Title

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Journal

Plant Science, 176(1)

ISSN

0168-9452

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Publication Date

2009

DOI

10.1016/j.plantsci.2008.09.016

Peer reviewed

Plant Science 176 (2009) 12–19

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Review

Regulatory genes involved in the determination of frost tolerance in temperate cereals

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ARTICLE INFO

Article history:
Received 5 August 2008
Received in revised form 25 September 2008
Accepted 26 September 2008
Available online 17 October 2008

Keywords:
Wheat
Frost tolerance
Vernalization
CBF
VRN1
FR2

ABSTRACT

Recent progress in the characterization of two groups of genes responsible for natural differences in frost tolerance in wheat and barley is reviewed here. The first group includes the vernalization genes that delay flowering until the end of the winter and protect sensitive floral primordia. This process is regulated mainly by differences in the regulatory regions of *VRN1* and *VRN3* genes or in the coding regions of *VRN2*. The second group includes a set of tandemly duplicated *CBF* (*C-repeat Binding Factors*) transcription factors at the *FR2* (*Frost Resistance 2*) locus. *CBF* transcription factors are known regulators of the *COR* genes (*COld Regulated* genes) which are induced by cold and confer tolerance to subsequent freezing temperatures (acclimation). Natural differences in frost tolerance in both wheat and barley have been mapped to the *FR2* locus, and are associated with differences in threshold induction temperatures and/or transcript levels of several *CBF* genes. Higher threshold induction temperatures result in earlier up-regulation of *COR* genes during the fall, whereas higher induction levels by cold are associated with faster cold acclimation rates. Both processes result in longer acclimation periods and improved frost tolerance. Increases in *VRN1* transcript levels in the leaves are associated with reduced responsiveness of *CBF* and *COR* genes to cold and with the end of the acclimation period. Therefore, delays in the induction of *VRN1* and in the transition to the reproductive stage can extend the acclimation period and improve frost tolerance. These observations suggest that the vernalization and cold acclimation regulatory gene networks are interconnected.

Contents

1. Introduction

The capability of fall-sown varieties to survive winter is often referred to as winter hardiness. Winter hardiness is a complex trait involving tolerance to freezing, desiccation, anoxia, ice-encasement,

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doi:10.1016/j.plantsci.2008.09.016

resistance to diseases, etc. However, tolerance to low freezing temperatures (frost tolerance) has been considered as the primary limiting factor in most regions [1]. Frost tolerance can be defined as the ability of plants to survive freezing temperatures, prevent damage to the vegetative tissues and minimize other negative effects of freezing temperatures on future yield potential. This trait is different from spring radiation frost, which occurs during the reproductive stage in the spring and can cause anther sterility, floret and spike abortion as well as damage to the developing grain [2]. Spring radiation frost is currently less understood than vegetative frost tolerance and will not be covered in this review.

Winter wheat varieties are planted in the fall and, if they have adequate tolerance to survive winter freezing temperatures, usually have higher yield potential than spring varieties planted later in the spring because of their longer growing period. Although varietal differences in winter hardiness have been documented, breeders have made limited progress in improving this trait in wheat and barley [3]. This slow progress is due partly to the variable nature of winter injuries. Selection in the field is unreliable because weather patterns fluctuate from season to season. An additional limitation for the selection of optimal allele combinations for frost tolerance is the complex genetic regulation of this trait. It is difficult to distinguish the effects of low temperature tolerance genes from the pleiotropic effects of developmental genes, which also affect the ability of the plants to survive freezing temperatures.

Reproductive meristems are more sensitive to frost damage than vegetative meristems and therefore, small differences in developmental stages can affect plant survival to freezing temperatures. As a consequence of this relationship, allelic differences in genes regulating the initiation of the reproductive phase by photoperiod (*PPD* genes) [4,5] or vernalization (*VRN* genes) [6–10] have a large impact on frost tolerance. *VRN* genes are of particular interest to this review as they are regulated by long exposures to cold but non-freezing temperatures, the same conditions required for plant acclimation to freezing temperatures. The recent identification of the main genes responsible for the natural variation in vernalization requirement and cold acclimation has provided new insights into the regulation of frost tolerance in temperate cereals. The roles of these genes and their possible interactions are discussed in this review.

2. The role of vernalization gene *VRN1* in development and frost tolerance

Temperate cereals are usually divided by their growth habit into winter and spring classes. Varieties with a winter growth habit require long exposures to cold temperatures to accelerate flowering (vernalization requirement) whereas those with a spring growth habit do not have such a requirement. The main sources of natural differences in vernalization requirement in both diploid and polyploid wheat are allelic differences at the *VRN1* locus. Mutations in the *VRN1* promoter region [10–12] or in the first intron [13–15] are completely linked to a dominant spring growth habit in barley and wheat. In hexaploid wheat, a dominant *VRN1* allele at any one of the three genomes is sufficient to confer a spring growth habit [16].

Deletions of the *VRN1* complete gene in two independent diploid wheat mutants have resulted in plants that remained vegetative indefinitely, demonstrating that this gene is essential for the transition to the reproductive stage [17]. *VRN1* codes for a MADS box protein similar to the duplicated meristem identity genes *AP1/CAL/FUL* in Arabidopsis [6,7,10] and is up-regulated (directly or indirectly) by vernalization and by long days (Fig. 1). *VRN1* has been mapped on the long arm of homologous group 5

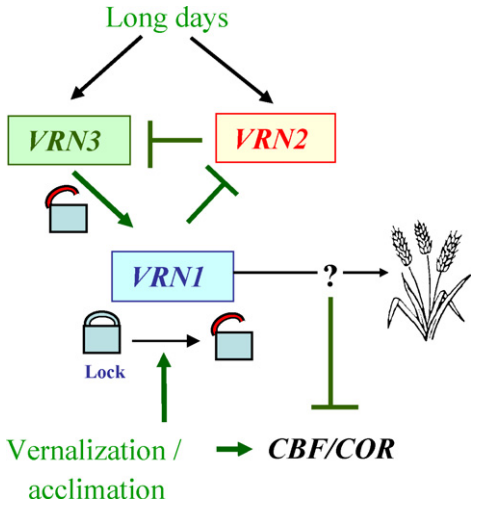

Fig. 1. Hypothetical model of flowering initiation showing the interactions among *VRN* genes and their putative effect on *COR*/*CBF* genes. The lock represents the repressed initial state of the meristem identity gene *VRN1*. Vernalization releases the *VRN1* repression (open lock) and initiates the transition of the vegetative apex to the double ridge stage. In photoperiod sensitive genotypes, flowering is accelerated by long days (LD), which induce *VRN3* and therefore promote *VRN1*. However, in the absence of vernalization, *VRN2* transcript levels are also high under long days and repress *VRN3* preventing flowering. Vernalization results in the down-regulation of *VRN2*, directly or indirectly through the action of *VRN1* [8,33]. Without *VRN2*, *VRN3* is induced promoting *VRN1* and flowering. Limin and Fowler (2006) [27] suggested that *VRN1* or genes induced by *VRN1* (question mark in the figure) can down-regulate the *CBF*/*COR* pathway (see text). Arrows indicate induction and lines ending in a crossed bar indicate repression (–|).

