Current Topics in Plant Biology

Review

Frost tolerance in cereals - from a molecular point of view

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ABSTRACT

Plant frost tolerance is a complex quantitative trait, influenced by the genotype, the physiological state and the environment. The molecular aspects of the regulation of the genes involved in cold stress adaptation have been most extensively studied in *Arabidopsis*, but an increasing amount of data is now available in cereals. This review summarises recent results achieved on the genetics of frost tolerance in bread wheat, einkorn and barley. In particular, it focuses on the genetics, expression and regulation of the *Cbf* transcription factors in cereals, which are key regulators of frost tolerance. As a consequence of this work over the last decade, candidate genes (*Cbf* genes) for frost tolerance have been suggested for the first time. The results from our research groups are discussed in the present paper and compared to the findings of other authors working in this area.

KEYWORDS: cereal, frost tolerance, gene regulation, *Cbf*

INTRODUCTION

Understanding the complex molecular mechanism of frost tolerance is a challenge but the increasing number of experimental data, published by molecular biologists and geneticists, is gradually making the picture clearer. We are becoming closer and closer to being able to explain why one plant is frost tolerant while the other is not, what kinds of genes are involved in this process and how these genes are regulated. Frost tolerance can be defined as the ability of a plant to survive frost effects without any considerable damage. From a genetic point of view, plant frost tolerance is a quantitative trait, influenced by the physiological state of the plant, by environmental factors and by the genetic background as well. The plants, studied most exhaustively on the subject, are *Arabidopsis thaliana* and cereals. This review focuses on recent results achieved in the regulation of frost tolerance in temperate cereals.

Genetics of frost tolerance - early years

Studies on the inheritance of frost resistance in wheat started at the beginning of the 20th century. The first conclusion was that this trait is controlled by polygenes. The next step was the description of gene interactions. Diallel crosses clarified dominant and recessive interactions. The study of special genetic materials, such as monosomic, disomic and single chromosome substitution lines, made it possible to identify the chromosomes involved in the genetic control of frost tolerance in wheat. The overall conclusion was that at least 11 of the 21 pairs of chromosomes are involved in the genetic control of wheat frost tolerance (a detailed list of relevant publications was published by Sutka and Veisz [1]). Substituting single chromosomes from the frost-tolerant wheat variety Cheyenne into the

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spring type Chinese Spring recipient proved the central role of the homeologous group 5 chromosomes in wheat [2].

QTLs for frost tolerance

In order to localize a gene on a chromosome, more specific genetic material is required. Several mapping populations to map frost tolerance genes, localised on different chromosomes, were developed. A gene for frost resistance on chromosome 5A of wheat was located using single chromosome recombinant lines from a cross between the substitution line Hobbit/*Triticum spelta* 5A and the variety Hobbit. In this sample of recombinant lines the locus for frost resistance, *Fr-A1* (formerly: *Fr1*), proved to be completely linked on the long arm of chromosome 5A to the locus *Vrn-A1* (formerly: *Vrn1*) controlling vernalization requirement [3]. However mapping data for a population originating from a cross between two single chromosome substitution lines (Chinese Spring/*Triticum spelta* 5A x Chinese Spring/Cheyenne 5A), showed that although the two loci are tightly linked (2 cM), they are nevertheless separable [4]. This result was confirmed by physical mapping using Chinese Spring deletion lines. The *Vrn-A1* gene was localised between the breakpoints 0.68 and 0.78, while the frost resistance gene *Fr-A1* was mapped between the deletion breakpoints 0.67 and 0.68 (Fig. 1) [5].

