tissue from the most recently deployed leaf was harvested at 4 p.m. on day one of the experiment. At 8 a.m. on day two, plants were transferred to 4 °C and leaf tissue harvested again at 4 p.m. Harvested tissue was immediately frozen in liquid nitrogen and then ground into a fine powder. Total RNA was extracted with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and 1 µg of the RNA equivalent of cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). Each 20 µl qRT-PCR reaction consisted of 1× USB® Veri-Quest™ SYBR® Green qPCR Master Mix (Affymetrix, Santa Clara, CA, USA) 0.5 µM of F and R primers and 10 ng of cDNA. qRT-PCR was carried out using an Applied Biosystems 7500 Fast Real-Time PCR System machine (Applied Biosystems, Foster City, CA, USA). *ACTIN* was used as an endogenous control. All qRT-PCR primers used in this study are listed in Supplemental Material Table S6. Primer efficiency and specificity were determined by analyzing amplification in a fourfold dilution series and checking dissociation curves for a single amplified product. Transcript levels are reported as linearized fold-*ACTIN* levels calculated by the formula  $2^{(ACTIN C_T - TARGET C_T)} \pm SE$ . The reported value represents the ratio between the initial number of molecules of the target gene and the number of molecules of *ACTIN* and therefore, the *Y* axes are comparable across genes.

### Frost tolerance test

For each genotype tested, 20 seeds were planted into a single cell of a six-cell pack with randomization, in soil-less potting mix (Sunshine Mix #1/LC1, SunGro Horticulture, Seba Beach, CA, USA). Eight of these cell packs were evaluated in each run of the programmable temperature cabinet, so that 48 genotypes were evaluated in each run. Prior to freezing, the seeds were germinated under a long-day regime (16 h light, 8 h dark) under cool-white fluorescent lights (200 µmol/m<sup>2</sup>/s) at 22/15 °C day/night temperatures, for 7 days in a Conviron PGR-15 growth chamber (Conviron, Manitoba, Canada) at the WSU Plant Growth Facility, and plants were then cold acclimated at a constant temperature of 4 °C for 5 weeks under a long-day regime. At the end of this acclimation period, the leaves of each plant were clipped 2.5 cm above the crown. The planting substrate was saturated with 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. Then, the plants were placed into a LU113 programmable temperature cabinet (Espec Na, Hudsonville, MI, USA) in darkness and a temperature probe (Sensatronics, Austin TX, USA) was placed into each cell of 20 plants, with a capacity of 48 cells per run. Because all of our panels were larger than 48, each panel was split into 2 blocks and the experiment for each panel was designed as a partially balanced incomplete block with 2–6 replications. Appropriate checks for each panel were included in each block. The progress of each freeze test was monitored at 2-min intervals using the temperature probes which were attached to a Senturion Environmental Monitor. The temperature of the programmable chamber was lowered from 4 to -3 °C over 1 h and held at -3 °C for 16 h to allow the heat produced during ice formation to dissipate. The temperature was then decreased to a target temperature at a rate of -4 °C/h, held at the target temperature for 1 h and then increased to 4 °C at a rate of 4 °C/h. Target temperatures were determined based on preliminary assays to consistently discern differences between susceptible and resistant check genotypes within each set of genotypes evaluated. After these steps, the trays were transferred into a growth chamber with a constant temperature of 4 °C under a long-day regime for the first 24 h, before being moved into a greenhouse at ~22 °C. Survival rate (survival/emergence) in each cell was scored 5 weeks after the freezing.

For the winter panel, two replications were assayed at a target temperature of -13 °C, and two replications assayed at a target temperature of -14 °C. For the spring panel, four replications were assayed at a target temperature of -6 °C. For the 'Eltan/ORFW'  $F_{4,5}$  segregating population, six replications were assayed at a target temperature of -10 °C.

### Statistical analysis

The survival data were analyzed within each panel using the GLM procedure of 'SAS 9.3' (SAS Institute, Cary, NC, USA) with the model  $Y_{ijk} = X + G_i(B_j) + R_k + e_{ijk}$ , where  $Y_{ijk}$  represents survival for genotype  $i$  in block  $j$  and replication  $k$ ,  $X$  represents the mean for survival within a panel,  $G_i$  represents the effect of genotype  $i$  in block  $(B)_j$ ,  $R_k$  represents the replication effect and  $e_{ijk}$  is the experimental error. In these models, genotypes were considered to be fixed effects, and replications were random effects. Least squares (LS) means for survival, adjusted for the incomplete blocks, were obtained for each genotype within each panel and these were used in subsequent analyses of *FR-A2* and *VRN-A1* allelic effects. Negative LS means were corrected to 0.

The statistical model used within each panel to determine *FR-A2* and *VRN-A1* allelic effects was  $Y_{ij} = X + F_i + V_j + FV_{ij} + e_{ij}$ , where  $Y_{ij}$  represents the

LS mean for percent survival of genotypes with *FR-A2* alleles indexed by  $i$ , and *VRN-A1* alleles indexed by  $j$ ,  $X$  represents the average survival within a panel as above,  $F_i$  represents the effect of allele  $i$  at *FR-A2*;  $V_j$  represents the effect of allele  $j$  at *VRN-A1*;  $FV_{ij}$  represents their interaction and  $e_{ij}$  is the experimental error. For the winter panel, the alleles tested at the *FR-A2* locus were designated *FR-A2-S* and *FR-A2-T*, while the alleles for *VRN-A1* were either  $V$  or  $W$  (*VRN-A1* SNP) or the CNV at *VRN-A1* (*VRN-A1* CNV) which we scored as 1, 2, or 3 based on the relative *VRN-A1:CO2* ratio obtained from the Taqman<sup>®</sup> assay. For the Eltan/ORFW population, the *FR-A2* locus alleles were scored as above and the *VRN-A1* CNV (2 or 3) was used to score the alleles at the *VRN-A1* locus because complete linkage was observed between the *VRN-A1* SNP and *VRN-A1* CNV in that panel. For the spring panel, the alleles at the *FR-A2* locus were scored as above but those at the *VRN-A1* locus were scored as the presence of either *Vrn-A1a* or *Vrn-A1b* conditioning spring growth habit (group A) or the absence of those two alleles (group B).