[18–20], in the same chromosome region associated with quantitative trait loci (QTL) for frost tolerance in both wheat and barley [21–26]. It is still unresolved whether this frost tolerance locus, designated as *FR1*, is just a pleiotropic effect of *VRN1* [27,28] or a separate gene [21–23]. In populations segregating for the vernalization gene *VRN2* [9], *FR1* is not detected, and a significant effect on frost tolerance is detected at the *VRN2* locus. This suggests that differences in vernalization requirement are sufficient to determine significant differences in frost tolerance [29].

The *VRN1* gene is positively regulated by *VRN3* (Fig. 1) a homologue of Arabidopsis *FT* [8,30], which is up-regulated by long days (Fig. 1). However, in unvernalized plants grown under long day, *VRN3* is down-regulated by *VRN2*, a zinc-finger CCT domain gene that is down-regulated during vernalization and short days [9,31,32]. Deletions of *VRN2* result in recessive spring growth habit [9].

A useful analogy to understand this regulatory network is to consider *VRN1* to be initially in a "locked" or repressed state (not-competent to flower) (Fig. 1). Vernalization gradually releases the strength of the lock and establishes competence to flower. Alternatively, mutations in the *VRN1* regulatory regions eliminate or reduce the strength of the lock resulting in dominant *Vrn1* alleles without vernalization requirement (Fig. 1). In the lock analogy, *VRN3* is a force that pushes to open the lock. Overexpression of *VRN3* in transgenic winter wheat plants was sufficient to induce flowering (open the lock) even in the presence of recessive *vrn1* alleles and the absence of vernalization [8]. Flowering is the result of a shift in the balance between the strength of the *VRN1* lock and the push of *VRN3* to open that lock. Once *VRN1* reaches a certain threshold, it represses *VRN2* [31–33], thereby releasing *VRN3* and leading to further up-regulation of *VRN1* transcript levels. These interactions result in an irreversible positive feedback loop that leads to flowering. It is currently not known if *VRN2* can repress

VRN1 independently of *VRN3* and also if vernalization can regulate *VRN2* independently of *VRN1*.

3. The role of the *FR2* locus in the regulation of cold induced genes

To survive freezing temperatures, many temperate plants use the low non-freezing temperatures before the first frost as a signal to increase their ability to withstand the subsequent freezing, a process known as cold acclimation or cold hardening [34]. Cold acclimation is accompanied by changes in many physiological and biochemical processes including increases in abscisic acid, soluble sugars, free proline, membrane lipid unsaturation, alterations in plasma membrane ultra structure and synthesis of antifreeze proteins (reviewed in [35]). It has been estimated that the expression of hundreds of genes may be altered when plants are exposed to low temperatures [36,37].

Among the cold-regulated genes, *COR14b* and *WCS120* are well characterized in wheat and barley. These genes are differentially expressed in freezing-sensitive and freezing-tolerant plants under both laboratory and field conditions. The *COR14b* gene encodes a polypeptide that accumulates in the stroma fraction of the chloroplasts in the presence of light, and its transcription is specifically induced by low temperature [38]. The COR14b protein helps to protect the photosynthetic apparatus from photodamage during light exposure following freezing [39]. WCS120 (=DHN5) belongs to the dehydrin group of proteins and is likely involved in low temperature-induced physiological dehydration. During cold acclimation, the accumulation of the WCS120 protein shows a positive correlation with freezing tolerance [40].

Increased transcript levels of *COR14b* and *WCS120* are observed in most winter cereals when plants are exposed to temperatures between 2 and 4 °C. However, when plants are exposed to milder cold temperatures (e.g. 12–15 °C) the induction profiles of these two genes show intraspecific variation. Crosses between accessions that differed in their *COR14b* and *WCS120* transcription levels at 12–15 °C were used to map these expression QTL in *T. monococcum* [29,41] and barley [25]. In both species the peak of the expression QTL for *COR14b* and *WCS120* mapped to the long arm of chromosome 5, approximately 30 cM proximal to *VRN1*. This chromosome region differs from the chromosome location of the *COR14b* and *WCS120* genes themselves, indicating that this is a *trans*-acting expression QTL [42].

The expression QTL for *COR14b* overlaps with a QTL for survival to freezing temperatures in both diploid wheat [29] and barley [25]. The locus under the peaks of these overlapping QTL was designated *FR2* (*Frost Resistance 2*)[29], and was shown to play also an important role in frost tolerance in polyploid wheat [43–45]. A high-density map of the *FR2* region in diploid wheat confirmed that the peaks of the QTL for frost tolerance and for *COR14b* and *WCS120* expression both map within a small 0.7 cM region [41]. This suggests that these two traits are either pleiotropic effects of the same gene or the result of tightly linked genes within this small genetic interval.

The molecular characterization of the *FR2* locus in both wheat and barley revealed a complex structure including multiple *CBF* genes [25,29,43,44,46–48]. The diversity of *CBF* genes among the temperate cereals and their roles in other plant species are described in the next section.

4. Roles and diversity of *CBF* genes

The *CBF* genes, also known as Dehydration Responsive Elements (DRE)-binding factors, were first studied in Arabidopsis. These genes encode for transcription factors that bind to the conserved core sequence CCGAC [C-repeat (CRT)/dehydration element (DRE)] in the promoters of many dehydration-responsive genes including those with early response to dehydration and cold [49,50]. The DNA-binding activity of some barley CBF proteins is observed only at low temperatures [51,52]. The *CBF* genes have no introns, and their proteins are characterized by a nuclear localization sequence, an AP2 binding domain flanked by conserved CBF family signature motifs, and an acidic C-terminal domain that may act as a transcriptional activation region. The C-terminal domain also exhibits conserved areas including several clusters of hydrophobic residues and an LWS(Y) motif at the end of most CBF proteins (reviewed by [51,53,54]).