It is still an open question, weather the frost tolerance locus *Fr-A1* really exists or the frost tolerance QTL mapped on the long arm of chromosome 5A in wheat is a pleiotropic effect of the vernalization gene, *Vrn-A1*. Genetic and physical mapping data [3, 4], detailed above, suggest the existence of two separable loci. On the other hand, the analysis of near-isogenic lines (NIL), differing for the *Vrn-A1* (*vrn*-A1 or *Vrn-A1* allele) allele, pointed out that the repression of the vernalization gene by short day photoperiod led to an increased level of low temperature tolerance in the spring habit NIL [18]. The authors concluded that the pleiotropic effect of the vernalization locus explains the higher level of tolerance, irrespective of the *Fr-A1* or the *Fr-A2* loci. The experimental data published up to now are insufficient to solve this problem; to answer this

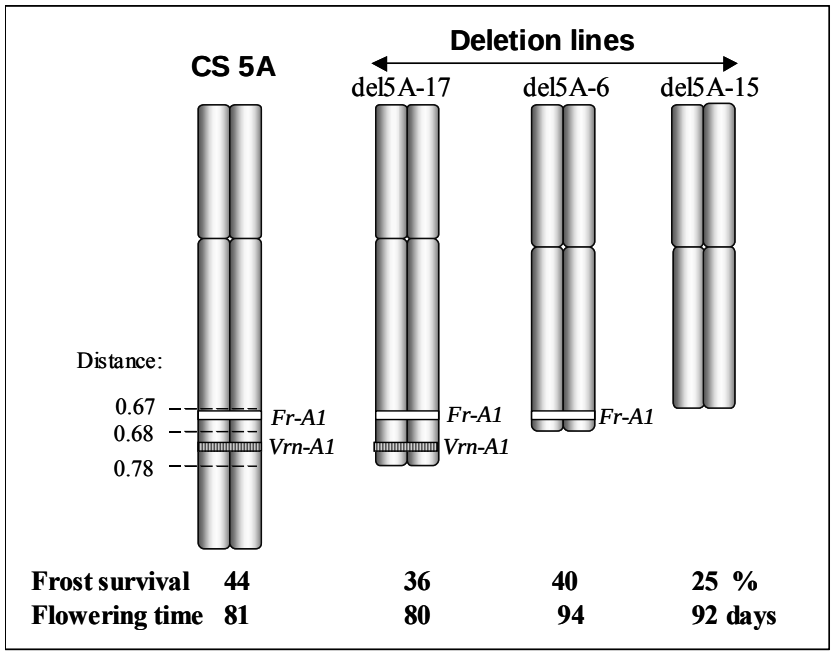

Fig. 1. Schematic representation of Chinese Spring chromosome 5A and three 5A deletion lines, illustrating the physical mapping of *Fr-A1* and *Vrn-A1* genes. Distances are given as Fraction Length (FL).

question further studies are necessary. To clarify the relationship between frost tolerance and the vernalization locus a new fine mapping population is being developed by selecting recombinant lines between the *Fr-A1* and *Vrn-A1* loci in bread wheat. Tests on frost tolerance and vernalization requirement of these recombinant lines may confirm the existence of the *Fr-A1* locus.

It is a well-established fact that the main frost tolerance QTL is located in the *Vrn-A1- Fr-A1* interval in bread wheat [6]. However, there is not only one locus determining cold hardiness on chromosome 5A. As early as 1990 Roberts [7] suggested that at least two loci on chromosome 5A had a major effect on cold hardiness. One of these loci is closely linked to the *Vrn-A1 - Fr-A1* region. The other locus, affecting cold hardiness, is linked to a locus with a major effect on the length of the leaves produced under cold-hardening conditions. The segregation data suggested that this gene and the *Vrn1* gene were not tightly linked, but more than 10 years passed before the presence of *Fr-A2* QTL was verified.