For all models, homogeneity of variances was tested using Levene's test and normality of residuals using the Shapiro–Wilk tests as implemented in SAS 9.3. For the three simple effect ANOVAs, in which Normality of the residuals could not be restored by transformation, we used non-parametric Kruskal–Wallis ANOVAs (Using the NPAR1WAY procedure in SAS 9.3) to test the significance of the differences. The results of the three non-parametric tests were consistent with the  $P$  values obtained in the corresponding parametric ANOVAs.

Accession numbers of the CBF genes sequenced in the current study

Twelve distinct sequences of the A, B and D genome homeologs of *CBF12*, *CBF14* and *CBF15* were submitted to the European Nucleotide Archive of European Molecular Biology Laboratory (EMBL) (<http://www.ebi.ac.uk/ena/>). Accession numbers: *CBF-A12* (ORFW), HG530924; *CBF-A12* (Eltan), HG530925; *CBF-B12*, HG530926; *CBF-D12*, HG530927; *CBF-A14* (Eltan), HG530928; *CBF-B14*, HG530929; *CBF-D14*, HG530930; *CBF-A15* (ORFW), HG530931; *CBF-A15* (Eltan), HG530932; *CBF-B15*, HG530933; *CBF-D15*, HG530934.

## Results

Copy number and sequence variation at the *VRN-A1* locus in winter wheat

The 65 varieties in the winter panel were originally selected to represent several global environments with severe

winters. Eight of these varieties carried the  $V$  allele and 57 the  $W$  allele at *Vrn-A1* (Table 2). We also sequenced the amplified fragment from this marker, which revealed that those lines carrying the  $V$  allele had only 'C' at the SNP position, while overlapping 'C' and 'T' peaks were detected in lines carrying the  $W$  allele, indicative of multiple copies of *vrn-A1* (see Table 1 for allele nomenclature). There were clear differences in *VRN-A1* copy number based on the ratio of fluorescent signal from the *VRN-A1/CO2* Taqman<sup>®</sup> assays. The 65 winter varieties were divided into three groups according to their *VRN-A1* copy number: the first group included only the variety 'Jagger' with a *VRN-A1:CO2* ratio of 0.4, corresponding to a haploid copy number of one. Eleven varieties had a ratio of 0.7–0.9 (~2 haploid copies), while the remaining 54 varieties had a ratio of 1.1–1.3 (~3 haploid copies) (Table 2).

*VRN-A1* copy number was associated with the presence of the  $V$  or  $W$  allele in this winter panel. The  $V$  allele was associated with a lower *VRN-A1* copy number and the  $W$  allele with a higher copy number ( $P < 0.0001$ ). In total, 53 of the 58 lines carrying the  $W$  allele had an estimated haploid copy number of 3, whereas 6 of the 8 lines carrying the  $V$  allele had an estimated copy number of 2. Of the other two varieties with the  $V$  allele, one carried a single copy of *VRN-A1* with the 'C' SNP (Jagger) and the other one three copies of *VRN-A1*, all with the 'C' SNP (Vakka) (Table 2).

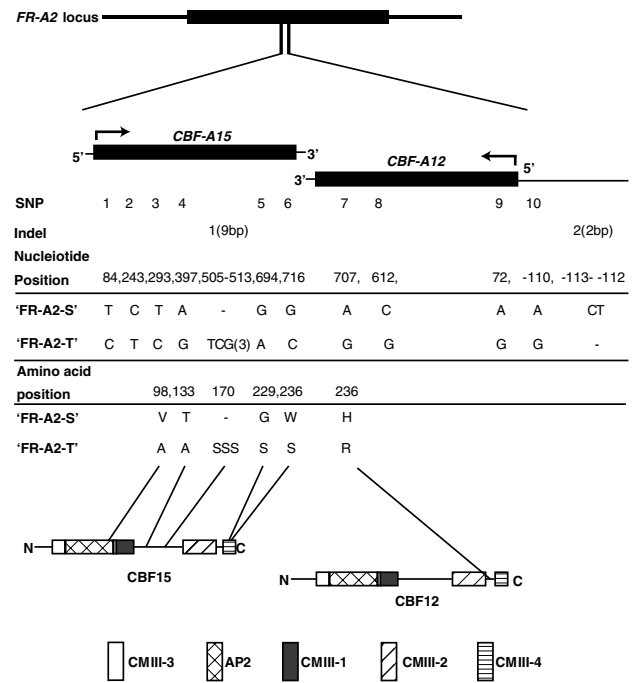
Two *FR-A2* haplotypes in the central *CBF* cluster

We characterized variation in the three central *CBF* genes of the *FR2* locus by sequencing the coding region and a short region of 5' and 3' UTR from the *CBF12*, *CBF14* and *CBF15* A, B and D homeologs for all 65 accessions in the winter panel (see "Materials and methods" for the accession numbers of all the sequences). We found no sequence diversity in either the B or D homeologs of any of these genes. On the A genome, we found a rare SNP polymorphism (A/G) at nucleotide 576 in *CBF-A14* (coordinates based on start codon at position '1' of the *CBF-A14* gene as a reference). Among the 65 accessions sequenced, only one—'Bussard'—had an 'A' at the SNP point, whereas the other 64 accessions all had a 'G' (EMBL accession number HG530928). However, at *CBF-A12* and *CBF-A15*, we identified four SNPs plus one indel polymorphism in *CBF-A12* and six SNPs plus one indel polymorphism in *CBF-A15*. These SNPs and indels at *CBF-A12* and *CBF-A15* were linked and grouped the winter panel into just two haplotypes (Fig. 1). Seven winter accessions 'Alma', 'Bussard', 'Malakov', 'Mira', 'ORFW', 'Sava' and 'Viking' carried the same haplotype at *FR-A2*, designated 'FR-A2-S' (EMBL accession numbers: *CBF-A12*, HG530924; *CBF-A15*, HG530931) (Supplemental Material Table S1). The second haplotype was shared by the remaining 58

**Table 2** Five alleles of recessive *vrm-A1* conditioning differential survival in freezing trials were identified among the winter panel varieties

