Regulation of Freezing Tolerance and Flowering in Temperate Cereals: The *VRN-1* Connection[1][W][OA]

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In winter wheat (*Triticum* spp.) and barley (*Hordeum vulgare*) varieties, long exposures to nonfreezing cold temperatures accelerate flowering time (vernalization) and improve freezing tolerance (cold acclimation). However, when plants initiate their reproductive development, freezing tolerance decreases, suggesting a connection between the two processes. To better understand this connection, we used two diploid wheat (*Triticum monococcum*) mutants, *maintained vegetative phase* (*mvp*), that carry deletions encompassing *VRN-1*, the major vernalization gene in temperate cereals. Homozygous *mvp/mvp* plants never flower, whereas plants carrying at least one functional *VRN-1* copy (*Mvp/−*) exhibit normal flowering and high transcript levels of *VRN-1* under long days. The *Mvp/−* plants showed reduced freezing tolerance and reduced transcript levels of several cold-induced *C-REPEAT BINDING FACTOR* transcription factors and *COLD REGULATED* genes (*COR*) relative to the *mvp/mvp* plants. Diploid wheat accessions with mutations in the *VRN-1* promoter, resulting in high transcript levels under both long and short days, showed a significant down-regulation of *COR14b* under long days but not under short days. Taken together, these studies suggest that *VRN-1* is required for the initiation of the regulatory cascade that down-regulates the cold acclimation pathway but that additional genes regulated by long days are required for the down-regulation of the *COR* genes. In addition, our results show that allelic variation in *VRN-1* is sufficient to determine differences in freezing tolerance, suggesting that quantitative trait loci for freezing tolerance previously mapped on this chromosome region are likely a pleiotropic effect of *VRN-1* rather than the effect of a separate closely linked locus (*FROST RESISTANCE-1*), as proposed in early freezing tolerance studies.

Exposure to low nonfreezing temperatures, a process known as cold acclimation, increases a plant's freezing tolerance (Thomashow, 1990, 1999). Freezing-tolerant plants that have not been cold acclimated are generally killed at approximately −3°C to −5°C, while cold-acclimated plants can survive much colder freezing temperatures. In addition, increasing the length of the cold acclimation period, up to a point, can also increase freezing tolerance. These two observations suggest that cold acclimation is an active process.

Freezing tolerance is essential for fall-planted temperate cereals (wheat [*Triticum* spp.], barley [*Hordeum vulgare*], and rye [*Secale cereale*]) to survive freezing temperatures during the winter. In contrast, spring-sown genotypes do not require high levels of freezing tolerance, since they are not exposed to the freezing temperatures of winter. One feature that distinguishes winter and spring genotypes is the requirement of the former for a long period (several weeks) at cold temperature to accelerate the transition from the vegetative growth phase to the reproductive growth phase, a process called vernalization. Spring genotypes do not have a vernalization requirement and flower in the absence of the extended low-temperature treatment (for review, see Trevaskis et al., 2007; Distelfeld et al., 2009).

The requirement for exposures to nonfreezing cold temperatures is common to both cold acclimation and vernalization, suggesting a potential connection between these two processes. Winter genotypes maintained under continuous cold, after an initial increase in freezing tolerance, exhibit a progressive decrease in their cold acclimation ability (Fowler et al., 1996a,

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1996b; Fowler and Limin, 2004). This progressive decrease inversely parallels the fulfillment of the vernalization requirement. A clear decrease in freezing tolerance occurs after the shoot apical meristem advances to the double ridge stage (Fowler et al., 1996a, 1999; Limin and Fowler, 2006). These studies suggest that a regulatory component of freezing tolerance is linked to a developmental shift between the vegetative and reproductive stages. Limin and Fowler (2006) suggested that the main vernalization gene, *VRN-1*, which is induced during vernalization, plays an important role in the decrease of the ability to cold acclimate with development.

Early genetic studies also revealed a correlation between growth habit and freezing tolerance; wheat genotypes having a spring growth habit were less freezing tolerant than genotypes having a winter growth habit (Hayes and Aamodt, 1927). Subsequent studies carried out using wheat chromosome substitution lines revealed that homeologous group 5 chromosomes, where *VRN-1* is located, have the largest effect (Roberts, 1986). The first major locus affecting freezing tolerance and winter hardiness on homeologous group 5 was designated *FROST RESISTANCE-1* (*FR-1*; Sutka and Snape, 1989). However, since *FR-1* cosegregates with *VRN-1* in most genetic studies, it is still not clear if *FR-1* is an independent gene or just a pleiotropic effect of *VRN-1* (Brule-Babel and Fowler, 1988; Sutka and Snape, 1989; Roberts, 1990; Hayes et al., 1993; Francia et al., 2004; Galiba et al., 2009).

More recently, a second locus associated with natural variation in freezing tolerance in wheat and barley was mapped on the long arm of homeologous group 5. This locus, designated *FR-2*, is approximately 30 centimorgans proximal to *VRN-1* and includes a cluster of 11 (or more) *C-REPEAT BINDING FACTOR* (*CBF*) genes (Vágújfalvi et al., 2003; Francia et al., 2004, 2007; Miller et al., 2006; Skinner et al., 2006; Knox et al., 2010). The *FR-2 CBF* gene cluster has surfaced as a major quantitative trait locus (QTL) affecting freezing tolerance in a number of wheat and barley mapping populations (Vágújfalvi et al., 2003; Francia et al., 2004, 2007; Båga et al., 2007).

The role of the *CBF* genes in freezing tolerance has been studied in detail in Arabidopsis (*Arabidopsis thaliana*). The CBFs are transcriptional activators that promote the expression of genes whose upstream regulatory sequences harbor the CRT/DRE low-temperature cis-acting DNA regulatory element (Stockinger et al., 1997). Approximately 20% of the Arabidopsis genes whose expression is altered during cold acclimation are directly or indirectly controlled by the CBF transcription factors (Vogel et al., 2005). Direct targets of the CBFs in Arabidopsis include the robustly induced *COLD REGULATED* (*COR*) genes (Jaglo-Ottosen et al., 1998). Similar candidate *CBF* target genes in the cereals, which also harbor CRT/DRE motifs in their upstream regulatory region, include *COR14b*, *DHN5*, and *DHN8* (Choi et al., 1999; Dal Bosco et al., 2003). Many of these *COR* genes are induced to higher levels in genotypes exhibiting greater freezing tolerance than in those having lesser freezing tolerance (Houde et al., 1992; Danyluk et al., 1994, 1998; Crosatti et al., 1996; Fowler et al., 1996b; Limin et al., 1997; Grossi et al., 1998; NDong et al., 2002). The use of *COR14b* as an expression QTL to map loci affecting *COR* expression levels revealed two major loci, one of which is coincident with *VRN-1* and the second one with *FR-2* (Vágújfalvi et al., 2000; Francia et al., 2004).

Notably, genotypes carrying the *vrn-1* allele for winter growth habit express certain *CBF* genes at higher levels than genotypes carrying the *Vrn-1* allele for spring growth habit (Stockinger et al., 2007). Moreover, once the winter genotypes carrying the *vrn-1* allele are vernalized, *CBF* transcript levels are dampened relative to levels detected in nonvernalized plants (Stockinger et al., 2007). This suggests that *VRN-1* somehow acts to repress expression of the *CBF*s at *FR-2* and in turn decrease freezing tolerance.

The molecular isolation of *VRN-1* revealed that this gene encodes a MADS box protein similar to the Arabidopsis meristem identity gene *APETALA1* (*AP1*; Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). The characterization of *VRN-1* alleles associated with winter and spring genotypes showed that the primary differences were insertions and deletions in regulatory regions located in the promoter and first intron (Yan et al., 2004; Fu et al., 2005; Pidal et al., 2009). Deletions in the *VRN-1* promoter affecting a small region tentatively designated the "VRN box" (Pidal et al., 2009) or large deletions/insertions in the *VRN-1* first intron are both associated with spring growth habit (Fu et al., 2005; Pidal et al., 2009). Genotypes with a winter growth habit (*vrn-1* allele) show very low *VRN-1* transcript levels until plants are vernalized. In contrast, spring genotypes (*Vrn-1* allele) constitutively express *VRN-1* to high levels. Flowering is initiated once *VRN-1* transcripts reach a critical threshold level (Loukoianov et al., 2005).