The expression of *CBF* genes in Arabidopsis is up-regulated after exposure of 15 min to 14 °C or lower temperatures [55], and then returns to basal levels after continuous exposure to cold temperatures. Overexpression of the *CBF* genes significantly altered transcript levels of approximately 35 cold-regulated genes in Arabidopsis microarray experiments [36] and 256 metabolites [56]. Although, *CBF* constitutive expression increased frost tolerance, it also had negative pleiotropic effects on plant growth in Arabidopsis [50,57,58]. The orthologous *CBF* genes from rice, maize, barley, rye, and wheat [29,59–62] can also be induced by cold and/or drought. In barley and wheat *CBF* transcript levels are also affected by photoperiod [28] and diurnal cycles [63]. Some *CBF* genes are expressed at low levels in non-stressed conditions [28,44,52,63,64] and may lead to continual, albeit low, accumulation of their target genes.

The *CBF* family is greatly expanded in the temperate cereals. Rice has five *CBF* members [61], whereas at least 13 *CBF* genes are present in *T. monococcum* [46] and 17 in barley [51]. Some *CBF* groups are found only in the Pooideae, suggesting that this expansion represents an adaptation of this group of species to colonize diverse temperate habitats [63]. At least 11 different *CBF* genes have been mapped within a small 0.7–0.8 cM region encompassing the *FR2* locus in both *T. monococcum* and barley [46–48,65]. Since the order of these genes is conserved between these two species (Fig. 2) a common gene-numbering system was adopted [46,51]. The different strategies used so far to determine the roles of individual *CBF* genes within the complex *FR2* locus are described in the next section.

5. Identification of candidate *CBF* genes for frost tolerance within the *FR2* locus

Transcription profiling and high-density mapping strategies have been used to identify possible candidate genes for frost tolerance within the *FR2-CBF* cluster. Quantitative PCR systems were first developed to study the transcription profiles of different *T. aestivum CBF* genes [44]. Most of the genes studied at this locus were induced by cold treatment at 2 °C for 2 h, with *TaCBF14* and *15* showing the highest transcript levels [44]. More importantly, *TaCBF14*, *15*, and *16* transcript levels were more than four-fold higher in lines carrying a frost tolerant *FR-A2* allele than in those carrying the frost sensitive allele (*TaCBF12* was not included in this study) [44]. A barley population segregating for frost tolerance also showed higher *CBF* transcript levels associated with the *FR-H2* allele from the frost tolerant parent, but the specific *CBF* genes (*HvCBF2* and *HvCBF4*) were different from those found in the previous wheat study [28]. Taken together these results suggest that although different combinations of *CBF* genes may be involved in different segregating populations, increases in *CBF* transcript levels may be frequently associated with increases in frost tolerance.

This seems to be also the case for the *CBF* genes identified in *T. monococcum* using a high-density mapping approach. Using lines

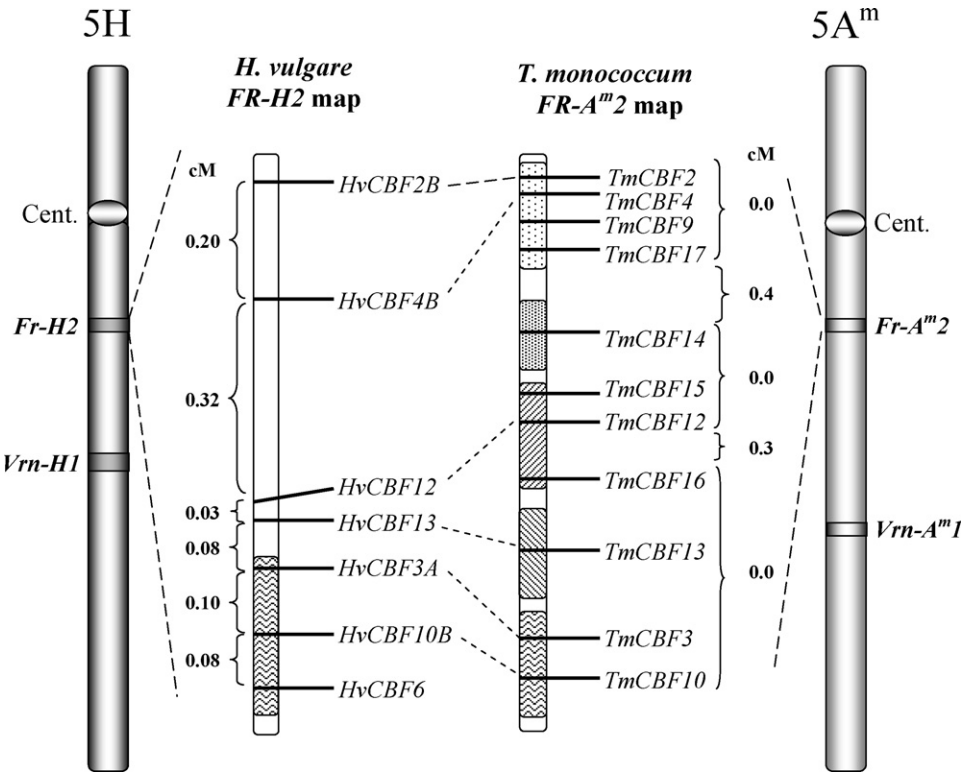

Fig. 2. Comparison of the high-density genetic map of the *FR2* locus in barley [65] and the physical and genetic map of the same region in diploid wheat [41,46]. The figure shows the relative positions of the mapped frost resistance (*FR*) and vernalization (*VRN*) genes on barley chromosome 5H and diploid wheat chromosome 5A^m. The *FR2* chromosome region is magnified. Genetic distances are in cM (Kosambi). Rectangles with different backgrounds represent different BAC clones. As no recombination events (0.0 cM) were detected between *TmCBF2* and *TmCBF17*, or between *TmCBF13* and *TmCBF10* the relative orientation of the flanking BACs was inferred from the order in barley. *HvCBF13* and *HvCBF3A* were found within a single genomic λ clone [65], whereas *HvCBF3A* and *HvCBF6* were found within a single BAC clone [51].

with recombination events within the *FR2* locus, the main differences in frost tolerance and in the differential regulation of *COR14b* and *WCS120* genes were mapped to the central region of the *CBF* gene cluster, completely linked to *TmCBF12*, *TmCBF14* and *TmCBF15* [41]. A smaller effect on frost tolerance was also associated with *TmCBF16*. This set of *CBF* genes partially overlaps with those described above in polyploid wheat [44].