The use of the diploid wheat *T. monococcum* (einkorn wheat) facilitated the discovery of *Fr-A^{m}2* [8]. Chromosome 5A from einkorn is collinear with chromosome 5A from wheat [9, 10], facilitating comparison of the results of the present study with those of previous studies on frost tolerance in polyploid wheats. The major gene for frost tolerance, *Fr-A1*, was mapped in hexaploid wheat on the long arm of chromosome 5A – closely linked to RFLP marker *Xwg644* [4, 5]. The same marker was 30 cM proximal to the *Fr-A^{m}2* frost tolerance locus in the *T. monococcum* mapping population used in this study, using parental lines DV92 and G3116 that to not differ at the *Vrn-A^{m}1-Fr-A^{m}1* locus. This lack of segregation at the *Fr-A1* locus made the QTL analysis more effective in detecting the new *Fr-A^{m}2* locus. The new frost tolerance locus *Fr-A^{m}2* was mapped in diploid wheat 30 cM proximally to RFLP marker *Xwg644*, which is known to be tightly linked to *Vrn-A1-Fr-A1* region in polyploid wheat. Although no frost tolerance locus has been described before in the *Fr-A2* region in polyploid wheats, it is possible that allelic variation at the *Fr-A2* locus might have been obscured by simultaneous variation at a linked *Fr-A1* locus.

In the case of bread wheat a locus (*Rcg1*) controlling cold-regulated gene expression was identified in 2000 [11], - for details see later -, tightly linked to the *Xpsr911* RFLP marker, which was later found to be in the same region as the *Fr-A^{m}2* in diploid wheat. The identity between this QTL and *Fr-A2* was validated in 2005 [12]. This QTL is in the same location as the QTL mapped by Båga *et al.* [13], 46 cM proximally to the *Vrn-A1* locus.

It has long been known that the homoeologous group 5 chromosomes of hexaploid wheat also carry loci both for frost tolerance and vernalization. Recently, frost tolerance and vernalization loci were mapped on chromosomes 5B and 5D in bread wheat. The same chromosome region where *Fr-A2* was located on 5A was found to affect frost tolerance on chromosome 5B of bread wheat [14]. The 5B locus, originally published as *Fr-B1*, was re-designated as *Fr-B2* in the 2004 supplement of the Catalogue of Gene Symbols for Wheat [15]. The QTL for frost tolerance on the long arm of chromosome 5D [16] was mapped at a location intermediate between *VRN-D1* and the 5D chromosome region orthologous to *Fr-2*. It was indicated that this population might have a bimodal distribution for the frost response, suggesting the possibility that the observed QTL might be a combination of the effects of two loci on chromosome 5D. The relative positions of the loci mentioned above are summarised in Fig. 2.

The orthologous relationships of these genes were mapped in barley. Two QTLs for frost tolerance were mapped on the long arm of chromosome 5H [17]. A comparison of molecular markers, mapped for both species indicated that the position of the first frost tolerance locus was homologous with the *Fr-2* locus described in bread wheat, while the second one corresponded to the *Vrn-1-Fr-1* region in wheat. A QTL for vernalization requirement was also mapped on 5H, and this *Vrn-H1* locus was found to be completely linked to the *Fr-H1* locus [17].

Cbf genes

The *Cbf* genes, first described in *Arabidopsis* [19, 20], code for transcription factors, which bind to the conserved core sequence CCGAC [C-repeat