In addition to vernalization, photoperiod also plays a role in *VRN-1* regulation. In photoperiod-sensitive genetic backgrounds, long-day photoperiods enhance *VRN-1* transcript accumulation while short-day photoperiods delay transcript accumulation. In both wheat and barley, the delay in the transition to floral initiation in plants grown under short-day photoperiods is associated with increased freezing tolerance (Fowler et al., 2001; Limin and Fowler, 2006). One of the cis-elements responsible for the lack of *VRN-1* expression under short days in diploid wheat (*Triticum monococcum*) is thought to reside within the *VRN-1* promoter CArG motif, a binding site for MADS box transcription factors located downstream of the VRN box (Dubcovsky et al., 2006; Pidal et al., 2009). Under short days, diploid wheat (*T. monococcum*) plants carrying deletions in this CArG motif (*Vrn-1f* and *Vrn-1g* alleles) show accumulation of *VRN-1* transcripts and a slow transition of the shoot apical meristem to the reproductive stage, whereas plants with intact CArG motifs (e.g. *Vrn-1h* and *vrn-1*) show no *VRN-1* expression in short days and

remain in the vegetative phase (Dubcovsky et al., 2006). Under long days, all accessions with *VRN-1* alleles for spring growth habit show accumulation of *VRN-1* transcripts and a rapid initiation of the transition to the reproductive stage (Dubcovsky et al., 2006).

T. monococcum mutants with deletions of the *VRN-1* gene fail to flower, indicating that this gene is indispensable for the transition to the reproductive phase (Shitsukawa et al., 2007b). Two independently induced nitrogen-ion-beam mutants, designated *maintained vegetative phase1* (*mvp-1*) and *mvp-2*, were generated in different *T. monococcum* genetic backgrounds. The deletions in these two mutants encompass the complete *VRN-1* gene and several closely linked genes (Distelfeld and Dubcovsky, 2010).

To investigate the role of *VRN-1* in freezing tolerance, we made use of the *mvp* mutants and of natural *T. monococcum* accessions that differ in their ability to express *VRN-1* under short days. We found that freezing tolerance and transcript levels of several *CBF* and *COR* genes were higher in the *mvp* mutants relative to the plants carrying at least one functional *VRN-1* copy. However, the expression of *VRN-1* under short days was not as effective as under long days to down-regulate *COR14b* gene transcription. Taken together, these results suggest that *VRN-1* transcription is necessary but not sufficient to down-regulate the *COR* genes.

RESULTS

Effect of the *mvp* Mutations on Freezing Tolerance

The homozygous *mvp-2* mutants (*mvp-2/mvp-2*) and the plants carrying at least one functional copy of *VRN-1* (*Mvp-2/−*) were identified using a dominant molecular marker for *VRN-1* as described in Supplemental Figure S1. Just before the freezing experiments, there were clear differences in apical development between plants from each group. The apices from the *mvp-2/mvp-2* mutant were at the vegetative stage, whereas those from the *Mvp-2/−* plants were already at the double ridge stage (Supplemental Fig. S2).

Significant differences in survival rates were detected between the *mvp-2/mvp-2* and *Mvp-2/−* plants in controlled freezing experiments. Differences between the two genotypic classes were detected at both −9°C and −11°C freezing temperatures (Table I). In the group frozen to −9°C, none of the *Mvp-2/−* plants survived, whereas 87% of the *mvp-2/mvp-2* mutants survived (Table I). In the group frozen to −11°C, none of the *Mvp-2/−* plants survived, whereas approximately half (46%) of the *mvp-2/mvp-2* mutants survived (Table I). In a second freezing experiment performed under slightly different acclimation and freezing conditions (Supplemental Fig. S3), approximately 70% of the *mvp-2/mvp-2* mutants survived −12°C freezing temperatures, whereas only 40% of the *Mvp-2/−* plants survived the same treatment ($P = 0.009$; Supplemental Fig. S3). In this second experiment, all plants from both genotypic classes were killed at −13°C (Supplemental Fig. S2).

To evaluate the effect of freezing on the functionality of PSII, the maximum quantum yield of PSII photochemistry was measured by the ratio of variable (F_v) to maximal (F_m) fluorescence in a dark-adapted state, F_v/F_m (Butler and Kitajima, 1975). F_v/F_m ratios taken 2 to 4 h after returning the plants to 20°C paralleled the survival results (high F_v/F_m values indicate low freezing damage). Homozygous *mvp-2/mvp-2* mutant plants showed significantly higher F_v/F_m values than the *Mvp-2/−* plants (Table I; $P < 0.0001$), in agreement with the greater freezing tolerance of the *mvp-2/mvp-2* plants.

Along with the F_v/F_m measurements, samples of the crown and adjoining tissues were collected to measure relative conductivity. This measurement estimates the cellular electrolytes leached from the freeze-damaged tissue as a proportion of the total cellular electrolytes and is based on the principle that the greater the damage to cells from freezing injury, the greater the exosmosis of cellular electrolytes into a water solvent (Dexter, 1956). The *Mvp-2/−* plants showed higher relative conductivity values than those from the homozygous *mvp-2/mvp-2* mutants, both at −9°C (19% increase) and −11°C (45% increase). However, the differences between genotypes were only marginally significant (Table I; $P = 0.05$), likely due to the limited

Table I. *Survival, relative conductivity, and F_v/F_m in mutant plants (mvp-2/mvp-2= m/m) and plants carrying at least one wild-type VRN-1 allele (Mvp-2/− = M/−)*

Sixty-day-old plants were acclimated at 10°C for 19 d, 4°C for 12 d, and –2°C for 12 h before the freezing treatment. *P* values correspond to factorial ANOVAs including temperature, genotype, and their interactions in the model. Experimental conditions are described in "Materials and Methods."

Parameter	Freezing Temperature				Factorial ANOVA (*P* between Genotypes)
	−9°C		−11°C		
	M/−	*m/m*	*M/−*	*m/m*	
F_v/F_m	0.56	0.78	0.56	0.81	<0.0001
Relative conductivity (%)	12.0	10.1	17.3	11.9	0.05
No. of plants that regrew per total (%)	0/50 (0%)	26/30 (87%)	0/59 (0%)	11/24 (46%)	NA[a]

[a]All *Mvp-2/−* plants failed to regrow; therefore, there is no variance within this class to perform an ANOVA. The differences between *mvp-2/mvp-2* and *Mvp-2/−* were obvious.

number of mutant plants sacrificed for relative conductivity measures in this first experiment (three *mvp-2/mvp-2* and six *Mvp-2/−* plants per temperature). A second experiment using leaves from nine plants per genotype-temperature combination confirmed the higher relative conductivity of the *Mvp-2/−* plants compared with the *mvp-2/mvp-2* plants (76% average increase over the three temperatures; $P = 0.003$; Supplemental Fig. S3).

Taken together, these three sets of data indicate that the presence of the *VRN-1* gene in the *Mvp-2/−* lines is associated with a decrease in freezing tolerance relative to the *mvp* mutants.

Effect of the *mvp* Mutations on *CBF* Transcript Levels

Quantitative reverse transcription (qRT)-PCR was used to compare transcript levels of 11 *CBF* genes in 4-week-old *Mvp-2/−* and *mvp-2/mvp-2* plants both before (20°C) and after 8 h of cold treatment at 4°C (Fig. 1). With the exception of *CBF2*, which showed very low levels of expression both at 20°C and 4°C, the other 10 *CBF* genes showed very low transcript levels at 20°C and were significantly up-regulated after 8 h of cold treatment ($P < 0.01$). Five *CBF* genes (*CBF2*, *CBF4*, *CBF9*, *CBF12*, and *CBF17*) showed significantly higher expression levels ($P < 0.01$) in the *mvp-2/mvp-2* homozygous mutant plants than in the *Mvp-2/−* plants (Fig. 1). The same difference was marginally significant for *CBF14* ($P = 0.03$) and not significant for the other *CBF* genes.