Copy number estimate		Type 'V'		Type 'W'	
	Variety names	Mean survival (% ±SE)	Variety names	Variety names	Mean survival (% ±SE)
1 Copy (0.4–0.6) <sup>a</sup>	Jagger	5.9 ± 14.9	Alma, Ilchevka, Mira, Simon, Stephens	AGS2000, Alabaskaja, Albatros Odesskii, Arthur, Bavaria, Besostaja 1, Boundary, Bus-sard, CDC Kestrel, CDC Clair, Centurk 78, Chukar, Crimson, Daws, Eltan, Endurance, Expedition, F26-67, Frankemuth, Freedom, Fundulea 133, Fundulea 174, Fundulea 63-70, Geneva, Harding, Hopewell, IDO621, Inna, Jerry, Karl, Kavkaz, Malakov, Millennium, Minhardi, Mironovskaja, Moldova, Moro, Norstar, Odesskaja, OH552, Purkov, Redwin, SD97250, Seward, Tiber, Trego, Viking, Volgogradskaja 84, Wahoo, Wanser, Westley, Xerpha	8.4 ± 5.4
2 Copies (0.7–0.9)	Chernova, Fundulea 174-71, Munstertaler, Olympia, ORFW, Sava	16.8 ± 6.4			
3 Copies (1.1–1.3)	Vakka	47.4 ± 14.9			

<sup>a</sup> Copy number based on average fold-change ratio between target gene relative to *CONSTANS2* based on four biological replicates



**Fig. 1** Nucleotide and amino acid sequence variation distinguishing *FR-A2* haplotypes. Nucleotide positions of the two indel and ten SNP polymorphisms of *CBF-A12* and *CBF-A15* are listed. Six of the 12 sequence polymorphisms lead to amino acid changes and the positions and detail of each amino acid substitution in CBF-12 and CBF-15 are displayed. AP2, CMIII-1, CMIII-2, CMIII-3 and CMIII-4 are the five conserved amino acid motifs which distinguish *CBF* genes from the other members of ERF family. These five domains are indicated by different colors and filling types in the diagrammatic sketch of CBF12 and CBF15 proteins

winter accessions and was designated 'FR-A2-T' (EMBL accession numbers: *CBF-A12*, HG530925; *CBF-A15*, HG530932).

In *CBF-A12*, one indel polymorphism and one SNP were located in the promoter region resulting in a change from 'CTAA' ('FR-A2-S') to 'AG' ('FR-A2-T') at nucleotides –110 to –113 (coordinates based on start codon at position '1' of the *CBF-A12* gene using the 'FR-A2-S' haplotype as a reference). There were two synonymous SNP mutations located at nucleotide positions 72 and 612 and one SNP that resulted in an H/R amino acid polymorphism at position 236 between the CBF conserved motif CMIII-2 and CMIII-4 (Fig. 1). This and other amino acid changes are designated with a two-letter and one number code (e.g. 'H236R'), where the first and last letters represent the amino acids at the 'FR-A2-S' and 'FR-A2-T' haplotypes, respectively, and the number the position from the initial methionine in the 'FR-A2-S' haplotype.

In *CBF-A15*, we identified two synonymous mutations at nucleotide positions 84 and 243. A three amino acid deletion (SSS) was detected in the 'FR-A2-S' haplotype after position 170 between the CMIII-1 and CMIII-2 motifs.



There were also four SNPs which resulted in conservative amino acid changes; 'V98A' in the AP2 DNA binding domain (BLOSUM 62 = 0), 'T133A' between the CMIII-1 and CMIII-2 motifs (BLOSUM 62 = 0), 'G229S' (BLOSUM 62 = 0) and 'W236S' (BLOSUM 62 = -3) both located in the CMIII-4 motif (Fig. 1). To summarize, we identified twelve polymorphisms distinguishing 'FR-A2-S' from 'FR-A2-T', six of which result in amino acid substitutions (one in *CBF-A12* and five in *CBF-A15*).

To determine if sequence diversity at the *FR-A2* locus also existed in the ancestors of modern wheat, we sequenced these three *CBF* genes in 25 accessions of *T. dicoccoides* [tetraploid wild emmer wheat (genomes AABB)] and two accessions of *T. urartu*, the diploid A-genome progenitor of tetraploid and hexaploid wheat. In contrast to modern hexaploid varieties, we identified some sequence variation in the *CBF-A14* gene. Three different versions of the *CBF-A14* gene were identified within the *T. dicoccoides* population, but, with the exception of the variety 'Bussard', which carries a G–A SNP at position 576, none of these polymorphisms were found within the hexaploid wheat varieties in our study (EMBL accession numbers CBF-A14 (Bussard), HG939430; CBF-A14 (Nesher), HG939431; CBF-A14 (Tabigha), HG939432). At the *CBF-A12* and *CBF-A15* genes, we identified the same *FR-A2* haplotypes as described in hexaploid wheat. *T. dicoccoides* accessions can be split into two geographically distinct sub-populations, one northern, centered on Iraq and Turkey, and one southern, centered on Israel and Syria (Luo et al. 2007). In both populations, we identified accessions with the 'FR-A2-S' and 'FR-A2-T' haplotypes (Supplemental Material Table S5). Within the northern sub-population, 8 out of 12 accessions carried the 'FR-A2-T' haplotype, whereas in the southern sub-population, the 'FR-A2-S' haplotype was more common (8 out of 13 accessions). We also detected both 'FR-A2' haplotypes in *T. urartu* with the accession 'G1812' (originating in Lebanon) carrying the 'FR-A2-S' haplotype and the accession 'ICWT 500612' (originating in Turkey) carrying the 'FR-A2-T' haplotype. These results suggest that these haplotypes at the *FR-A2* locus are ancient and pre-date the origin of tetraploid wheat. Based on the 10 SNPs that differentiate the 'FR-A2-S' and 'FR-A2-T' haplotypes (8 transitions and 2 transversions in 1,810 aligned bases), we calculated a minimum divergence time of  $661,000 \pm 209,000$  years using Kimura's two-parameter model (Kimura 1980) and a conservative divergence rate of  $4.2 \times 10^{-9}$  substitutions/nt/year (Ramakrishna et al. 2002). This estimated divergence time places the origin of the 'FR-A2-S' and 'FR-A2-T' haplotypes before the origin of tetraploid wheat [ $<500,000$  years (Huang et al. 2002)]. We developed two CAPS markers to distinguish the two *FR-A2* haplotypes (Supplemental Material Fig. S1) and used them to screen the 81 varieties in the

spring wheat panel. The frequency of the *FR-A2* alleles differed significantly between the winter and spring panels ( $\chi^2$  test  $P < 0.0001$ ). The 'FR-A2-T' haplotype was more frequent among winter wheat varieties (89.3 %) and the 'FR-A2-S' haplotype was more frequent among spring wheat varieties (66.7 %) (Supplemental Material Tables S1, S2).

