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Permalink
https://escholarship.org/uc/item/7xq5s770

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
Review

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

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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.

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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,
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 gene 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 [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 VrnI 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...
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 dehydron 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-repeats (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 Pooidaeae, 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
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 hardness, 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].

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 TmCBF12 protein from the frost tolerant 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 or initiate the cold acclimation process.

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.

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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 from Cheyenne into Chinese Spring results in a significant correlation observed between the abundance of VRNI 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 Vrn1 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

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 (API) 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 API [76]. The absence of FD in Arabidopsis leaves explains the limited expression of API in this tissue, as demonstrated by high API 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 Vrn1 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
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 will 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.

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