A second experiment was carried out using 8-week-old *Mvp-1/−* and *mvp-1/mvp-1* plants and, as before, measurements of *CBF* transcript levels after 8 h at 4°C (Supplemental Fig. S4). However, in this second experiment, no samples were collected at 20°C because of the negligible *CBF* transcript levels observed in the first experiment at this temperature. In the *Mvp-1/−* plants, the shoot apical meristems were between the double ridge and terminal spikelet stages, whereas in the *mvp-1/ mvp-1* mutants, the shoot apical meristems were in the vegetative stage. As in the 4-week-old *Mvp-2* plants used in the previous experiment, the older 8-week-old *Mvp-1* plants showed significantly lower transcript levels of *CBF2*, *CBF4*, *CBF9*, *CBF12*, and *CBF17* in the *Mvp-1/−* plants relative to the homozygous *mvp-1/mvp-1* mutants after the cold treatment. Whereas *CBF14* transcript level differences between *Mvp-2/−* and *mvp-2/mvp-2* genotypes were marginally significant in the first experiment, in this second experiment the differences in *CBF14* transcript levels between *Mvp-1/−* and *mvp-1/mvp-1* were not

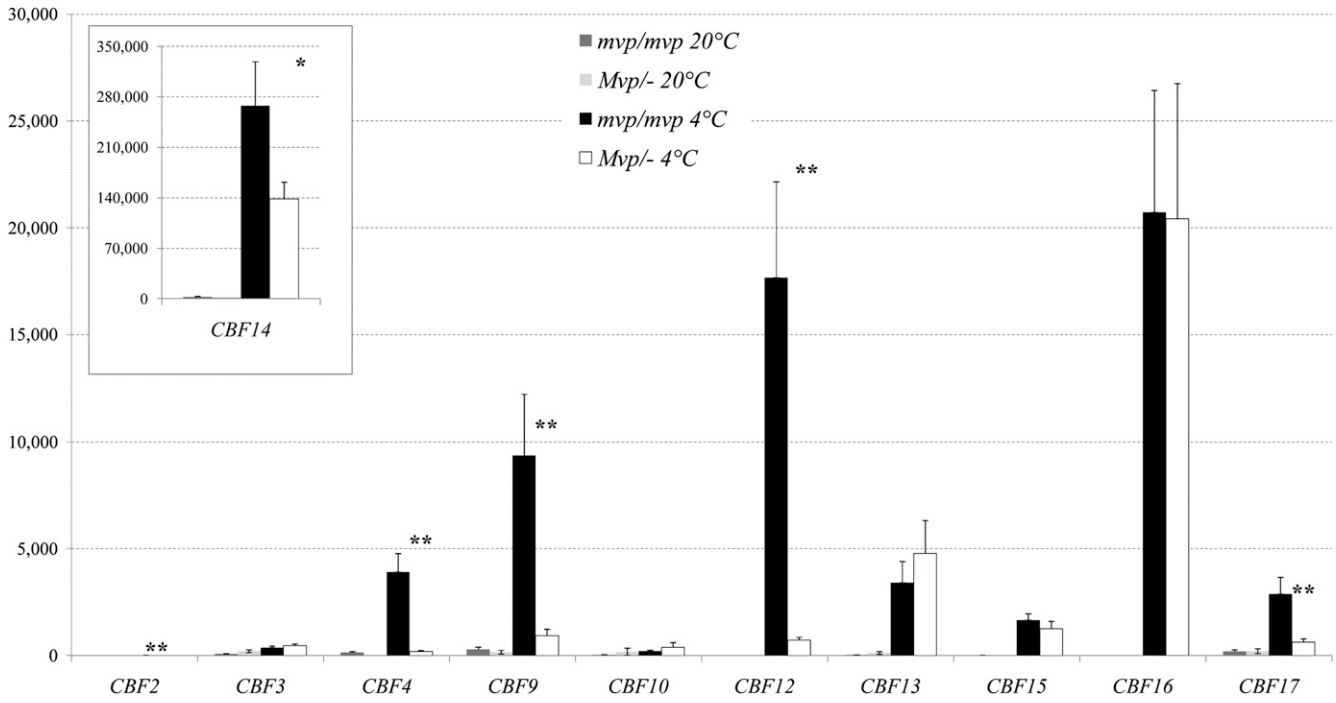

Figure 1. qRT-PCR analysis of transcript levels of the *CBF* genes present at the *FR-2* locus relative to the *ACTIN* endogenous control. Samples were collected from leaves of 4-week-old *mvp-2/mvp-2* and *Mvp-2/−* plants (20°C) and again 1 d later at the same time following an 8-h cold treatment at 4°C. Values on the *y* axis were normalized and calibrated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The same calibrator was used for all genes, so scales are comparable across genes. Values are averages of eight biological replications ± SE. The inset shows *CBF14* transcript levels, which were significantly higher than the other genes at this locus. *P* values for the differences between *mvp/mvp* and *Mvp/−* after the cold treatment were calculated using ANOVA and are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.

significant. Curiously, transcript levels of *CBF12* and *CBF16* were much lower relative to the other genes in the 8-week-old *Mvp-1* plants relative to the 4-week-old *Mvp-2* plants.

Quantitative PCR measurements of *VRN-1* in both experiments revealed high levels of *VRN-1* transcripts in the *Mvp-1/−* and *Mvp-2/−* plants, which were even higher than those of the highly expressed *TRANSLATION ELONGATION FACTOR1* (*TEF1*) endogenous control gene in both cases. As expected, no *VRN-1* transcripts were detected in the homozygous *mvp/mvp* mutants. Taken together, these results suggest that the presence of *VRN-1* (or genes regulated by *VRN-1*) modulates the response of several *CBF* genes to cold.

Effect of the *mvp* Mutations on *COR* Gene Transcript Levels

Eight hours after transferring 8-week-old plants from 20°C to 4°C, *COR14b* transcripts were 2-fold higher ($P < 0.05$) in the homozygous *mvp-1/mvp-1* plants than in the *Mvp-1/−* plants (Supplemental Fig. S4). Two additional experiments, in which plants were exposed to longer periods of cold temperatures, were performed to further characterize the differences in *COR14b* transcript levels between mutants and nonmutants.

In the first experiment, 4-week-old *mvp-2/mvp-2* and *Mvp-2/−* plants were transferred from room temperature to 4°C and kept at that temperature for 12 d. Leaf samples for RNA analysis were collected on the day prior to the cold treatment and 8 h, 32 h, 4 d, and 12 d after transferring the plants to 4°C. All samples were collected at 2 PM to avoid differences that might be caused by circadian effects. In this qRT-PCR experiment, transcript levels from both genotypes peaked at 32 h and then decayed slowly during the next 11 d. At each of the last three sampling points, the *COR14b* transcript levels were significantly higher ($P < 0.001$) in the *mvp-2/mvp-2* homozygous plants than in *Mvp-2/−* plants (Fig. 2).

In the second experiment, steady-state transcript levels of *COR14b* and two additional *COR* genes (*DHN5* and *DHN8*) were evaluated by RNA-blot analysis using a more gradual decrease in temperatures and longer exposure times to the inductive temperatures (19 d at 10°C followed by 12 d at 4°C, all under long days; Fig. 3). At the beginning of the cold induction, the *Mvp-2/−* plants were already induced to flower and showed high levels of *VRN-1* transcripts, whereas the *mvp-2/mvp-2* plants were in the vegetative stage and, as expected from the homozygous deletion, showed no *VRN-1* transcripts (Fig. 3).