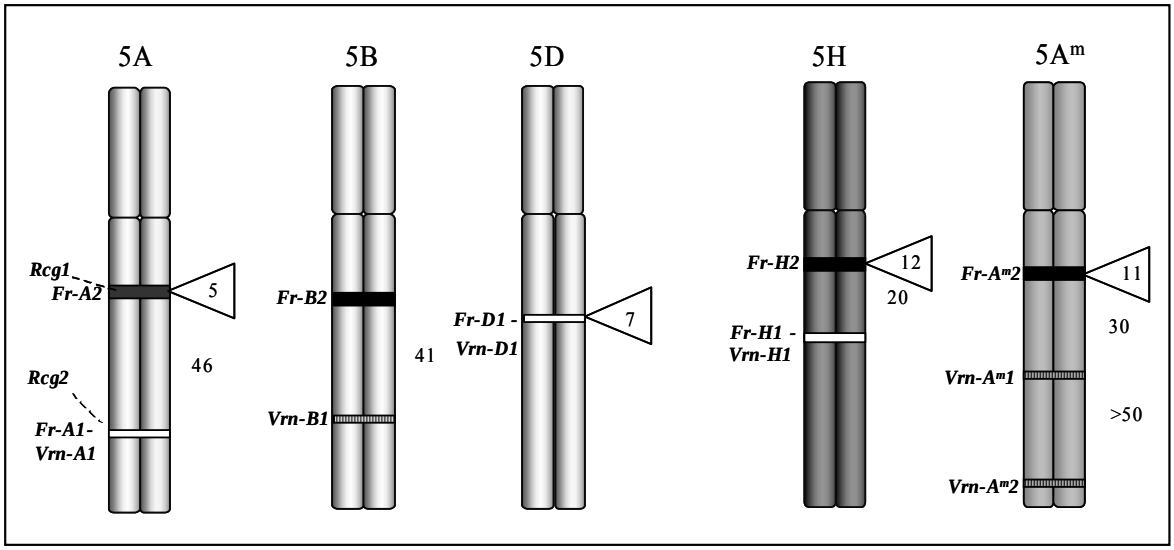

Fig. 2. Relative positions of frost tolerance (*Fr*) and vernalization (*Vrn*) loci mapped on the homeologous chromosome 5 in bread wheat, on barley chromosome 5H and on $5A^m$ of einkorn. Numbers are the distances between the loci in cM, while the numbers in triangles represent the number of *Cbf* genes mapped in the given position. Since the genetic control of frost tolerance QTLs mapped on the distal part of the long arms of wheat homeologous chromosomes 5 and on chromosome 5H in barley is still an open question (detailed in the text) the *Fr-A1 - Vrn-A1* is tentatively presented as a single region. The two regulatory regions of *Cor14b* gene expression (*Rcg1*, *Rcg2*) identified on 5A chromosome are also indicated.

(CRT) dehydration element (DRE)], found in many cold-regulated (*Cor*) genes [19]. The *Cbf* genes have been described by two teams independently, so each gene has two names: *CBF1* (*DREB1B*), *CBF2* (*DREB1C*) and *CBF3* (*DREB1A*). The *DREB1* genes involved in low temperature stress tolerance, are induced rapidly and transiently, and are part of the ABA-independent pathway; the *DREB2* genes are induced by osmotic and salinity stress. Since several *DREB1* genes are induced not only by cold but also by osmotic or salinity stress, cross-talk is assumed to exist between the two groups. *Cbf* homologous genes have been found in many dicot and monocot plant species including einkorn [21], bread wheat [12, 13, 22], barley [17, 23, 24], maize [25], rye [26] and rice [27]. The *DREB/Cbf* genes belong to the Apetala 2/ethylene responsive element binding protein (*AP2/EREBP*) family of transcription factors (reviewed by Van Buskirk and Thomashow [28], and by Yamaguchi-Shinozaki and Shinozaki [29]). A detailed structural analysis of the *DREB* genes was presented by Agarwal *et al.* [30]. The phylogenetic characterization and grouping of barley *HvCbf* genes is presented by Skinner *et al.* [31], while a detailed cluster analysis of CBF proteins from wheat, einkorn, barley, rice and *Arabidopsis* was published by Miller *et al.* [21]. The most recent classification of *Cbf* genes was discussed by Badawi *et al.* [22], who classified the *Poaceae Cbf*s into 10 groups, 6 of which are only found in the *Pooideae.*

Cbfs are clustered in cereals on chromosome 5

The cold-inducible *Cbf* genes in *Arabidopsis*, namely *CBF1* (*DREB1B*), *CBF2* (*DREB1C*) and *CBF3* (*DREB1A*), are clustered in a tandem arrangement on chromosome 4 [32].