#### *CBF* CNV is correlated with *FR-A2* haplotype

Previous studies have shown that CNV exists at the *FR-A2* locus in temperate cereals, so we analyzed the *CBF-A12*, *CBF-A14* and *CBF-A15* gene copy number and found evidence of CNV for all three genes in both winter and spring panels. In the winter panel, significant correlations between CNV and frost tolerance were discovered for both *CBF-A12* ( $R = 0.36$ ,  $P = 0.0035$ ) and *CBF-A14* ( $R = 0.35$ ,  $P = 0.0040$ ). In the spring panel, correlations between *CBF* CNV and frost tolerance were not significant. In both spring and winter panels, we observed a good correlation between *CBF-A12* and *CBF-A15* CNV within both 'FR-A2-S' and 'FR-A2-T' haplotype groups (Table 3). Both these genes were also highly correlated individually with *CBF-A14* CNV in the 'FR-A2-T' haplotype, but strikingly, this correlation was greatly reduced in the 'FR-A2-S' haplotype (Table 3). These patterns of correlation suggest that a key component of the *FR-A2* haplotypes is a change in *CBF-A14* copy number relative to the other genes at this locus.

The average copy number ratio of all three *CBF* genes was significantly different between *FR-A2* haplotypes in the winter panel, with the strongest association observed for *CBF-A14* ( $P = 0.0002$ ) (Table 4). The 'FR-A2-T' haplotype was associated with increased *CBF-A12* and *CBF-A14* copy number, and reduced *CBF-A15* copy number relative to the 'FR-A2-S' haplotype. Within the spring panel, differences in copy number between the two haplotypes were also significant for *CBF-A14* and *CBF-A15*, but there was no significant difference in *CBF-A12* copy number ( $P = 0.38$ ) (Table 4).

In summary, this data show that the 'FR-A2-S' and 'FR-A2-T' haplotypes are associated with differences in copy number of all three *CBF* genes in the central region of the *FR-A2* locus, and that the 'FR-A2-T' haplotype can be defined as having an increased *CBF-A14* copy number and a decreased *CBF-A15* copy number relative to the 'FR-A2-S' haplotype.

Interactions between the *VRN-A1* and *FR-A2* loci and their association with frost tolerance

#### Winter panel

The survival rates of the 65 lines in the winter panel after exposure to freezing temperatures were associated with

**Table 3** Pearson correlations among the CNVs of three *CBF* genes within each *FR-A2* haplotype in the winter and spring panels

CBF genes	Winter			Spring		
	FR-A2-S <i>n</i> = 7	FR-A2-T <i>n</i> = 58	All <i>n</i> = 65	FR-A2-S <i>n</i> = 53	FR-A2-T <i>n</i> = 28	All <i>n</i> = 81
A12 vs A15	0.55 ( <i>P</i> = 0.19)	0.48 ( <i>P</i> = 0.0002)	0.33 ( <i>P</i> = 0.0008)	0.81 ( <i>P</i> = 0.0001)	0.87 ( <i>P</i> = 0.0001)	0.81 ( <i>P</i> = 0.0001)
A12 vs A14	−0.04 ( <i>P</i> = 0.19)	0.50 ( <i>P</i> = 0.0001)	0.56 ( <i>P</i> = 0.0001)	0.33 ( <i>P</i> = 0.0184)	0.72 ( <i>P</i> = 0.0001)	0.35 ( <i>P</i> = 0.0015)
A14 vs A15	0.02 ( <i>P</i> = 0.27)	0.59 ( <i>P</i> = 0.0001)	0.20 ( <i>P</i> = 0.10)	0.09 ( <i>P</i> = 0.4995)	0.83 ( <i>P</i> = 0.0001)	0.14 ( <i>P</i> = 0.2244)

**Table 4** Least squares means of *CBF* copy number within each *FR-A2* haplotype in the winter and spring panels

CBF gene	Winter			Spring		
	FR-A2-S	FR-A2-T	Probability of a significant difference	FR-A2-S	FR-A2-T	Probability of a significant difference
<i>CBF-A12</i>	0.77	1.02	0.005	0.59	0.55	0.38
<i>CBF-A14</i>	1.01	1.98	0.0002	0.78	1.19	0.0001
<i>CBF-A15</i>	3.10	2.48	0.03	1.94	1.48	0.0085

**Table 5** Significance of the effects of the *VRN-A1* and *FR-A2* loci and their interactions on percent survival in freezing tests

Population	VRN-A1	FR-A2	Factorial ANOVA <sup>a</sup>	<i>R</i> <sup>2</sup> (%)	<i>P</i> (VRN-A1 X FR-A2)	<i>P</i> (VRN-A1)	<i>P</i> (FR-A2)
Winter panel	1, 2 or 3 copies	S vs T	3 × 2	31.97	0.0246	0.4243	0.0223
Eltan/ORFW	<i>V</i> = 2 copies vs <i>W</i> = 3 copies	S vs T	2 × 2	45.26	0.0026	0.1485	<0.0001
Spring panel	Group A: Vrn-A1 vs group B: VrnB1+D1	S vs T	2 × 2	20.72	0.0247	<0.0001	0.0562