At all time points after the cold induction, the transcript levels of *COR14b* and *DHN5* were higher in the *mvp-2/mvp-2* plants (no *VRN-1* transcripts) than in the *Mvp-2/−* plants (high *VRN-1* transcripts). The *DHN8* gene did not show this alternate pattern between genotypes (Fig. 3). In *mvp-2/mvp-2* homozygous mutants, *COR14b* and *DHN5* levels remained high throughout the sampling time course (Fig. 3).

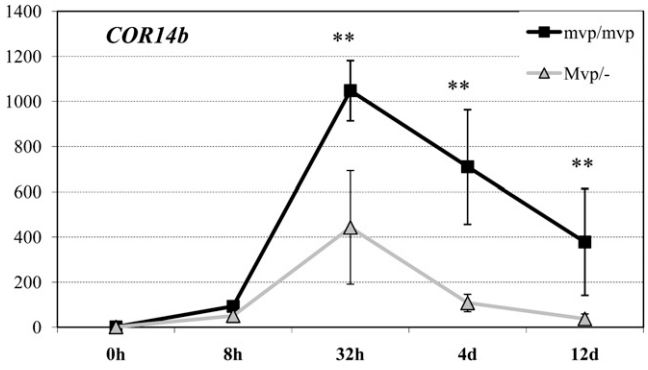

Figure 2. qRT-PCR transcript levels of *COR14b* relative to *TEF1* endogenous control. Plants were 4 weeks old at the beginning of the experiment and were exposed to 4°C for 12 d. Values on the *y* axis were normalized and calibrated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Homozygous *mvp-2/mvp-2* plants (null *VRN-1*) are indicated by black squares and lines, and *Mvp-2/−* plants (one or two *VRN-1* copies) are indicated by gray triangles and lines. Values are averages of eight biological replications in the untransformed scale ± SE. *P* values were calculated using ANOVA of $\log_{(n+1)}$-transformed values for each time point: ** $P < 0.01$.

Taken together, the expression data from these experiments showed that in plants with high *VRN-1* transcript levels, several *CBF* and *COR* genes are down-regulated. To test if the down-regulation of these genes was a direct effect of the increase in *VRN-1* transcript levels or a result of the transition to the reproductive phase, the relationship between *VRN-1* and *COR14b* transcript levels was studied in *T. monococcum* lines that, under short days, differ in the expression of *VRN-1* but show similar delays in the progression to the reproductive phase.

Effect of *VRN-1* Transcription on *COR14b* Transcript Levels under Short Days

T. monococcum lines carrying a "wild-type" *vrn-A1* allele and recessive *vrn-A2* alleles have a spring growth habit and show no expression of *VRN-1* under short days (Dubcovsky et al., 2006). However, *T. monococcum* lines carrying a 1-bp deletion in the *VRN-1* promoter CArG box and an insertion in intron 1 (*Vrn-1f* allele), or a 34-bp deletion encompassing the complete CArG box (*Vrn-1g* allele), show high *VRN-1* transcript levels under short days (Dubcovsky et al., 2006). These plants show a transition of the shoot apical meristem to the double ridge stage under short days, but further development of the spike is delayed until the plants are transferred to long days (Dubcovsky et al., 2006).

Expression profiling of three independent accessions of *T. monococcum* lines carrying each of the three genotypes confirmed previously published results (Dubcovsky et al., 2006). Under short days, lines having the *Vrn-1f* or *Vrn-1g* allele showed high *VRN-1* transcript levels, whereas those having the wild-type

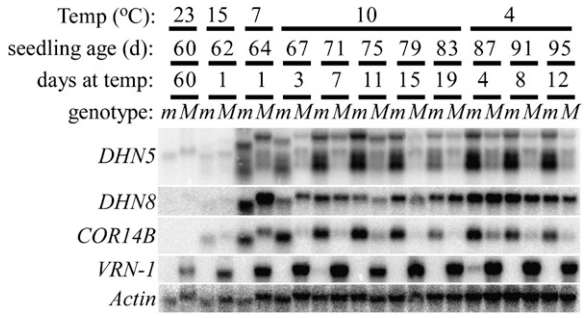

Figure 3. Time courses of *VRN-1* and *COR* genes *COR14b, DHN5,* and *DHN8* in *mvp-2/mvp-2* plants homozygous for a *VRN-1* deletion (*m*) and *Mvp-2/−* plants with one or two functional *VRN-1* copies (*M*). The presence of a faint *VRN-1* hybridization in the 87-d mutant sample is suspected to be due to cross-contamination.

vrn-1 allele showed no *VRN-1* transcripts (Fig. 4A). As expected, transcript levels of *VRN-1* were higher at 6 weeks than at 2 weeks, and *COR14b* transcripts were high after cold temperatures and nearly absent in plants maintained at warm temperature (Fig. 4A).

In the 6-week-old *Vrn-1f* and *Vrn-1g* lines grown under short days, *COR14b* was highly responsive to cold temperatures, despite relatively high *VRN-1* transcript levels in these lines (Fig. 4A, arrows). In contrast, when 6-week-old plants of these same genotypes were grown under long-day conditions, the same cold treatment failed to induce *COR14b* to high levels (Fig. 4A). This result indicates that, under short days, the expression of *VRN-1* was not sufficient to down-regulate *COR14b*.

Analyses of the 2-week-old plants (Fig. 4A) further confirmed the inverse correlation between *VRN-1* and *COR14b*. Under short days, the cold treatments resulted in a strong up-regulation of *COR14b* (low *VRN-1* transcript levels in all genotypes), but under long days, the down-regulation of *COR14b* was not as strong as in the 6-week-old plants, likely because the *VRN-1* transcript levels in 2-week-old plants was not as high as in the 6-week-old plants (Fig. 4A). To confirm these results, this experiment was repeated using qRT-PCR and the same genotypes at the same developmental stage (Fig. 4B). In plants grown under short days and exposed for 32 h to 4°C, *COR14b* transcript levels showed no significant differences ($P = 0.95$) between the wild type and *Vrn-1* mutant lines (*Vrn-1f* and *Vrn-1g*), despite significant differences in *VRN-1* transcript levels. In contrast, highly significant differences in *COR14b* transcript levels ($P = 0.006$) were detected under long days, in which *COR14b* transcript levels in the *Vrn-1f* and *Vrn-1g* mutant lines were lower than in the wild type (Fig. 4B).

An additional experiment was carried out to compare *COR14b* expression levels in *T. monococcum* lines carrying the *Vrn-1f* and *Vrn-1g* alleles (early spring growth habit) with those in a *T. monococcum* accession carrying the *Vrn-1h* allele (late spring growth habit). The *Vrn-1h* allele has an almost identical repetitive element insertion in the first intron as *Vrn-1f* but lacks the CArG box mutation (Dubcovsky et al., 2006). As reported previously, the line with the *Vrn-1h* allele showed low *VRN-1* transcripts under short days and maintained low *VRN-1* transcript levels even when plants were grown under long days for 6 weeks. The results presented in Figure 4C confirmed that high *VRN-1* transcript levels under short days (*Vrn-1f* and *Vrn-1g*) were not sufficient to suppress the induction of *COR14b*. In contrast, when plants were grown under long days, the *Vrn-1h* line (low *VRN-1* transcript levels) showed a significantly stronger ($P = 0.006$) induction of *COR14b* than the *Vrn-1f* and *Vrn-1g* mutants (Fig. 4C). As in the previous experiments, both high *VRN-1* transcript levels and long days were necessary for the down-regulation of *COR14b*.

Taken together, the results from this and previous experiments suggest that *VRN-1* expression is required to initiate the developmental processes that reduce the ability of *COR14b* to respond to cold temperatures but that *VRN-1* transcription alone is not sufficient to produce this effect.

DISCUSSION

Homozygous *mvp/mvp* Mutants Show More Freezing Tolerance Than *Mvp/−* Plants

When the shoot apical meristem of the *Mvp/−* plants transitions to the reproductive phase, it stops producing new leaves. Therefore, *Mvp/−* plants are expected to exhibit a reduced ability to generate new leaves after freezing as they transition to the reproductive phase. In contrast, the shoot apical meristem of the *mvp/mvp* homozygous mutants never transitions to the reproductive phase; thus, their ability to generate new leaves after freezing should not be drastically altered with time.