Sequence comparison of the CBF proteins revealed that the *Tm*CBF12 protein from the frost susceptible parent contains a deletion of five amino acids in the AP2 DNA binding domain relative to the same protein in the frost tolerant parent and other Triticeae species. Electrophoretic Mobility Shift Assays confirmed the inability of this mutant protein to interact with the *COR* gene promoters, providing a possible explanation for the differences in frost tolerance observed between these *T. monococcum* lines [41]. Similar studies will be required to determine which *CBF* genes are responsible for interspecific and intraspecific differences in frost tolerance, as mutations in different *CBF* genes may be present in different species and accessions within species. It is interesting to point out here that the frost susceptible *T. monococcum* parent is a cultivated accession from a region with mild winters (Titograd, Montenegro), suggesting the possibility of reduced selective pressures for frost tolerance alleles.

6. Threshold induction temperatures and acclimation rates

Maximum frost tolerance is attained after several weeks of subjecting plants to temperatures low enough to induce the acclimation process [66]. Due to the long period required to reach maximum frost hardiness, an earlier start of the cold acclimation process has the potential to increase survival to a subsequent frost event. Indeed, different cereal genotypes start to acclimate at different temperatures under field conditions [67]. These differences are associated with differences in the induction temperatures of some cold-regulated genes [68]. The term "threshold induction temperature" will be used hereafter to indicate temperatures that are low enough to up-regulate cold-regulated genes or initiate the cold acclimation process.

Threshold induction temperatures for cold acclimation in wheat, barley and rye are positively correlated with freezing tolerance at full acclimation both between and within species [69]. For example, after 2 days of acclimation, the cold-hardy winter variety Norstar has a 5.4 °C warmer activation temperature than a tender winter-Manitou line [69]. Differences in threshold induction temperatures for the *CBF*-regulon seem to play an important role in the determination of frost tolerance differences in *T. monococcum* [41]. These two traits were both mapped to a small chromosome region of 0.7 cM in a cross between frost tolerant and frost sensitive *T. monococcum* lines. Three of the *CBF* genes mapped to this region (*TmCBF12*, *TmCBF15* and *TmCBF16*) were induced at 12–15 °C in the leaves and crowns of recombinant lines carrying the *FR2* allele from the frost tolerant parent but not in those with the allele from the frost susceptible parent [41]. The fact that all three *CBF* genes differ in their threshold induction temperatures can be explained either by independent mutations in the regulatory regions of the three genes or, by differences in a single gene within the *FR2* region that regulates these three *CBF* genes.

Since CBF proteins bind to the *COR* gene promoters and regulate their transcription [49–51], the differential threshold induction temperatures of the *TmCBF* alleles provide a simple explanation for the differences in *COR14b* and *WCS120* induction at 12 °C observed between the two *T. monococcum* parental lines [41]. The

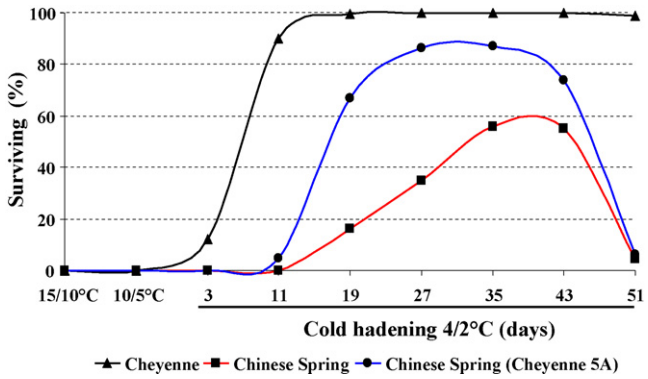

Fig. 3. Effect of cold hardening duration on wheat frost tolerance. Frost tolerant Cheyenne and Chinese Spring(Cheyenne 5A) chromosome substitution line and frost sensitive Chinese Spring plants were cold hardened for different number of days and subjected to frost tests (−10 °C). Frost tolerance was evaluated as percent survival. Plants were initially grown at 15/10 °C (day/night), then pre-hardened at 10/5 °C and finally exposed to cold hardening at 4/2 °C for 3, 11, 19, 27, 35, 43 and 51 days before the frost test [72].

differences in threshold induction temperatures of these genes were precisely mapped to the same 0.7 cM region including the differentially regulated *CBF* genes. Higher threshold induction temperatures of *COR14b* in frost resistant genotypes relative to frost susceptible ones have been also reported for common wheat [68] and barley [70,71]. The differences in *COR14b* induction between frost susceptible and frost tolerant genotypes observed at mild cold temperatures disappear when plants are exposed to lower temperatures (<4 °C) [41,68].

In addition to the differences in threshold induction temperatures, wheat frost tolerant genotypes usually acclimate faster than frost susceptible ones at constant cold temperatures [27,72]. Fig. 3 shows that the percent survival at −10 °C for the frost tolerant variety Cheyenne starts to increase 3 days after exposure to 2 °C, whereas it takes 11 days at the same temperature for the frost susceptible Chinese Spring. The replacement of chromosome 5A from Cheyenne into Chinese Spring results in a significant acceleration of the acclimation process (Fig. 3). Since both Cheyenne and Chinese Spring chromosomes 5A have recessive *vrn-A1* alleles, the differences between the two lines cannot be attributed to differences in vernalization requirement. Polymorphisms at the *FR-A2* locus seem to be a more likely explanation for the different rates of cold acclimation because transcript levels of three *CBF* genes (see Section 5) were more than four-fold higher in lines carrying the Cheyenne *FR-A2* allele than in those carrying the Chinese Spring allele [44]. Differences in initial rates of cold acclimation have been also mapped to the *FR-A2* locus in a cross between frost tolerant Norstar and frost susceptible winter-Manitou. In this cross the *FR-A2* QTL explained 40% of the variation in frost tolerance [43]. These results suggest that higher transcript levels of *CBF* genes might be associated with faster cold acclimation rates.

The *FR2* locus seems to play a major role in the determination of both the initial rates of cold acclimation and the threshold induction temperatures, but the decay in frost tolerance observed at the end of the acclimation period seems to be associated mainly with differences at the *VRN1* locus. Fig. 3 shows a slower decay in cold acclimation in the winter variety Cheyenne (*vrn-A1 vrn-B1 vrn-D1*) than in Chinese Spring or Chinese Spring (Cheyenne 5A) substitution line, which are both spring with dominant *Vrn-D1* alleles. The first genotype has a winter growth habit (recessive *vrn1* alleles at all three homoloci), whereas the last two have a spring growth habit (*Vrn-D1*). The effects of the dominant *Vrn1* alleles on cold acclimation are discussed in detail in the next section.