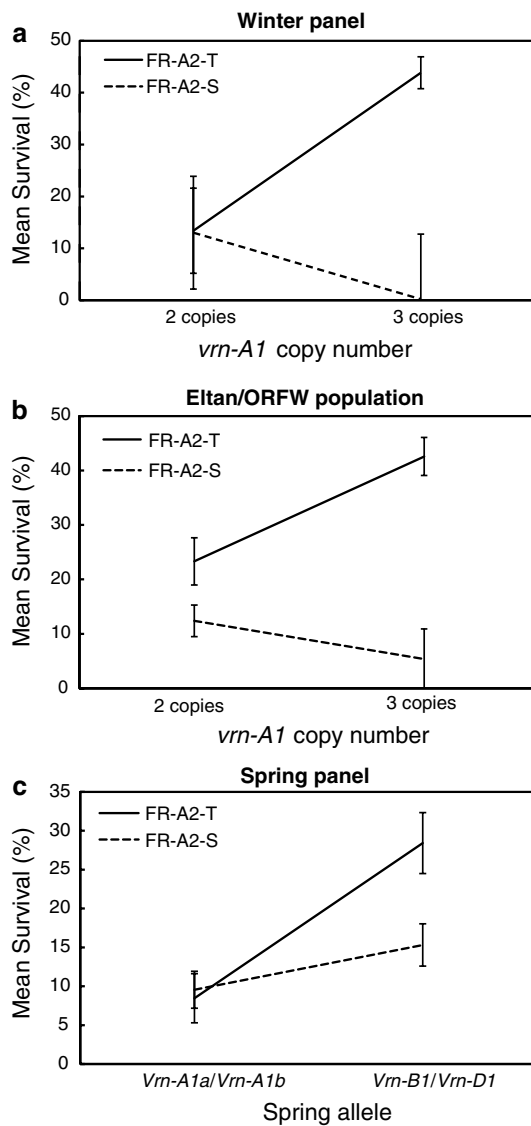
<sup>a</sup> ANOVAs were based on factorial models within each of the three populations

different alleles at the *VRN-A1* and *FR-A2* loci. The 3 × 2 factorial ANOVA using *VRN-A1* CNV (1, 2, 3) and *FR-A2* haplotype (S, T) as classification variables (Table 5) explained 32.0 % of the variation in frost tolerance in the winter panel. We observed significant differences in frost tolerance between the *FR-A2* alleles (*P* = 0.022) but not between the *VRN-A1* copy number classes (*P* = 0.424). The interaction between these two factors was significant (*P* = 0.025) (Table 5) reflecting the contrasting effects of the *FR-A2* alleles on frost tolerance in the presence of the different *VRN-A1* alleles.

To analyze the simple effects, we ran four one-way ANOVAs within each *FR-A2* haplotype and within *VRN-A1* CNV groups 2 and 3. The effect of *FR-A2* within the *VRN-A1* class with one copy could not be tested statistically because only one variety was found in this class (Jagger). Within the ‘FR-A2-S’ group, lines that carried two copies of *vrn-A1* had greater survival after freezing (13.03 ± 10.86 %) than lines that carried three copies (0.20 ± 12.54 %), but the differences were not significant (*P* = 0.152) (Fig. 2a). In contrast, within the ‘FR-A2-T’ group, lines that carried three copies of *vrn-A1*

(43.83 ± 3.07 %) had about threefold greater survival than lines that carried two copies of *vrn-A1* (13.40 ± 8.21 %) and the differences were highly significant (*P* = 0.004). In the reciprocal analysis, differences in frost survival between the ‘FR-A2-S’ and ‘FR-A2-T’ alleles were only significant within the *VRN-A1* ‘three copy’ group (*P* = 0.005) and not within the ‘two copy’ group (*P* = 0.864, Fig. 2a).

We then performed an additional factorial ANOVA using *FR-A2* and the *VRN-A1* SNP allele (*V/W*) rather than *VRN-A1* CNV as a classification variable, to investigate which of the two factors was more predictive of the differences in frost tolerance. When *VRN-A1* CNV was used as a classification variable, the model explained 32.0 % of the variation in frost tolerance, whereas when the two *VRN-A1* SNP classes were used, the model explained just 21.6 % of the variation. In addition, the ANOVA using *VRN-A1* CNV classes showed a more significant interaction with *FR-A2* (*P* = 0.0246) than the ANOVA using *VRN-A1* SNP as classification criteria (*P* = 0.123). These results suggest that the differences in frost tolerance were more closely associated with the differences in copy number at *VRN-A1* than with the SNP differences.



**Fig. 2** Interaction between *VRN-A1* and *FR-A2* within the winter panel (a), Eltan/ORFW  $F_{4:5}$  population (b) and the spring panel (c). For each class and allele combination, average LS means survival rates  $\pm$ SE are presented. All the interactions were highly significant ( $P < 0.05$ , based on ANOVA including *VRN-A1* and *FR-A2* for each population, Table 5)

This result was further validated by two additional one-way ANOVAs within the ‘FR-A2-T’ haplotype. First, we compared the effects of *VRN-A1* CNV among varieties fixed for the *VRN-A1* W allele. The 49 varieties carrying three copies of *vrn-A1* (and *W/FR-A2-T* alleles) exhibited average frost survival rates ( $41.68 \pm 3.68$  %) fourfold higher than the five varieties carrying two *vrn-A1* copies (and *W/FR-A2-T* alleles) ( $9.63 \pm 2.65$  %,  $P = 0.0116$ ). In contrast, no significant differences in frost tolerance were detected between V and W alleles among the varieties carrying two copies of *vrn-A1* and the ‘FR-A2-T’ haplotype ( $P = 0.5111$ ). Taken together, the above analysis

consistently suggests that it is the CNV at *VRN-A1* rather than the SNP variation that is responsible for the observed differences in frost tolerance. However, we cannot rule out the possibility of an effect from other closely linked genes.