While changes in the susceptibility of the shoot apical meristem to freezing damage may account for the observed differences in regrowth after freezing, the *mvp/mvp* homozygous mutants also exhibited greater freezing tolerance in the existing leaves after acclimation than the *Mvp/−* plants, as suggested by higher F_v/F_m values and lower relative conductivity after the freezing treatment. This suggests that the presence of *VRN-1* transcript levels and the concomitant dampening of *COR14b* induction by cold contributed to a reduction of freezing tolerance in the existing leaves of the *Mvp/−* plants.

Barley COR14b and the related Arabidopsis COR15 are hydrophilic proteins targeted to the chloroplast stromal compartment (Crosatti et al., 1995). In Arabidopsis, increasing the levels of COR15 results in increased freezing tolerance (Artus et al., 1996). COR15 appears to stabilize membranes from freeze-induced injury, which would account for the reduced electro-

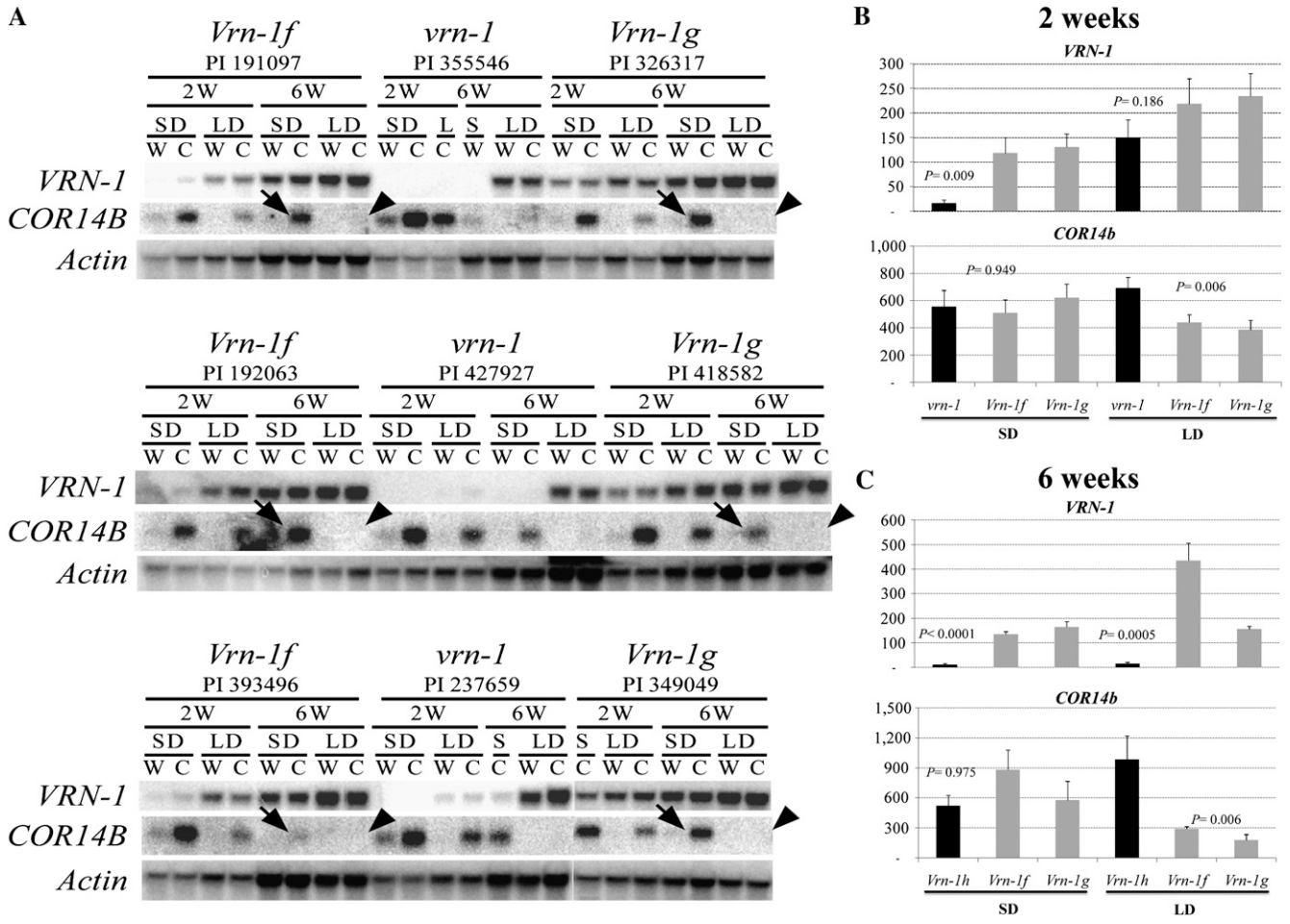

Figure 4. *VRN-1* and *COR14b* transcript levels in a set of *T. monococcum* lines differing in *VRN-1* expression under short days. Abbreviations are as follows: W, warm conditions; C, decrease from 18°C to 6°C (occurring at daybreak); 2W, 2 weeks old; 6W, 6 weeks old; SD, short day; LD, long day; *vrn-1*, wild type; *Vrn-1f*, allele with a 1-bp deletion in the CArG box coupled with the *VRN-1* intron 1 insertion; *Vrn-1g*, allele with a 34-bp deletion encompassing the CArG box; *Vrn-1h*, allele with an insertion in *VRN-1* intron 1. The three accessions with the *Vrn-1f* allele carry the dominant *Vrn-2* allele, whereas all the other accessions carry nonfunctional *vrn-2* alleles. All the accessions have a spring growth habit. A, mRNA-blot analyses of three genotypes per promoter class (indicated by different PI numbers). Arrows and arrowheads identify the presence and absence, respectively, of *COR14b* transcripts in 6-week-old plants in the *Vrn-1f* and *Vrn-1g* natural mutants under short days and long days. B and C, qRT-PCR validation of *VRN-1* and *COR14b* transcript levels relative to the *ACTIN* endogenous control at 4°C. Values on the *y* axes were normalized and calibrated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Lines carrying the wild-type allele (*vrn-1*) or *Vrn-1h* (spring, not induced in short days) are indicated by black bars, and lines carrying the *Vrn-1f* and *Vrn-1g* alleles (spring, induced in short days) are indicated by gray bars. Values are averages of five biological replications ± SE. *P* values were calculated using contrasts between either *vrn-1* or *Vrn-1h* and the average of the lines carrying the *Vrn-1f* and *Vrn-1g* alleles. Samples were collected when the plants were 2 weeks old (B) and 6 weeks old (C).

lyte leakage and higher F_v/F_m values (Artus et al., 1996). In addition, the higher transcript levels of dehydrin genes such as *DHN5* (Fig. 3) in the leaves of the *mvp/mvp* homozygous mutants relative to the *Mvp/−* plants likely contribute to their improved freezing tolerance. Dehydrins have a highly conserved 15-amino acid segment (the "K segment") that interacts with acidic phospholipids in lipid vesicles. This interaction results in a conformational change of the protein structure that is hypothesized to stabilize membrane integrity (Koag et al., 2003, 2009).

The Increased Freezing Tolerance of the *mvp* Mutants Is Likely Caused by the *VRN-1* Deletion

The MADS box meristem identity gene *VRN-1* plays an essential role in the regulation of the transition between vegetative and reproductive phases, and its deletion results in plants that fail to flower. In Arabidopsis, gene duplications of the *VRN-1* homolog that occurred after the monocot-dicot divergence resulted in three paralogous genes, *AP1*, *CAULIFLOWER* (*CAL*), and *FRUITFULL* (*FRU*), that have retained partial ability to promote the transition of the vegetative shoot apical

meristem to the reproductive phase. Simultaneous deletions of all three genes are required to generate nonflowering Arabidopsis plants (Ferrandiz et al., 2000).