7. Interaction between frost tolerance and vernalization

In the temperate cereals, up-regulation of *VRN1* by vernalization is observed in both apices and leaves [10,73]. *VRN1* transcripts are also abundant in the leaves of other non-grass monocot species [74]. This contrasts with Arabidopsis or rice, in which transcripts of the homologous gene (*AP1*) are mainly restricted to the apices and are not detected or present at very low levels in some specialized leaves (e.g. in the vascular tissues of cotyledons [75]). In Arabidopsis the FD protein binds to FT (the VRN3 homologue) in the apex and together they induce *AP1* [76]. The absence of FD in Arabidopsis leaves explains the limited expression of *AP1* in this tissue, as demonstrated by high *AP1* transcript levels in transgenic Arabidopsis lines overexpressing *FD* in the leaves [76]. In wheat, a homologue of *FD* (*TaFDL2*) is expressed at high levels in the leaves. TaFDL2 has the ability to interact with VRN3 and to bind to the *VRN1* promoter *in vitro* [30], and that explains how *VRN1* is induced in the wheat leaves. However, it does not explain why the expression of a meristem identity gene has been retained in the leaves of all temperate grass species studied so far (wheat, barley, oats and ryegrass) [6,7,10,77,78].

A tentative answer to this question was proposed by Limin and Fowler [27] based on the observation that frost tolerance increases during the acclimation of wheat plants in the vegetative stage, but decreases after the transition from vegetative to reproductive apices, the time point when *VRN1* transcript levels increase. Near-isogenic lines for the *VRN1* gene carrying the recessive *vrn1* allele (winter growth habit) can tolerate 11 °C lower freezing temperatures than lines carrying the dominant *Vrn1* allele (spring growth habit). Similarly, spring lines grown under short days, which down-regulates the *VRN1* transcript levels, can tolerate 8.5 °C lower temperatures than the same lines cultivated under long days. Based on these results Limin and Fowler [27] concluded that the association between freezing tolerance and winter growth habit can be explained by pleiotropic effects of the *VRN-A1* locus.

The previous hypothesis is supported by the negative correlation observed between the abundance of *VRN1* transcripts and the ability of *COR* transcripts and proteins to accumulate in response to cold [6,64,79]. In addition to the down-regulation of several *COR* genes, the presence of dominant *Vrn1* alleles seems to affect the regulation of some *CBF* genes. *TaCBF2* is down-regulated in spring wheat isogenic lines expressing dominant *Vrn1* alleles relative to near-isogenic winter lines [64] and several other *CBF* genes have been reported to have lower transcript levels in spring relative to winter common wheat varieties [63]. This negative correlation has been also observed in barley, where double-haploid lines carrying recessive *vrn-H1* alleles had higher *CBF* transcript levels than those carrying dominant *Vrn-H1* alleles [28]. In addition, when these lines were grown under short day conditions (reduced *VRN-H1* levels) *CBF* transcript levels were about two-fold higher than when the same lines were grown under long day [28]. Based on these results it is tempting to speculate that the temperate cereals have evolved the ability to use the presence of VRN1 in the leaves as a signal to down-regulate the frost/cold-tolerance genes (Fig. 1). This hypothesis does not necessarily imply a direct interaction between *VRN1* and the *CBF* or *COR* genes, as several genes are up- or down-regulated after the expression of *VRN1* in the leaves and can act as intermediaries in such interaction.

A better understanding of the molecular mechanisms involved in the association between the up-regulation of *VRN1* and the

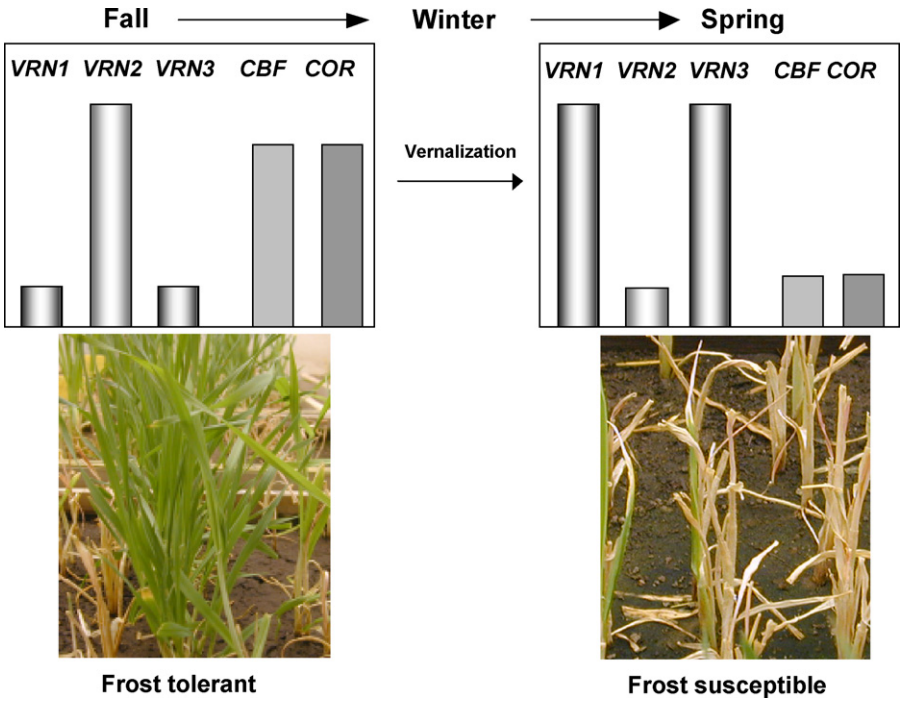

Fig. 4. Schematic representation of *VRN1* (meristem identity), *VRN2* (flowering repressor) and *VRN3* (flowering promoter) transcript levels during fall and spring, and their potential effect on the ability of *CBF* and *COR* genes to respond to cold temperatures. Individual genes are in different scales, so comparisons among genes are not valid. In the fall, the apical meristem is in a vegetative stage. After vernalization and induction of *VRN1*, the apex transitions to the double ridge stage that marks the beginning of the reproductive development. This transition is correlated with a decrease in frost tolerance. Pictures below each season correspond to frost tested wheat plants (−10 °C) before and after the transition to the reproductive phase.

down-regulation of the *COR* genes, will be necessary to determine if it is possible to uncouple these two mechanisms. Even though this may result in improved frost tolerance in spring varieties, it would also have a cost associated with the induction of a large number of cold-regulated genes when they are not necessary.