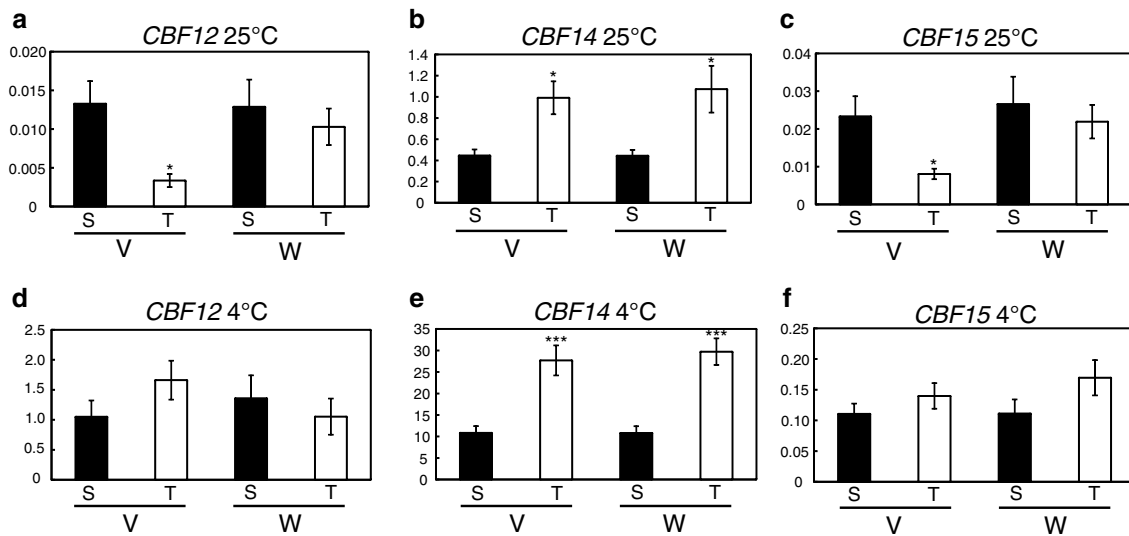
#### Bi-parental ‘Eltan/ORFW’ $F_{4:5}$ population

To validate the interaction between *VRN-A1* and *FR-A2* observed in the winter panel, we analyzed the data from the ‘Eltan/ORFW’  $F_{4:5}$  population using a  $2 \times 2$  factorial ANOVA. In this population, *VRN-A1* CNV co-segregated with the *VRN-A1* SNP which distinguished V and W alleles. The ANOVA using *VRN-A1* ( $V = 2$  vs  $W = 3$  in this panel) and *FR-A2* haplotype (S, T) as classification variables and their respective interaction (Table 5) explained 45.3 % of the variation in frost tolerance. As in the winter panel, significant differences were detected for *FR-A2* ( $P < 0.0001$ ) and no significant differences were detected between the two *VRN-A1* classes ( $P = 0.1485$ ) in the bi-parental population (Table 5). This ANOVA showed a highly significant interaction ( $P = 0.0026$ , Fig. 2b) due to the differential responses of the *VRN-A1* alleles within the different *FR-A2* classes.

The analysis of the simple effects, showed that lines within the ‘FR-A2-S’ group that carried the *VRN-A1* V allele (=2 copies) had ~twofold greater survival ( $12.39 \pm 2.91$  %) than the lines that carried the *VRN-A1* W allele (=3 copies,  $5.38 \pm 5.53$  %), but the differences were not significant ( $P = 0.131$ ). In contrast, within the ‘FR-A2-T’ group (Fig. 2b) lines that carried the *VRN-A1* W allele ( $42.57 \pm 3.50$  %) had twofold greater frost survival than lines that carried two copies of *VRN-A1* ( $23.31 \pm 4.34$  %) and the differences were highly significant ( $P = 0.0123$ , Fig. 2b). In the reciprocal analyses, the *FR-A2* haplotypes showed significant differences in frost survival within both *VRN-A1* classes, but the differences were larger within the W allele class ( $P < 0.0001$ ) than within the V allele class ( $P = 0.0387$ ).

#### Spring panel

In spring wheat varieties, different dominant *VRN-A1* alleles have significantly different effects on frost tolerance and survival. Therefore, we divided the varieties in the spring panel into two groups. The first group (group A, 47 accessions) included accessions that carried the strongest dominant *Vrn-A1a* or *Vrn-A1b* alleles, which completely eliminate the vernalization requirement. The second group (group B, 34 accessions) included the remaining accessions carrying the dominant *Vrn-B1* or *Vrn-D1* spring alleles, which exhibit a residual vernalization requirement and a slightly later flowering time than the *Vrn-A1* alleles. The interaction between these two loci was analyzed using a



**Fig. 3** Expression levels of *CBF12*, *CBF14* and *CBF15* at 25 °C (a–c) and after 8 h exposure to 4 °C (d–f). Y axis values represent the ratio between the initial number of molecules of the target gene and the number of molecules of *ACTIN* and are the average of six biological replicates  $\pm$ SE. V/W, *Vrn-A1* allele; S, ‘FR-A2-S’; T, ‘FR-A2-T’.

\* $P \leq 0.05$ , \*\*\* $P \leq 0.001$ , significance of differences between ‘FR-A2-S’ and ‘FR-A2-T’

$2 \times 2$  factorial ANOVA with *VRN-A1* groups (A, B) and *FR-A2* haplotype (S, T) as classification variables (Table 5). This model explained 20.7 % of the variation in frost tolerance. In this analysis, *VRN-A1* ( $P < 0.0001$ ) and the interaction between *FR-A2* and *VRN-A1* ( $P = 0.032$ ) both showed significant differences in frost tolerance, but *FR-A2* alone did not ( $P = 0.056$ ) (Table 5). Taken together, our results show that there is a significant interaction between the *VRN-A1* and *FR-A2* loci in both winter and spring wheat and that, therefore, models considering both loci and their interaction are necessary to correctly classify wheat genotypes for frost tolerance.

Within the ‘FR-A2-S’ group, lines that carried *Vrn-B1* or *Vrn-D1* (group B) showed twofold higher survival ( $15.31 \pm 2.71$  %) than lines that carried *Vrn-A1a* or *Vrn-A1b* alleles (group A) ( $9.54 \pm 2.38$  %), and the difference was significant ( $P = 0.05$ ). Within the ‘FR-A2-T’ group, lines that carried the *Vrn-B1* or *Vrn-D1* alleles (group B) showed 3.4-fold greater frost survival ( $28.41 \pm 3.92$  %) than the lines which carried the *Vrn-A1a* or *Vrn-A1b* alleles (group A— $8.45 \pm 3.16$  %, Fig. 2c) and the differences were highly significant ( $P = 0.0005$ ). Differences between the *FR-A2* alleles were not significant in the presence of *Vrn-A1a* or *Vrn-A1b* alleles ( $P = 0.3976$ ), but were highly significant in the presence of the *Vrn-B1* or *Vrn-D1* alleles ( $P = 0.0266$ ).