An additional difference between the temperate cereals and Arabidopsis meristem identity genes is their spatial expression profile. *VRN-1* transcripts are detected at high levels in the leaves of wheat (Danyluk et al., 2003; Yan et al., 2003; Li and Dubcovsky, 2008), barley (Schmitz et al., 2000; Trevaskis et al., 2003), *Lolium* (Petersen et al., 2004), and oat (*Avena sativa*; Preston and Kellogg, 2008), suggesting a similar profile among the temperate cereals. In contrast, the Arabidopsis meristem identity homologs are expressed primarily in the apical meristem and reproductive tissues. *AP1* and *CAL* transcripts are abundant in the induced shoot apical meristem and floral primordia in Arabidopsis but are undetectable or present at much lower levels in some vegetative tissues such as the vascular tissues of cotyledons (Abe et al., 2005). *FUL* is also expressed primarily in the meristem and floral tissue, but it is also detected in cauline leaves (Teper-Bamnolker and Samach, 2005). In the winter cereals, the expression of *VRN-1* in the leaves (and apices) occurs only after vernalization, providing a potentially useful regulatory signal to down-regulate the cold acclimation pathway in this tissue in the spring.

Winter wheat lines exposed to continuous cold temperatures improve their freezing tolerance during the first 3 to 4 weeks of the treatment but then gradually start losing those gains. The inflection point in this freezing tolerance curve coincides with the transition of the shoot apical meristem to the double ridge stage and high *VRN-1* transcript levels in the leaves (Danyluk et al., 2003). Limin and Fowler (2006) found that wheat near-isogenic lines for *VRN-1* carrying the allele for winter growth habit tolerate freezing temperatures 11°C lower than lines carrying the *VRN-1* allele for spring growth habit. The authors also showed that when the same near-isogenic spring lines were grown under short days, which are less promotive of *VRN-1* expression than long days, the plants tolerated temperatures 8.5°C colder than the same lines grown under long days (Limin and Fowler, 2006). Based on these results, these authors hypothesized that the expression of *VRN-1* might be an important signal to regulate the freezing tolerance pathway.

Previous studies using near-isogenic lines (Limin and Fowler, 2006), and others using QTL mapping for freezing tolerance, all point to the *VRN-1* region as important in the regulation of freezing tolerance (Sutka and Snape, 1989; Roberts, 1990; Hayes et al., 1993; Francia et al., 2004; Galiba et al., 2009). However, the recombination points flanking the *VRN-1* gene in the lines used in these studies are not known and can encompass large chromosome regions including a large number of genes. The use of deletion mutants in this study provides a more precise delimitation of the chromosome region responsible for the differences in freezing tolerance.

Nonetheless, some caution is still required in the interpretation of the *mvp* results, since the deletions present in these lines include other genes flanking *VRN-1*. A screening with probes for multiple genes in the *VRN-1* region showed that the *mvp* deletions include the *AGLG1*, *CYS*, and *PHYC* genes but exclude flanking genes *CYB5* and *ADA2* (Distelfeld and Dubcovsky, 2010). The exclusion of *ADA2* from the deleted regions is relevant because this gene is critical for *CBF*-mediated transactivation (Stockinger et al., 2001). Based on colinearity with *Brachypodium*, the *mvp* deletion is also predicted to include two additional genes, an oligopeptide transporter (*Bradi1g08420*) and a proteinase inhibitor I9 (*Bradi1g08450*; Distelfeld and Dubcovsky, 2010). Most of these additional genes are unlikely candidates for the improved freezing tolerance observed in the *mvp* mutants, with the exception of phytochrome *PHYC*, since phytochromes B and D have been shown to affect the CBF regulon in Arabidopsis (Franklin and Whitelam, 2007). Thus, we cannot rule out the possibility of the presence of additional genes with an effect on freezing tolerance in the *mvp* deleted region until it is completely sequenced.

However, the expression studies provided an independent source of evidence pointing to *VRN-1* as the best candidate gene for the down-regulation of the cold acclimation response. In all the *T. monococcum* accessions carrying the *VRN-1f* or *VRN-1g* allele, the higher transcript levels of *VRN-1* were always associated with a significant down-regulation of *COR14b* when plants were grown in long-day conditions (Fig. 4, B and C). In addition, larger differences in *VRN-1* transcript levels during development were correlated with larger differences in *COR14b* transcript levels (Fig. 4, B and C). All the expression results presented here support the hypothesis that *VRN-1* is the best candidate for the dampening of the cold acclimation response among the genes present within the *mvp* deletions. We are currently developing TILLING mutants of the *VRN-A1* and *VRN-B1* genes in tetraploid wheat (Uauy et al., 2009) to provide an independent validation of this hypothesis.

Homozygous *mvp* Mutants Exhibit Higher Transcript Levels of Several *CBF* and *COR* Genes after a Short Cold Treatment

A negative association between *VRN-1* and *COR* genes *COR14b* and *DHN5* (=*WCS120*) transcript levels has also been reported in previous wheat and barley studies (Vágújfalvi et al., 2000; Danyluk et al., 2003; Knox et al., 2008). In a doubled-haploid barley population segregating for *VRN-H1*, the lines carrying the recessive *vrn-H1* allele showed higher transcript levels of *CBF* and *COR* genes than those carrying the dominant *Vrn-H1* allele (Stockinger et al., 2007). In addition, lines grown under short days (reduced *VRN-H1* levels) showed higher *CBF* and *COR* transcript levels than lines grown under long days when plants were transferred to the cold (Stockinger et al., 2007). The reductions in the transcript levels of multiple *CBF* genes and their downstream *COR* gene

targets in plants with high *VRN-1* transcript levels provide a simple explanation for the gradual decrease in freezing tolerance observed after the initiation of the reproductive phase.

The *mvp* mutants characterized in this study exhibited a similar negative association between *VRN-1* and both *CBF* and *COR* transcription profiles. Five of the 11 *CBF* genes tested by qRT-PCR and the *COR14b* gene showed significantly higher transcript levels 8 h after moving the plants to 4°C in the *mvp* deletion homozygotes than in those carrying at least one functional copy of *VRN-1* (Fig. 1; Supplemental Fig. S4). In plants maintained at 4°C for 12 d, the *COR14b* transcript levels were still 10-fold higher in the plants homozygous for the *mvp* deletion (Fig. 2). However, analyses of two additional *COR* genes, *DHN5* and *DHN8*, showed that not all *COR* genes respond in the same way. Whereas *DHN5* showed the same negative correlation with *VRN-1* as *COR14b*, the *DHN8* gene was not significantly affected by the change in the level of *VRN-1* transcripts, which indicates that not all *COR* genes are down-regulated by *VRN-1*. Similarly, for half of the 11 *CBF* genes present in the *FR-2* cluster, no significant differences in transcript levels were detected between *Mvp/−* and *mvp/mvp* lines (Fig. 1; Supplemental Fig. S4), suggesting that cold activation of these genes is not regulated by *VRN-1*. Thus, it appears that this *VRN-1*-mediated mechanism may play a role in the regulation of a specific subset of cold-responsive genes.

Allelic Differences in *VRN-1* Are Likely Sufficient to Explain Differences in Freezing Tolerances Previously Assigned to a Separate *FR-1* Locus

In earlier studies, differences in freezing tolerance mapped to the *VRN-1* region in wheat were considered to be the result of a closely linked gene designated *FR-1*. However, only two studies have reported recombination between *FR-1* and *VRN-1*, and they differ in the relative positions of these two genes, with *FR-1* distal to *VRN-1* in the initial mapping studies (Galiba et al., 1995, 1997) and proximal to *VRN-1* in a later mapping study using deletion lines (Sutka et al., 1999). Although the differences in freezing tolerance across the deletion lines used in the latter study were clear, it is still possible that the reduced freezing tolerance observed in the larger deletion used to map *FR-1* to a proximal deletion bin than *VRN-1* was the result of the loss of a larger number of genes and an overall reduction in plant vigor rather than the effect of a single *FR-1* gene. It is also possible that simultaneous segregation at the linked *FR-2* locus, which was not known at the time of these two studies, affected the mapping results.