To determine the chromosomal localization of *Cbf* genes in einkorn the *T. monococcum* BAC library was first screened with two *Cbf* probes isolated from barley. Twenty positive clones were identified, which were organised into seven contigs. Based on the sequence, CAPS markers were designed for each *Cbf* gene and mapping was performed on two F_2 einkorn mapping populations. The locus of the*TmCbf5* gene was mapped on chromosome

$7A^m$, and that of *TmCbf18* on $6A^m$. The remaining 11 genes were mapped on chromosome $5A^m$, at the *Fr-Am2* frost tolerance locus. The genes were found to be tightly clustered: all 11 genes were localised within a 0.8 cM region [21]. In hexaploid wheat, a total of 37 *Cbf* genes were identified and grouped into at least 15 different orthologous groups [22]. The chromosomal localization was determined for 27 genes, 26 of which were localized on the homeologous group 5 chromosomes. The most precise determination was achieved by studying 5AL and 5DL deletion lines. It was found that 5 genes could be localised in the same bins on chromosome 5A, and seven in the same bins on chromosome 5D [22]. It seems likely that these genes are also clustered, but more research will be required to confirm this. Twenty *Cbf* genes were identified in barley by Skinner *et al.* [24], 11 of which were found to be arranged in two clusters on the long arm of chromosome 5H (a chromosome homologous to wheat chromosome 5). These results correspond to the findings of another team working on barley, who found 2 clusters on chromosome 5H, containing one and six members of the *Cbf* gene family [33]. Similarly, four clustered *Cbf* genes were mapped [34] within 2.2 cM on chromosome 5 (LG5) of perennial ryegrass (*Lolium perenne*). A 10 kb region of rice chromosome 9, which is collinear with the *Cbf*-containing region of chromosome 5 in the *Triticeae*, includes three *Cbf* genes (*OsDREB1A*, *OsDREB1B* and *OsDREB1H*) [27, 31]. According to the mapping data presented above, the majority of the *Cbf* genes are clustered in the *Poaceae*; this could be simply a historical result of tandem duplication, but it also possible that the tight linkage between these transcription factors involved in the same regulatory process may confer an evolutionary advantage.

Expression of *Cbf* genes

The expression pattern of the *Cbf* genes in cereals was most widely studied by Skinner *et al.* [31] in barley and by Badawi *et al.* [22] in wheat.

All the *Cbf* genes studied so far are induced by at least one abiotic stress (drought, cold or salt stress) in barley. The different expression patterns and timing of the individual gene expression suggests that different sets of *Cbf* genes are involved in each stress response, though a certain level of interference exists. The majority of *Cbf* genes are induced rapidly and transiently, though two *HvCbf* genes showed delayed induction, and a high level of these transcripts was maintained for a longer period. This phenomenon might explain the maintenance of low temperature tolerance in over-wintering cereal. It was also suggested that the cold-induced expression of the genes was genotype dependent, with a higher level of induction in varieties tolerant of low temperature [31]. Quantitative Real Time RT-PCR experimental data [22] suggest that the expression level of the *Cbf* genes is correlated with the level of low temperature tolerance in five (out of six) *Poaceae* *Cbf* groups.

The duplication events of an ancestral *Cbf* gene (more than 50 million years ago) resulted in the clustered, tandem arrangement of the genes in the *Triticeae* [21, 24]. It is tempting to speculate that the amplification of the genes was maintained since it resulted in a higher level of frost tolerance. Winter-type genotypes have the genetic capacity to induce a higher level of these transcription factors, which regulate the effector genes responsible for cold acclimation and improved frost tolerance [22]. A circadian fluctuation of the *Cbf* transcript accumulation has been found in wheat [22] and in *Arabidopsis* [35]. The periodical daily accumulation of transcripts, without low temperature stimulus, is likely to be advantageous when a sudden drop in temperature occurs [22]. The previous study suggest that a sudden decrease in temperature leads to the fast increase in the binding capacity of transcription factors already accumulated in the cells, which rapidly promotes the transcription of cold-inducible genes.