#### *FR-A2* haplotype and *CBF* transcript levels

To determine if the detected differences in *CBF* copy number associated with *FR-A2* haplotypes result in differential

expression of these genes, we compared *CBF* transcript levels in ‘FR-A2-S’ and ‘FR-A2-T’ genotypes within the segregating Eltan/ORFW  $F_{4,5}$  population after exposure to a short period of cold (8 h). Transcript levels of *CBF14* were more than tenfold higher than those of *CBF12* or *CBF15* and all three *CBF* genes were significantly induced in response to cold treatment in all genotypic combinations (Fig. 3). At 4 °C, we found no significant differences in *CBF12* or *CBF15* expression levels between the ‘FR-A2-S’ or ‘FR-A2-T’ genotypes (Fig. 3d, f), despite the association of ‘FR-A2-S’ with a higher copy number of *CBF-A15*. However, *CBF14* expression was significantly higher ( $P \leq 0.001$ ) in plants carrying the *FR-A2-T* allele than those carrying the *FR-A2-S* allele, both in genotypes carrying the V or the W allele at *VRN-A1* and both at 25 °C (Fig. 3c) and 4 °C (Fig. 3e). These results show that the increased *CBF-A14* genomic copy number associated with the ‘FR-A2-T’ genotype is associated with an increase in *CBF14* transcript levels in response to cold treatment.

## Discussion

### *VRN-A1* allelic diversity and copy number

Previous studies describing variation at the *VRN-A1* locus among winter varieties identified different *vrn-A1* alleles, which are distinguished by differences in both sequence and copy number (Table 1). In addition to the three alleles described in previous studies (Díaz et al. 2012), we identified six ‘V’ individuals carrying two copies and one variety

with three copies of the ‘C’ form of *vrn-A1*. We demonstrate here that CNV at the *VRN-A1* locus is closely associated with the *V* and *W* allele classes. By taking advantage of a small number of historic recombination events that have separated these two sources of variation, and carrying out separate ANOVAs within each allele and copy number class, we show that *VRN-A1* CNV is more likely responsible than the *V* and *W* alleles for the differences in frost tolerance associated with this locus. It is therefore possible that the previously observed association between *vrn-A1* *V* and *W* alleles and flowering time (Chen et al. 2009) is also an indirect effect of the close association between these alleles and *VRN-A1* CNV.

#### Copy number and sequence variation at the *FR-A2* locus

We also identified sequence and CNV at the central cluster of the *FR-A2* locus. Previous studies have documented *CBF-A14* CNV in wheat, but the association between this variation and frost tolerance was not determined (Knox et al. 2010; Dhillon and Stockinger 2013). In addition to differences in *CBF-A14* copy number, we also identified CNV in the two other genes in the central *FR-A2* cluster, *CBF-A12* and *CFB-A15*. Despite sequencing many varieties with multiple copies of these two genes, we did not identify double peaks in the sequencing traces at the SNP or indel positions which define the *FR-A2* *S/T* alleles. This indicates that the events resulting in differences in *CBF* gene copy number between the *FR-A2* haplotype classes occurred after the divergence of the ‘FR-A2-S’ and ‘FR-A2-T’ haplotypes.

Furthermore, the large number of linked polymorphisms between ‘FR-A2-S’ and ‘FR-A2-T’ suggests that these are relatively old haplotypes. In support of this hypothesis, we identified both *FR-A2* haplotypes within *T. dicoccoides* accessions originating from both northern and southern sub-populations and within different *T. urartu* accessions, demonstrating that this sequence diversity at the *FR-A2* locus already existed within the wild donor of the A genome in polyploid wheat before the polyploidization event that originated tetraploid wheat <500,000 years ago (Huang et al. 2002). This agrees with the estimation of the divergence times between the ‘FR-A2-S’ and ‘FR-A2-T’ haplotypes at least 600,000 years ago. The presence of ‘FR-A2-S’ and ‘FR-A2-T’ haplotypes in both diploid and tetraploid wheat suggests either multiple origins of tetraploid wheat involving different *T. urartu* accessions or gene flow between the sympatric populations of *T. urartu* and tetraploid wheat. Interestingly, we did not identify any polymorphisms in any of the B or D homoeologous copies of these genes within the modern wheat panels (we did find some instances of sequence diversity within the *CBF-A14* gene among *T. dicoccoides* accessions). *CBF-A14* CNV

was closely associated with ‘FR-A2-S’ and ‘FR-A2-T’ haplotypes in both winter and spring panels, but *CBF-A12* CNV was associated with the *FR-A2* haplotypes only in the winter panel. The tight association between *CBF-A14* CNV and *FR-A2* haplotypes within the varieties tested in the current study limited our ability to determine if the observed differences in frost tolerance were associated with *CBF* CNV or with the *FR-A2* S and T haplotypes.

We compared *CBF* expression levels between ‘FR-A2-S’ and ‘FR-A2-T’ genotypes within the segregating Eltan/ORFW *F*<sub>4.5</sub> population and found that the ‘FR-A2-T’ genotype exhibited significantly higher *CBF14* expression levels in response to cold treatment than the ‘FR-A2-S’ genotype. Despite the association of ‘FR-A2-S’ genotypes with a higher *CBF-A15* copy number, this did not result in higher transcript levels of this gene under the conditions tested and neither did we detect differences in *CBF-A12* transcript levels. These findings suggest that the increased frost tolerance associated with the ‘FR-A2-T’ haplotype may be explained in part by an increase in *CBF14* expression levels in response to cold treatment. However, we cannot rule out a potential role of the observed amino acid changes in CBF12 and CBF15 as alternative causes for the differences in cold tolerance associated with these three tightly linked genes.