The improved freezing tolerance and higher transcript levels of *CBF* and *COR* genes in the *mvp* mutants suggest that *VRN-1* allelic differences are likely sufficient to explain differences in freezing tolerance previously considered to be the result of a separate *FR-1* gene. Therefore, our results support the hypothesis that *FR-1* is a pleiotropic effect of *VRN-1* rather than a separate gene. This hypothesis is also supported by experiments showing that the repression of *VRN-1* by short days in spring wheat genotypes is associated with increased freezing tolerance (Limin and Fowler, 2006) and that *VRN-1* transcript levels in the different Triple Dirk near-isogenic lines are inversely correlated with freezing tolerance (Koemel et al., 2004).

VRN-1 Transcription Is Not Sufficient to Promote the Down-Regulation of *COR* Genes

The experiment using the *T. monococcum* lines carrying the *VRN-1f* and *VRN-1g* alleles (Fig. 4) shows that up-regulation of *VRN-1* transcript levels under short days is insufficient to produce a significant down-regulation of *COR14b* as that observed under long days. Under short days, 6-week-old plants carrying these alleles show high transcript levels of *VRN-1* and a transition of the vegetative apex to the double ridge stage. However, under continuous short days, spike development proceeds slowly and stems fail to elongate. Once plants are transferred to long days, genotypes having the *VRN-1f* and *VRN-1g* alleles complete their spike development faster and head earlier than genotypes with the wild-type *VRN-1* allele due to their more advanced developmental state (Dubcovsky et al., 2006).

When grown under long days, plants carrying the *VRN-1f* and *VRN-1g* alleles showed significantly lower levels of *COR14b* than lines carrying the *vrn-1* or *Vrn-1h* allele. These results were consistent across three independent accessions for each of the *VRN-1* alleles, supporting the hypothesis that the differences in *COR14b* were associated with the differences in the *VRN-1* alleles. However, no differences in *COR14b* were observed among the same genotypes under short days, despite large differences in *VRN-1* transcript levels. These results suggest that the down-regulation of the *COR14b* requires the presence of additional factors that are activated under long days and that require the expression of *VRN-1*. Taken together, the *mvp* mutant and *VRN-1f* and *VRN-1g* experiments suggest that *VRN-1* expression is necessary but not sufficient to down-regulate several *COR* genes and reduce freezing tolerance in the leaves of wheat plants.

A similar phenomenon has recently been described in Arabidopsis, where the floral activator MADS box gene *SOC1* functions as a negative regulator of the cold response pathway through the direct repression of the *CBF* genes (Seo et al., 2009). In the Columbia wild type, *SOC1* was expressed most strongly in leaves but was also detected in vegetative apices, inflorescences, stems of flowering plants, and roots (Lee et al., 2000). A microarray experiment comparing 7-d-old seedlings of a *soc1* knockout mutant and a *SOC1*-overexpressing line with wild-type plants revealed that six *COR* genes were among the 20 genes most negatively regulated in the *SOC1*-overexpressing line. In addition, the expression

level of the three Arabidopsis *CBF* genes increased in the *soc1* mutants and decreased in *SOC1*-overexpressing lines, without affecting the transcript levels of the *CBF* regulatory genes *ICE1*, *HOS1*, or *ZAT12*. A chromatin immunoprecipitation experiment using a SOC1 antibody revealed that the CArG box regions in the *CBF* promoters were enriched in the *SOC1*-overexpressing line relative to the *soc1* knockout, which suggests that SOC1 negatively regulates cold response through direct repression of the transcription of the *CBF* genes (Seo et al., 2009). It is interesting that the expression of the wheat homolog of Arabidopsis *SOC1*, *WSOC1*, is not affected by vernalization or photoperiod, suggesting different functions in these two species (Shitsukawa et al., 2007a).

Although both *SOC1* in Arabidopsis and *VRN-1* in the temperate cereals seem to be associated with the down-regulation of the *CBF* and *COR* genes in the leaves, the effect of *VRN-1* on the *CBF* genes does not seem to be as direct as the effect of *SOC1* in Arabidopsis. The results from the experiments using *T. monococcum* accessions with differential expression of *VRN-1* under short days suggest that additional genes operating downstream of *VRN-1* and that are regulated by long days are required to mediate the negative effect of *VRN-1* on freezing tolerance. The identification of these downstream genes and the understanding of their regulatory mechanisms could potentially lead to novel strategies to prevent the premature dampening of the cold acclimation pathway in environments where the premature activation of *VRN-1* may increase the risk of freezing damage.

Do Temperate Cereals Respond Differently to the Same Cool Temperatures in the Fall and the Spring?

The system described above provides the temperate cereals with the ability to differentiate the same cool temperature in the fall and the spring. A cool temperature in the fall, when plants have low *VRN-1* transcript levels, results in the induction of the *CBF* and the downstream *COR* genes, initiating the acclimation of the plants to cold temperatures. This is essential in the fall, when cool temperatures are an indication of the approaching freezing temperatures of the winter. The same cool temperature in the spring, when *VRN-1* transcript levels in the leaves increase significantly in response to lengthening photoperiod, would result in a significantly lower up-regulation of several *CBF* and *COR* genes. Since cool temperatures in spring are generally not a prelude of coming freezing temperatures, a robust up-regulation of the CBF pathway response in the spring would likely not be advantageous for plant survival.

A similar system seems to be operating in Arabidopsis. Arabidopsis *soc1* null mutants show increased responsiveness of the *CBF* genes to cold and improved freeing tolerance, suggesting that low levels of *SOC1* transcripts during the fall may favor plant acclimation to cold temperatures (Seo et al., 2009). *SOC1* transcript levels increase significantly by the time of the initiation of Arabidopsis flowering (Lee et al., 2000), indicating that high *SOC1* transcript levels will be present in the leaves in the spring, down-regulating the *CBF* genes and their downstream *COR* targets.

The activation of the *CBF* regulon has a potentially high energetic cost to plants, since numerous *COR* genes are up-regulated in the leaves by these transcription factors (Fowler and Thomashow, 2002). In addition, *CBF* genes have also been shown to repress plant growth (Achard et al., 2008). Therefore, the down-regulation of the *CBF* genes during the spring has a potential adaptive value, ensuring the plant's rapid development under optimal conditions, and may explain the presence of related systems in Arabidopsis and the temperate cereals.

MATERIALS AND METHODS

Plant Materials

mvp Mutants

Two independent *Triticum monococcum* mutants (*mvp-1* and *mvp-2*) that remain indefinitely in the vegetative state (Shitsukawa et al., 2007b) were used in this study. Since the two mutants carry similar deletions (Distelfeld and Dubcovsky, 2010), they were alternated among experiments depending on seed supply. Seeds from these mutants were kindly provided by K. Murai. These mutants were generated by ion-beam radiation, and both have large deletions that include *VRN-1* (Shitsukawa et al., 2007b) and several flanking genes (Distelfeld and Dubcovsky, 2010). The *mvp-1* mutation was generated in the KU104-2 background and the *mvp-2* mutation in the KU104-1 background (Shitsukawa et al., 2007b). When grown in the greenhouse under long-day conditions (16-h photoperiods), KU104-2 flowered 10 weeks after planting while KU104-1 flowered 3 weeks later.

Homozygous *mvp* individuals do not flower and therefore must be maintained in a heterozygous state. Genotyping was carried out using a dominant *VRN-1* molecular marker based on a set of three primers that are described in Supplemental Figure S1. Using this assay, the lines carrying one or two functional *VRN-1* copies are detected as a single genotypic class, referred throughout the text as *Mvp/−*.