The first candidate gene for frost tolerance is a *Cbf* gene in cereals

The relationship between the expression of *Cbf* genes and frost tolerance was first demonstrated in *Arabidopsis*. The constitutive overexpression of the *AtCbf1* gene increased both the level of *Cor* gene transcripts and the level of frost tolerance [36]. It was proved that *Cor* genes, especially *Cor15a,* enhance *in vivo* freezing tolerance, stabilizing the chloroplasts membranes in non-acclimated plants [37, 38]. Later it was proved

[39, 40] that the overexpression of all three *Arabidopsis Cbfs* increased frost tolerance, probably as a result of the increase detected in the level of osmotically active solutes, such as proline and carbohydrates, which act as cryoprotectants.

These results prompted us to study the relationship between the expression of *Cbf* genes, the accumulation of the cold-inducible gene *Cor14b* and the level of frost tolerance in cereals. *Cor14b*, like *Cor15a* in *Arabidopsis,* codes for a small protein (14 kDa), and is cold-inducible, the transcript being targeted to the chloroplast [41]. The study of single chromosome substitution lines clearly proved that the expression of the *Cor14b* gene was controlled by chromosome 5A in bread wheat. More exact localization was achieved using selected single chromosome recombinant lines. Two regions on the long arm of 5A were found to be responsible for differential accumulation, the first, *Rcg1* (regulator for *cor g*enes) linked to the locus for RFLP marker *Xpsr911*, and the second, *Rcg2* located between *Xpsr2021* (*ABA2*) and the *Fr-A1* loci [11]. The expression of the *Cor14b* gene was mapped in einkorn (*T. monococcum*) RIL (Recombinant Inbred Line) populations. A single QTL (LOD score: 3.5) was found, which was mapped with a peak at the locus of the RFLP marker *Xbcd508*, localised on the long arm of chromosome $5A^m$. A *Cbf* gene from barley, namely *HvCbf3*, was used as an RFLP probe to check whether any *Cbf* genes could be localised on the same chromosome. The successful mapping indicated that the probe mapped to the *Xbcd508* locus. Frost tolerance was also mapped using the same RIL population. The combined data of two independent frost tests showed a QTL with a LOD score of 8.9 with a confidence interval of 7 cM for a peak centred on the *Xbcd508* - *Cbf3* locus. This novel frost tolerance locus, designated *Fr-*A^m*2*, was localised 40 cM from the centromere and was shown by comparative mapping data to be 30 cM proximal to the vernalization locus *Vrn-A1*. Since a *Cbf* gene was mapped to the peak of this QTL, the expression of a *Cor* gene (*Cor14b*) was also tested and mapped as a QTL with a peak at the exact same position as *Cbf3* and the peak for frost tolerance. The *Cbf* gene was suggested as a candidate gene for frost tolerance [8]. The same results were found in barley: a *Cbf* gene, and QTLs for frost tolerance and *Cor14b* gene expression were mapped in the same position on the long arm of chromosome 5H. Comparative mapping data confirmed that the above-mentioned regions are orthologous in einkorn and barley [12].

Cbf genes and frost tolerance

Since the relationship between frost tolerance and the expression of *Cbf* genes had already been proved in *Arabidopsis*, several groups begun to characterize the expression of *Cbf* genes in bread wheat. As a first step, an unspecific *Cbf* probe (containing the *AP2-Cbf* signature conserved domain) was used for Northern analysis. Studying the expression of the *Cbf* genes in a set of Chinese Spring/Cheyenne single substitution lines, subjected to cold stress, clearly showed the key role of chromosome 5A in the regulation of these genes. Assuming, that the *Rcg1* and/or *Rcg2* region is likely to be involved in the regulation of *Cbf* gene expression, a selected set of single chromosome recombinant lines was studied by Northern analysis. The analysis of the parental alleles of the lines studied - in correlation to the transcript level - led to the conclusion that the *Rcg1* of chromosome 5A is the regulatory region for the *Cbf* genes (Fig. 3). This experiment also proved that the expression level was correlated with the level of frost tolerance. The use of an aspecific *Cbf* probe provided an overall view of the *Cbf* gene expression.