8. Conclusions and future directions

The down-regulation of the *COR* genes by *VRN1* can explain how plants differentiate mild cold temperatures in the fall from similar temperatures in the spring (Fig. 4). This distinction is important because mild cold temperatures in the fall preclude future frost events, whereas that is not the case for similar temperatures in the spring. In the fall, when *VRN1* is transcribed at low basal levels in the leaves, a mild cold temperature will result in the normal up-regulation of the *CBF*-regulon and the initiation of the acclimation process for the winter. In contrast, the same mild cold temperatures during the spring, when *VRN1* and its downstream target genes are up-regulated in the leaves, will not result in the unnecessary up-regulation of the *COR* genes. According to this hypothesis, the *VRN1* gene or one of its downstream targets in the leaves (question sign in Fig. 1) are responsible for the reduced responsiveness of the *CBF* and *COR* genes to cold. Transgenic or mutant lines for *VRN1* will be required for a final validation of this hypothesis.

In addition, the generation of mutants for the different *CBF* genes would be useful to investigate their individual roles in initial rates of cold acclimation and threshold induction temperatures of *COR* genes. However, functional redundancy between some of the *CBF* genes may complicate the interpretation of the effect of individual mutations. The tandem organization of these genes will also limit the possibility to use crosses to combine mutations in different *CBF* genes into double or triple mutants. RNAi strategies may be used to simultaneously down-regulate several *CBF* genes and avoid the limitations imposed by gene redundancy (for a review of RNAi in wheat see [80]). The functional characterization of the different *CBF* genes and alleles, and the generation of improved haplotypes combining optimum *CBF* alleles may provide a useful tool to enhance frost tolerance and hardiness in wheat and barley breeding programs.

Acknowledgements

This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, project number 2008-01010, and by the Hungarian, Plant Resource" OMFB—000515/2007 (2006–2009) German–Hungarian Research Collaboration and the Hungarian Research Fund 'OTKA' No. K68894.

References

[1] D.B. Fowler, A.E. Limin, Breeding for winter hardiness in cereals, Acta Agronomica Hungarica 45 (1997) 301–309.
[2] J.L. Reinheimer, A.R. Barr, J.K. Eglinton, QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.), Theor. Appl. Genet. 109 (2004) 1267–1274.
[3] A.E. Limin, D.B. Fowler, Breeding for cold hardiness in winter-wheat—problems progress and alien gene-expression, Field Crop Res. 27 (1991) 201–218.
[4] A. Turner, J. Beales, S. Faure, R.P. Dunford, D.A. Laurie, The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley, Science 310 (2005) 1031–1034.
[5] J. Beales, A. Turner, S. Griffiths, J.W. Snape, D.A. Laurie, A pseudo-response regulator is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.), Theor. Appl. Genet. 115 (2007) 721–733.
[6] J. Danyluk, N.A. Kane, G. Breton, A.E. Limin, D.B. Fowler, F. Sarhan, *TaVRT-1*, a putative transcription factor associated with vegetative to reproductive transition in cereals, Plant Physiol. 132 (2003) 1849–1860.
[7] B. Trevaskis, D.J. Bagnall, M.H. Ellis, W.J. Peacock, E.S. Dennis, MADS box genes control vernalization-induced flowering in cereals, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 13099–13104.