VRN-1f, VRN-1g, and VRN-1h Alleles

T. monococcum lines with four different *VRN-1* alleles were used to test the effect of their differential regulation under short days on *COR14b* transcript levels. *T. monococcum* lines PI355546, PI427927, and PI237659 carry the "wild-type" *vrn-1* allele and a recessive *vrn-2* allele that confers spring growth habit. Lines with the *vrn-1* allele showed no expression under short days in previous studies (Dubcovsky et al., 2006). *T. monococcum* lines PI191097, PI192063, and PI393496 carry the *Vrn-1f* allele, which has a 1-bp deletion in a CArG box located in the promoter plus an insertion of a repetitive element in the first intron. *T. monococcum* lines PI326317, PI418582, and PI349049 carry the *Vrn-1g* allele, which has a 34-bp deletion including the promoter CArG box. Both the *Vrn-1f* and *Vrn-1g* alleles confer spring growth habit and show high levels of *VRN-1* transcripts under short days (Dubcovsky et al., 2006). *T. monococcum* accession PI306540 has the *VRN-1h* allele, which has the same intron 1 insertion as *Vrn-1f* but lacks the CArG box mutation. This allele is not expressed under short days and confers a spring phenotype but with later flowering than the *Vrn-1f* and *Vrn-1g* alleles (Dubcovsky et al., 2006). Although the shoot apical meristem transitions to the double ridge stage, spike development progresses slowly and spikes fail to elongate if these plants are left under short days (Dubcovsky et al., 2006).

Growth, Cold Acclimation, and Freezing Assays

Freezing experiments with the *mvp* mutants were all carried out using long-day conditions (16 h of light/8 h of dark). Experiments with the

T. monococcum lines with different *VRN-1* alleles were carried out using both long-day and short-day (8 h of light/16 h of dark) photoperiod cycles.

The RNA-blot analysis and the freezing experiment of the *mvp-2* mutants were done in parallel. Seeds collected from *Mvp-2/−* heterozygotes were grown under cool-white fluorescent lamps in the laboratory at room temperature for 13 d using a light intensity of 50 μmol m^{-2} s^{-1}. Genotyped seedlings were transplanted to wooden boxes having internal dimensions measuring 42 cm (length) × 22 cm (width) × 14 cm (height) and having 9.5-cm soil depth. The boxes were placed into a Conviron growth chamber (model PGW36; Controlled Environments) for an additional 47 d under cool-white fluorescent and incandescent bulbs using a light intensity of 130 μmol m^{-2} s^{-1} and a constant temperature of 23°C. The growth chamber temperature was decreased to 10°C and held at this temperature for 19 d.

Wooden boxes were then transferred to Percival growth chambers (model CU-36L2X; Geneva Scientific) where the seedlings were held at 4°C for 12 d (the PGW36 growth chamber can only cool to +10°C). After cold acclimation, the CU-36L2X growth chamber temperatures were decreased to −2°C and held for 12 h. Ice nucleation was induced by spraying the leaves with ice water. Following 12 h at −2°C, the temperature was then decreased at 1°C h^{-1} to the target temperatures of −9°C and −11°C and held at these temperatures for 24 h. Afterward, the temperature was returned to +2°C for 12 h. During this time, the plants were kept in the dark. The chamber temperature was then raised to 20°C and returned to a 16-h photoperiod.

Chlorophyll Fluorescence (F_v/F_m)

Chlorophyll fluorescence measurements were made using a hand-held portable pulse amplitude-modulated fluorometer (model OS-30p; Opti Sciences). F_v/F_m measurements were taken 2 to 4 h after the plants were returned to normal growth conditions. Leaves were dark adapted for approximately 10 min prior to taking the measurements.

Relative Conductivity

Conductivity measurements were taken on crown tissue consisting of a 1- to 1.5-cm segment of the white, nonphotosynthetic tissue between the upper photosynthetic green shoot and the primary root. Because the use of the crowns required the destruction of the plant, the number of replications in the first experiment was limited to three *mvp/mvp* homozygotes and six *Mvp/−* plants. The second experiment used leaves instead of crown regions and nine plants from the temperature/genotype combination. The electrolyte leakage assay methods are described in detail in the methods used for Supplemental Figure S3. Tubes were shaken for 1 h at 300 rpm before reading the conductivity with an Accumet Basic AB30 electrical conductivity meter (Fisher Scientific). Tubes were then autoclaved for 20 min, cooled to room temperature, and shaken for 1 h at 300 rpm before measuring the total potential conductivity. Values were adjusted by subtracting the conductivity of the deionized water. Relative conductivity represents the adjusted mean ion leakage as a percentage of the total adjusted leakage from frozen-killed samples (for formula, see methods used for Supplemental Fig. S3).

CBF qRT-PCR Experiments

Eight *mvp-2/mvp-2* and *Mvp-2/−* plants and 10 *mvp-1/ mvp-1* and *Mvp-1/−* plants were selected using the *VRN-1* molecular marker and were grown in the greenhouse for 4 weeks and 8 weeks, respectively (20°C–25°C, long days). Plants were then transferred to a growth chamber at 4°C for 8 h. RNA samples were collected from leaves from eight *mvp-2* and eight *Mvp-2/−* plants before (20°C) and after (4°C) the cold treatment in the first experiment and from 10 *mvp-1* and 10 *Mvp-1/−* plants only after the cold treatment in the second experiment.

COR14b qRT-PCR Time-Course Experiment

Eight *mvp-2* and eight *Mvp-2/−* plants were selected using the *VRN-1* molecular markers and grown in the greenhouse under the same conditions described above. After 4 weeks, when the *Mvp-2/−* plants were still at the vegetative stage, plants were transferred to 4°C and were kept at this temperature for 12 d at the same light intensity indicated above (long days). Leaf samples for RNA analysis were collected 1 d before the cold treatment and after 8 h, 32 h, 4 d, and 12 d at 4°C. Samples were always collected at 2:00 PM (8 h after the subjective daybreak) to avoid potential differences at different times of the day.

VRN-1/COR14b qRT-PCR Experiment

Five plants with each *VRN-1* allele were grown for either 2 or 6 weeks under short- or long-day conditions before transferring to 4°C. RNA samples were collected from leaves after 32 h of cold treatment.

For all qRT-PCR experiments, RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). First-strand cDNA was synthesized from 1 μg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen). Primers for qRT-PCR expression analyses are presented in Supplemental Table S1. The *TEF1* and *ACTIN* genes were used as endogenous controls (Distelfeld and Dubcovsky, 2010).

RNA-Blot Analyses

Tissue samples were collected 13 to 14 h after the subjective daybreak from both cold-acclimating plants and nonacclimated plants. Total RNA was isolated using RNeasy Plant Mini kits (Qiagen). Seven micrograms of total RNA was loaded per lane. RNA samples consisted of RNAs pooled from the crown tissue of 10 plants (*Mvp-2/−* experiment) or five plants (*mvp-1* and *VRN-1* promoter deletion mutant experiments).

Fragments used as probes were generated by PCR amplification of cloned cDNA inserts. Radiolabeled probes were generated by random priming (Feinberg and Vogelstein, 1983). Overnight hybridizations were at 42°C in 50% formamide, 5× SSC, and 20 mM Na-phosphate buffer, pH 6.8, containing 100 μg mL^{-1} herring sperm DNA, 1× Denhardt's solution, 0.1% SDS, and 10% dextran sulfate. Three to four 1-h moderate-stringency washes were performed at 62°C to 65°C in 0.2× SSC, 0.05% SDS, and 0.01% Na-pyrophosphate. Images were generated using the Molecular Dynamics Storm840 PhosphorImager (GE Healthcare) and phosphor autoradiography.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Dominant *VRN-1* molecular marker.

Supplemental Figure S2. Apices of *Mvp-2/−* and *mvp-2/mvp-2* plants.

Supplemental Figure S3. Comparison of freezing tolerance and relative conductivity between *mvp-1/mvp-1* and *Mvp-1/−* plants.

Supplemental Figure S4. qRT-PCR analysis of transcript levels of the *CBF* genes.

Supplemental Table S1. Primers used for qRT-PCR.

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