The next step was the characterization of the individual *Cbf* genes. Based on available sequence [21], gene-specific primers were designed to quantify the expression of individual *Cbf* genes by Quantitative RT-PCR (QRT-PCR). The results showed that the expression of 3 *Cbf* genes, *Cbf14, Cbf15* and *Cbf16* (formerly *1A*, *1C* and *7*, respectively) was higher in the frost resistant wheat genotypes subjected to cold stress (2°C). It was concluded that differences in expression were controlled by the *Rcg1* region of chromosome 5A [12]. These results were confirmed by Båga *et al.* [13]. The analysis of bread wheat NILs (Near Isogenic Lines) proved the existence of a second frost tolerance locus in the *Fr-A2* region (46 cM proximally to the *Vrn-A1* locus). They also mapped two *Cbf* genes at the peak of this QTL, namely *Cbf14* and *Cbf15*.

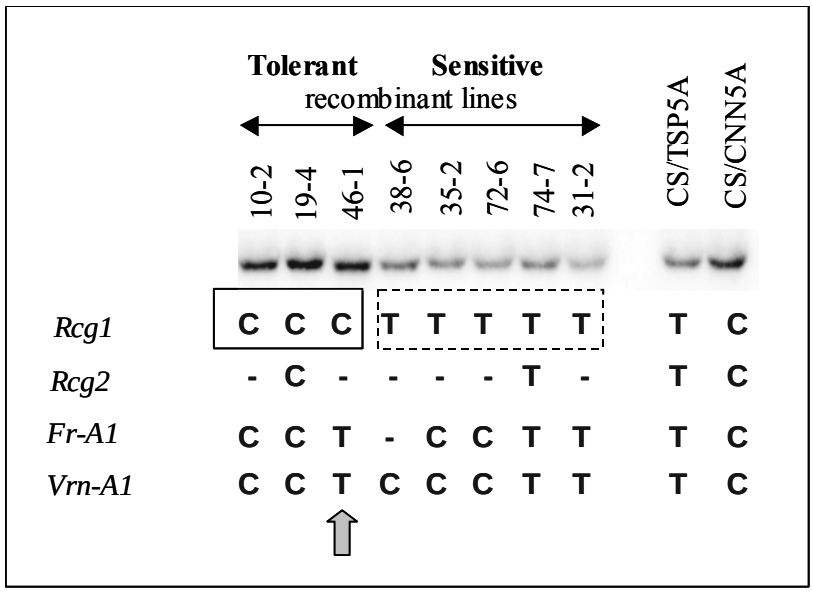

Fig. 3. Northern analysis of *Cbf* gene expression of the parental lines CS/TSP5A and CS/CNN5A, moreover some selected single chromosome recombinant lines. **C** represents the allele from the frost tolerant variety Cheyenne, while **T** from the sensitive *T. spelta*. All the lines showing high level of *Cbf* expression share the common feature: **C** allele at the *Rcg1* locus, while the lines with low expression carry **T** allele at this locus (boxed). The line 46-1 (arrow) has **T** allele at the frost tolerance locus *Fr-A1* and **C** at the *Rcg1* locus. Since this line showed an increased level of frost tolerance we presumed the presence of a second frost tolerance locus, *Fr-A2* in bread wheat and concluded, that the expression of *Cbf* genes is linked to the level of frost tolerance.