To study the potential effect of allelic differences on *CBF12* and *CBF15* gene function, we analyzed those changes resulting in amino acid substitutions within the conserved domains of these proteins. Mutations with negative BLOSUM 62 scores (negative values indicate different amino acid properties) are generally more likely to disrupt the protein function. However, amino acid substitutions in critical conserved positions may have a disruptive effect even if they have a neutral BLOSUM 62 score. For example, an A37V (BLOSUM 62 = 0) substitution in a critical amino acid of the conserved AP2 domain of DREBIII-4 in *Brassica napus* abolished the protein’s function by preventing it from binding either the CRT/DRE element or the GCC box (Liu et al. 2006). In our study, the ‘FR-A2-S’ haplotype carries a ‘V’ at position 98, which corresponds to the same position in the AP2 domain as in the substitution described by Liu et al. (2006), whereas the ‘FR-A2-T’ haplotype carries an ‘A’. The amino acid ‘A’ is conserved in CBF2, CBF3, CBF6, CBF9, CBF13 and CBF14 in common wheat and is also largely conserved in CBF proteins from *Arabidopsis*, rice, oat, barley, perennial ryegrass and rye (data not shown). The CBF15 ‘FR-A2-S’ haplotype also has a unique T133A (BLOSUM62 = 0) in a position that is conserved in barley, rye and other diploid and polyploid wheat CBF15 proteins. It would be interesting to investigate if the CBF12 and CBF15 proteins encoded by the ‘FR-A2-S’ haplotype are functional.

In *Arabidopsis*, the last 98 amino acids of the CBF1 C-terminal region have been shown to be important for the trans-activation function of this protein (Wang et al. 2005). In our study, two amino acid changes in the conserved CMIII-4 motif (S229G, BLOSUM 62 = 0 and S236 W, BLOSUM 62 = -3) were detected in the C-terminal regions of the CBF15 ‘FR-A2-T’ haplotype (Fig. 1), compared to the CBF15 protein sequences of diploid and hexaploid wheat and barley. This result suggests that the amino acid changes in the ‘FR-A2-T’ haplotype are derived. Since this allele is associated with increased frost tolerance in hexaploid wheat varieties with three *VRN-A1* copies, it is likely that selection for frost tolerance resulted in an increase of the frequency of these allele combination in winter wheat.

The C-terminal mutation between motif CMIII-2 and CMIII-4 predicted in the CBF12 protein resulted in the change from a histidine to an arginine at position 236 (H236R). The ‘H’ allele is also present in barley suggesting that this amino acid represents the ancestral state. Interestingly, the H236R polymorphism in CBF12 is also present in *T. monococcum* with the frost susceptible accession DV92 allele carrying the residue ‘H’ and the frost-tolerant G3116 accession carrying the ‘R’ (Knox et al. 2008). This result suggests that this polymorphism might precede the divergence of the A and A<sup>m</sup> genomes ~1 million years ago. Because of the complete linkage between the *CBF-A12* and *CBF-A15* polymorphisms in the varieties analyzed in this study, it is not possible to separate the effect of the detected mutations in the individual genes on frost survival.

#### Interaction between *VRN-A1* and *FR-A2* in hexaploid wheat

Within all three analyzed populations, we identified a significant interaction between the effects of *VRN-A1* and *FR-A2* loci on frost tolerance. Previous studies of this interaction compared the effect of spring and winter *VRN1* alleles on the *FR2* alleles. For example, the expression of *CBF2* and *COR/LEA* protein levels was lower in isogenic lines with dominant *Vrn-1* alleles (spring growth habit) than in those with recessive *vrn-1* alleles (winter growth habit) both in wheat (Kobayashi et al. 2005) and barley (Stockinger et al. 2007). Similarly, Limin and Fowler (2006) found that NILs carrying the recessive *vrn-A1* allele showed greater frost tolerance than lines carrying the dominant *Vrn-A1* allele. Dhillon et al. (2010) showed that the expression of *VRN1* was necessary but not sufficient to induce the down-regulation of the *CBF* and *COR* genes. It was only after the initiation of the reproductive development that the cold acclimation process was interrupted.

Our study expands upon previous interaction experiments by characterizing *FR-A2* haplotypes and by studying their effects within different dominant *Vrn-1* alleles and different recessive *vrn-A1* alleles. Within the spring panel, results were consistent with previous analyses in that the strongest spring alleles have a larger epistatic effect on *FR-A2*, eliminating or greatly reducing the effect of the *FR-A2* alleles on frost survival. This could be related to the earlier and higher levels of expression of *Vrn-A1* relative to *Vrn-B1* and *Vrn-D1*, which results in an earlier transition to the reproductive stage in the plants carrying the *Vrn-A1* allele (Loukoianov et al. 2005).

Among the winter varieties, CNV in *VRN-A1* showed opposite effects on frost survival depending on the *FR-A2* haplotype. Increased *VRN-A1* copy number was associated with increased frost tolerance in varieties carrying the ‘FR-A2-T’ haplotype, but with decreased frost tolerance in varieties carrying the ‘FR-A2-S’ haplotype. This interaction, validated using a bi-parental segregating population, highlights the significant crosstalk that exists between these two loci and provides opportunities for future research to investigate the molecular mechanisms responsible for these interactions.

The interactions between *VRN-A1* and *FR-A2* on frost tolerance also have practical implications. For example, our results suggest that combining the *vrn-A1* haplotype with three copies with the ‘FR-A2-T’ haplotype may be a good strategy for winter wheat breeders selecting for high frost tolerance. An important conclusion from this study is that *VRN-A1* and *FR-A2* must be considered together in the design of strategies to increase frost tolerance in winter wheat.

In contrast, since increased expression of *CBF* genes is associated with negative pleiotropic effects, including slower growth, delayed flowering and lower grain yields (Morran et al. 2011), in regions where cold does not pose a major threat for survival, varieties carrying the ‘FR-A2-S’ haplotype in a spring background may have a selective advantage. This could potentially explain the higher observed frequency of the ‘FR-A2-S’ in spring as opposed to winter wheat, since spring-sown plants are not exposed to severe low temperatures.

The models including *VRN-A1*, *FR-A2* and their interactions explain a considerable proportion of frost tolerance variation in each of our experiments suggesting that selection for the correct allele combination of these two genes can have a significant impact on frost tolerance improvement in winter wheat breeding programs while these alleles are segregating. The genetic markers for the *FR-A2* haplotypes developed in this study together with previous markers for *VRN1* (Yan et al. 2004; Fu et al. 2005; Chen et al. 2009) will aid breeder’s efforts to select the best allele combinations for their particular environment.

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**Conflict of interest** The experiments in this manuscript comply with the current laws of the United States. The authors declare that they have no conflict of interest.

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