[8] L. Yan, D. Fu, C. Li, A. Blechl, G. Tranquilli, M. Bonafede, A. Sanchez, M. Valarik, J. Dubcovsky, The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 19581–19586.
[9] L. Yan, A. Loukoianov, A. Blechl, G. Tranquilli, W. Ramakrishna, P. SanMiguel, J.L. Bennetzen, V. Echenique, J. Dubcovsky, The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization, Science 303 (2004) 1640–1644.
[10] L. Yan, A. Loukoianov, G. Tranquilli, M. Helguera, T. Fahima, J. Dubcovsky, Positional cloning of wheat vernalization gene *VRN1*, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 6263–6268.
[11] L. Yan, M. Helguera, K. Kato, S. Fukuyama, J. Sherman, J. Dubcovsky, Allelic variation at the *VRN-1* promoter region in polyploid wheat, Theor. Appl. Genet. 109 (2004) 1677–1686.
[12] B. Pidal, L. Yan, D. Fu, F. Zhang, G. Tranquilli, J. Dubcovsky, The *CArG*-box in the promoter region of wheat vernalization gene *VRN1* is not necessary to mediate the vernalization response. J. Hered., in press.
[13] D. Fu, P. Szücs, L. Yan, M. Helguera, J. Skinner, P. Hayes, J. Dubcovsky, Large deletions in the first intron of the *VRN-1* vernalization gene are associated with spring growth habit in barley and polyploid wheat, Mol. Genet. Genomics 273 (2005) 54–65.
[14] J. vonZitzewitz, P. Szücs, J. Dubcovsky, L. Yan, E. Francia, N. Pecchioni, A. Casas, T.H.H. Chen, P.M. Hayes, J.S. Skinner, Molecular and structural characterization of barley vernalization genes, Plant Mol. Biol. 59 (2005) 449–467.
[15] J. Cockram, E. Chiapparino, S.A. Taylor, K. Stamati, P. Donini, D.A. Laurie, D.M. O'Sullivan, Haplotype analysis of vernalization loci in European barley germplasm reveals novel *VRN-H1* alleles and a predominant winter *VRN-H1/VRN-H2* multi-locus haplotype, Theor. Appl. Genet. 115 (2007) 993–1001.
[16] A.F. Stelmakh, Growth habit in common wheat (*Triticum aestivum* L. em Thell.), Euphytica 36 (1987) 513–519.
[17] N. Shitsukawa, C. Ikari, S. Shimada, S. Kitagawa, K. Sakamoto, H. Saito, H. Ryuto, N. Fukunishi, T. Abe, S. Takumi, S. Nasuda, K. Murai, The einkorn wheat (*Triticum monococcum*) mutant, *maintained vegetative phase*, is caused by a deletion in the *VRN1* gene, Genes Genet. Syst. 82 (2007) 167–170.
[18] C.N. Law, A.J. Worland, B. Giorgi, Genetic-control of ear-emergence time by chromosomes-5A and chromosome-5D of wheat, Heredity 36 (1976) 49–58.
[19] J. Dubcovsky, D. Lijavetzky, L. Appendino, G. Tranquilli, Comparative RFLP mapping of *Triticum monococcum* genes controlling vernalization requirement, Theor. Appl. Genet. 97 (1998) 968–975.
[20] D.A. Laurie, N. Pratchett, J.H. Bezant, J.W. Snape, RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter × spring barley *Hordeum vulgare* (L.) cross, Genome 38 (1995) 575–585.
[21] G. Galiba, S.A. Quarrie, J. Sutka, A. Morgounov, RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat, Theor. Appl. Genet. 90 (1995) 1174–1179.
[22] P.M. Hayes, T. Blake, T.H.H. Chen, S. Tragoonrung, F. Chen, A. Pan, B. Liu, Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winterhardiness, Genome 36 (1993) 66–71.
[23] J. Sutka, G. Galiba, A. Vagujfalvi, B.S. Gill, J.W. Snape, Physical mapping of the *Vrn-A1* and *Fr1* genes on chromosome 5A of wheat using deletion lines, Theor. Appl. Genet. 99 (1999) 199–202.
[24] J. Sutka, J.W. Snape, Location of a gene for frost resistance on chromosome 5A of wheat, Euphytica 42 (1989) 41–44.
[25] E. Francia, F. Rizza, L. Cattivelli, A.M. Stanca, G. Galiba, B. Tóth, P.M. Hayes, J.S. Skinner, N. Pecchioni, Two loci on chromosome 5H determine low-temperature tolerance in a Nure (winter) × Tremois (spring) barley map, Theor. Appl. Genet. 108 (2004) 670–680.
[26] A.E. Limin, D.B. Fowler, Developmental traits affecting low-temperature tolerance response in near-isogenic lines for the vernalization locus *Vrn-A1* in wheat (*Triticum aestivum* L. em Thell), Ann. Bot. 89 (2002) 579–585.
[27] A.E. Limin, D.B. Fowler, Low-temperature tolerance and genetic potential in wheat (*Triticum aestivum* L.): response to photoperiod, vernalization, and plant development, Planta 224 (2006) 360–366.
[28] E.J. Stockinger, J.S. Skinner, K.G. Gardner, E. Francia, N. Pecchioni, Expression levels of barley *Cbf* genes at the *Frost resistance-H2* locus are dependent upon alleles at *Fr-H1* and *Fr-H2*, Plant J. 51 (2007) 308–321.
[29] A. Vágújfalvi, G. Galiba, L. Cattivelli, J. Dubcovsky, The cold regulated transcriptional activator *Cbf3* is linked to the frost-tolerance gene *Fr-A2* on wheat chromosome 5A, Mol. Genet. Genomics 269 (2003) 60–67.
[30] C. Li, J. Dubcovsky, Wheat FT protein regulates *VRN1* transcription through interactions with FDL2, Plant J. 55 (2008) 543–554.
[31] A. Loukoianov, L. Yan, A. Blechl, A. Sanchez, J. Dubcovsky, Regulation of *VRN-1* vernalization genes in normal and transgenic polyploid wheat, Plant Physiol. 138 (2005) 2364–2373.
[32] B. Trevaskis, M.N. Hemming, W.J. Peacock, E.S. Dennis, *HvVRN2* responds to daylength, whereas *HvVRN1* is regulated by vernalization and developmental status, Plant Physiol. 140 (2006) 1397–1405.
[33] B. Trevaskis, M.N. Hemming, E.S. Dennis, W.J. Peacock, The molecular basis of vernalization-induced flowering in cereals, Trends Plant Sci. 12 (2007) 352–357.
[34] M.A. Hughes, M.A. Dunn, The molecular biology of plant acclimation to low temperature, J. Exp. Bot. 47 (1996) 291–305.
[35] R.S. Pearce, Molecular analysis of acclimation to cold, Plant Growth Regul. 29 (1999) 47–76.
[36] S. Fowler, M.F. Thomashow, Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the *CBF* cold response pathway, Plant Cell 14 (2002) 1675–1690.
[37] C.L. Guy, Cold-acclimation and freezing stress tolerance—role of protein-metabolism, Annu. Rev. Plant Physiol. Plant Mol. Biol. 41 (1990) 187–223.
[38] C. Crosatti, C. Soncini, A.M. Stanca, L. Cattivelli, The accumulation of a cold-regulated chloroplastic protein is light-dependent, Planta 196 (1995) 458–463.
[39] M. Rapacz, B. Wolanin, K. Hura, M. Tyrka, The effects of cold acclimation on photosynthetic apparatus and the expression of *COR14b* in four genotypes of barley (*Hordeum vulgare*) contrasting in their tolerance to freezing and high-light treatment in cold conditions, Ann. Bot. 101 (2008) 689–699.
[40] F. Sarhan, F. Ouellet, A. Vazquez-Tello, The wheat *wcs120* gene family. A useful model to understand the molecular genetics of freezing tolerance in cereals, Physiol. Plantarum 101 (1997) 439–445.
[41] A.K. Knox, C. Li, A. Vágújfalvi, G. Galiba, E.J. Stockinger, J. Dubcovsky, Identification of candidate *CBF* genes for the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*, Plant Mol. Biol. 67 (2008) 257–270.
[42] R.C. Jansen, J.P. Nap, Genetical genomics: the added value from segregation, Trends Genet. 17 (2001) 388–391.
[43] M. Båga, S.V. Chodaparambil, A.E. Limin, M. Pecar, D.B. Fowler, R.N. Chibbar, Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat, Funct. Integr. Genomics 7 (2007) 53–68.
[44] A. Vágújfalvi, A. Aprile, A. Miller, J. Dubcovsky, G. Delugu, G. Galiba, L. Cattivelli, The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat, Mol. Genet. Genomics 274 (2005) 506–514.
[45] B. Tóth, G. Galiba, E. Fehér, J. Sutka, J.W. Snape, Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat, Theor. Appl. Genet. 107 (2003) 509–514.
[46] A.K. Miller, G. Galiba, J. Dubcovsky, A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*, Mol. Genet. Genomics 275 (2006) 193–203.
[47] A. Tondelli, E. Francia, D. Barabaschi, A. Aprile, J.S. Skinner, E.J. Stockinger, A.M. Stanca, N. Pecchioni, Mapping regulatory genes as candidates for cold and drought stress tolerance in barley, Theor. Appl. Genet. 112 (2006) 445–454.
[48] J. Skinner, P. Szücs, J. von Zitzewitz, L. Marquez-Cedillo, T. Filichkin, E.J. Stockinger, M.F. Thomashow, T.H.H. Chen, P.M. Hayes, Mapping of barley homologs to genes that regulate low temperature tolerance in Arabidopsis, Theor. Appl. Genet. 112 (2006) 832–842.
[49] E.J. Stockinger, S.J. Gilmour, M.F. Thomashow, Arabidopsis thaliana *CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 1035–1040.
[50] Q. Liu, M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, K. Shinozaki, Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis, Plant Cell 10 (1998) 1391–1406.
[51] J.S. Skinner, J. von Zitzewitz, P. Szücs, L. Marquez-Cedillo, T. Filichkin, K. Amundsen, E.J. Stockinger, M.F. Thomashow, T.H.H. Chen, P.M. Hayes, Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley, Plant Mol. Biol. 59 (2005) 533–551.
[52] G.P. Xue, The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature, Plant J. 33 (2003) 373–383.
[53] H.A. Van Buskirk, M.F. Thomashow, Arabidopsis transcription factors regulating cold acclimation, Physiol. Plantarum 126 (2006) 72–80.
[54] K. Yamaguchi-Shinozaki, K. Shinozaki, Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses, Annu. Rev. Plant Biol. 57 (2006) 781–803.
[55] D.G. Zarka, J.T. Vogel, D. Cook, M.F. Thomashow, Cold induction of Arabidopsis *CBF* genes involves multiple *ICE* (inducer of *CBF* expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature, Plant Physiol. 133 (2003) 910–918.
[56] D. Cook, S. Fowler, O. Fiehn, M.F. Thomashow, A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 15243–15248.
[57] K.R. Jaglo-Ottosen, S.J. Gilmour, D.G. Zarka, O. Schabenberger, M.F. Thomashow, Arabidopsis *CBF1* overexpression induces *COR* genes and enhances freezing tolerance, Science 280 (1998) 104–106.
[58] Y. Ito, K. Katsura, K. Maruyama, T. Taji, M. Kobayashi, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice, Plant Cell Physiol. 47 (2006) 141–153.
[59] K.R. Jaglo, S. Kleff, K.L. Amundsen, X. Zhang, V. Haake, J.Z. Zhang, T. Deits, M.F. Thomashow, Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species, Plant Physiol. 127 (2001) 910–917.
[60] D.-W. Choi, E.M. Rodriguez, T.J. Close, Barley *Cbf3* gene identification, expression pattern, and map location, Plant Physiol. 129 (2002) 1–7.
[61] J.G. Dubouzet, Y. Sakuma, Y. Ito, M. Kasuga, E.G. Dubouzet, S. Miura, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression, Plant J. 33 (2003) 751–763.
[62] D. Kizis, M. Pages, Maize DRE-binding proteins DBF1 and DBF2 are involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway, Plant J. 30 (2002) 679–689.