Direct proof of the involvement of the *Cbf* genes in frost tolerance could be obtained in several ways, one of which is based on RNA silencing. Unfortunately, RNA silencing appears to be inhibited at low temperature [42]. Another method, which has been successfully applied, is based on transformation. The transformation of the selected transcription factor under the control of an appropriate promoter may lead to an enhanced level of transcripts of the gene, and finally, to an increase in phenotypic phenomena such as abiotic stress tolerance. This method successfully proved the role of *Cbf* genes in the regulation of cold or frost tolerance in cereals. Because of methodological advantages, rice is the preferred species for transformation in cereals. Various *Cbf* genes isolated from *Arabidopsis* were transformed into rice plants. The *AtCbf1* (*DREB1b*) gene was introduced into rice driven by the maize ubiquitin promoter, and the induction of several cold-inducible genes was detected; however, no significant increase in frost tolerance was observed [43]. Indeed, higher tolerance of frost and also of drought and salinity was obtained when the rice *OsDREB1A* gene was transformed into *Arabidopsis* plants [27]. The transformation of *AtCbf3* (*DREB1A*) resulted in a slight improvement in frost tolerance and a more pronounced increase in drought and salinity tolerance in rice [43]. Improved tolerance to salinity, drought and low temperature was reported when rice plants were transformed either with *OsDREB1* gene from rice or with the *DREB1* genes from *Arabidopsis* [44]. The *DREB1A* gene from *Arabidopsis* was transformed into bread wheat under the control of a stress-inducible promoter, and the transformants showed substantial resistance to water stress [45].

Regulators of the *Cbf* regulators

Transcription factors regulate the effector *Cor* genes, directly involved in the development of frost tolerance. In the case of frost tolerance, the most important transcription factors seem to be the *Cbf* genes. But what kind of gene regulates the

*Cbf*s themselves? Since not many data are available in cereals yet, a brief summary will be given of the knowledge obtained from studies on *Arabidopsis*, which will be compared with the results available in cereals.

Ice1, the master regulator, or master switch, as it is often called, has been described in *Arabidopsis*. This constitutively expressed gene encodes a MYC-type bHLH transcription factor, which is a positive regulator for *Cbf* genes [46]. Binding to the promoter of *Cbf* genes, the ICE1 regulator promotes their transcription. The level of *Ice1* transcript is regulated by the *Hos1* gene, which mediates its ubiquitination and degradation [47]. The *Ice1* homologous gene, *HvIce1*, was mapped in barley [33] on chromosome 7H, and *HvICE2*, a closely related but distinct gene, was mapped on 3H [24]. It was found that the *Ice1* gene is also expressed constitutively in barley [48].

A negative regulator for *Cbf* genes has also been described in *Arabidopsis*. Besides regulating the *Cbf* genes, the repressor gene, *Zat12* acts on many cold-inducible genes [49], independently of the *Ice1-Cbf* regulation pathway [50]. Using the 24k Affimetrix GeneChip array, the majority of the genes highly induced by cold were found to belong to the *Cbf* and *Zat12* regulons [49]. A *Zat12* homologous gene (*HvZfp16*) has been mapped in barley on chromosome 1H [31], but the expression of the transcription factors mentioned above has not been studied in cereals yet.

A hypothetical model was suggested [51] in *Arabidopsis*, namely that the expression level of the *Cbf* genes is influenced by the *Cbf* genes themselves. According to the model, under control conditions the steady-state level of the *AtCbf2* gene represses the *AtCbf1* and *AtCbf3* genes. The exposure of the plants to cold stress induces certain regulators, such as *Ice1*, leading to the induction of *AtCbf1* and *Cbf3*. The accumulation of a certain quantity of transcripts represses the transcription of *Cbf1* and *AtCbf3*. The increased level of these genes leads to the induction of downstream genes, finally leading to an increased level of frost tolerance. Moreover, a feedback mechanism has also been proposed [52]: the expression of downstream target genes influences the expression level of regulators (such as *Cbf*s). More detailed regulation mechanisms were described recently [50, 53, 54], but the explanation of these models exceeds the limits of this review.

ACKNOWLEDGEMENTS

J. Dubcovsky acknowledges support by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2004-01783. The Hungarian research was funded by grants from the Hungarian National Research Fund (OTKA T046573) and the Hungarian Wheat Spike Consortium (NKFP 406404).

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