[63] M. Badawi, J. Danyluk, B. Boucho, M. Houde, F. Sarhan, The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBF*s, Mol. Genet. Genomics 277 (2007) 533–554.
[64] F. Kobayashi, S. Takumi, S. Kume, M. Ishibashi, R. Ohno, K. Murai, C. Nakamura, Regulation by *Vrn-1/Fr-1* chromosomal intervals of CBF-mediated *Cor/Lea* gene expression and freezing tolerance in common wheat, J. Exp. Bot. 56 (2005) 887–895.
[65] E. Francia, D. Barabaschi, A. Tondelli, G. Laidò, F. Rizza, A.M. Stanca, M. Busconi, C. Fogher, E.J. Stockinger, N. Pecchioni, Fine mapping of a *HvCBF* gene cluster at the frost resistance locus *Fr-H2* in barley, Theor. Appl. Genet. 115 (2007) 1083–1091.
[66] A.L. Brule-Babel, D.B. Fowler, Use of controlled environments for winter cereal cold hardiness evaluation - controlled freeze tests and tissue water-content as prediction tests, Can. J. Plant Sci. 69 (1989) 355–366.
[67] D.B. Fowler, R.J. Carles, Growth, development, and cold tolerance of fall-acclimated cereal grains, Crop Sci. 19 (1979) 915–922.
[68] A. Vágújfalvi, C. Crosatti, G. Galiba, J. Dubcovsky, L. Cattivelli, Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *cor14b* gene in frost-tolerant and frost-sensitive genotypes, Mol. Gen. Genet. 263 (2000) 194–200.
[69] D.B. Fowler, Cold acclimation threshold induction temperatures in cereals, Crop Sci. 48 (2008) 1147–1154.
[70] C. Crosatti, E. Nevo, A.M. Stanca, L. Cattivelli, Genetic analysis of the accumulation of COR14 proteins in wild (*Hordeum spontaneum*) and cultivated (*Hordeum vulgare*) barley, Theor. Appl. Genet. 93 (1996) 975–981.
[71] C. Crosatti, D. Pagani, L. Cattivelli, A.M. Stanca, F. Rizza, Effects of growth stage and hardening conditions on the association between frost resistance and the expression of the cold-induced protein COR14b in barley, Env. Exp. Bot. 62 (2008) 93–100.
[72] A. Vágújfalvi, I. Kerepesi, G. Galiba, T. Tischner, J. Sutka, Frost hardiness depending on carbohydrate changes during cold acclimation in wheat, Plant Sci. 144 (1999) 85–92.
[73] J. Schmitz, R. Franzen, T.H. Ngyuen, F. Garcia-Maroto, C. Pozzi, F. Salamini, W. Rohde, Cloning, mapping and expression analysis of barley MADS-box genes, Plant Mol. Biol. 42 (2000) 899–913.
[74] J.C. Preston, E.A. Kellogg, Reconstructing the evolutionary history of paralogous *APETALA1/FRUITFULL*-like genes in grasses (Poaceae), Genetics 174 (2006) 421–437.
[75] M. Abe, Y. Kobayashi, S. Yamamoto, Y. Daimon, A. Yamaguchi, Y. Ikeda, H. Ichinoki, M. Notaguchi, K. Goto, T. Araki, FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex, Science 309 (2005) 1052–1056.
[76] P.A. Wigge, M.C. Kim, K.E. Jaeger, W. Busch, M. Schmid, J.U. Lohmann, D. Weigel, Integration of spatial and temporal information during floral induction in Arabidopsis, Science 309 (2005) 1056–1059.
[77] K. Petersen, T. Didion, C.H. Andersen, K.K. Nielsen, MADS-box genes from perennial ryegrass differentially expressed during transition from vegetative to reproductive growth, J. Plant Physiol. 161 (2004) 439–447.
[78] J.C. Preston, E.A. Kellogg, Discrete developmental roles for temperate cereal grass *VRN1/FUL*-like genes in flowering competency and the transition to flowering, Plant Physiol. 146 (2008) 265–276.
[79] D.B. Fowler, L.P. Chauvin, A.E. Limin, F. Sarhan, The regulatory role of vernalization in the expression of low-temperature-induced genes in wheat and rye, Theor. Appl. Genet. 93 (1996) 554–559.
[80] D. Fu, C. Uauy, A. Blechl, J. Dubcovsky, RNA interference for wheat functional gene analysis, Transgenic Res. 16 (2007) 689–701.