THE NOVEL APPROACH TOWARDS ESTIMATION OF FROST TOLERANCE IN BARLEY

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ABSTRACT

We developed a convenient, efficient and reliable PCR-based technique to distinguish the frost tolerant and sensitive genotypes in barley. Based on artificial freeze test, significant differences among genotypes were found. In these plants, frost tolerance, expressed as LT_{50}, was strongly correlated with the existence of cold-induced genes. Comparison of LT_{50} values and the PCR-detected pattern of cold-induced genes showed that Hudson, Dictoo and Krusevac occupied three first positions in terms of a high frost tolerance. After 35 and 49 days of cold acclimation, the Nortstar was found to be the most tolerant wheat genotype (LT_{50}: -16°C). Low correlation between LT_{50} and existence of cold-induced genes was found for rye and wheat genotypes. Diploid forms of rye plants were found to be more frost tolerant than tetraploid forms.

Keywords: artificial freezing test, cold-induced genes, PCR-based frost resistance test

Introduction

Freezing is one of the most widespread environmental stresses and crucial factor restricting the crop production. Major challenge to plant breeders is to improve plant tolerance to cold (8). Field experiments are commonly used to test the frost tolerance in crop plants. The main limitation of such experiments in the determination of the frost-tolerance potential of cultivars is that results are often inconclusive, due either to complete winterkill or a lack of it (19). Furthermore, variation in factors such as soil moisture, soil fertility, ice encasement and smothering, can have an indirect effect on survival by limiting the level of cold hardiness that a plant can attain (19). To prevent the above limitations it is necessary to repeat experiments in diverse locations. Such experiments are, however, rather expensive and the experimental errors made on such occasions are usually large (7). The limitations mentioned above have led to a search for rapid and efficient laboratory methods for evaluating frost tolerance.

Genotypes with large differences in cold hardiness potential can be readily distinguished by estimation of their LT_{50} (temperature at which 50% of the plants are killed in artificial freeze test. Such tests have the advantages of speed, greater control over experimental conditions, and an opportunity for replication of experiments over time) (13).

It was reported that many genes are induced in frost tolerant barley during cold acclimation (24). They are involved in control of different cellular functions and in the context of frost resistance some of them may protect chloroplast and cell functions under cold (6), while others may be involved in the metabolic adjustment to cold (27). Testing the existence of cold-induced genes could be a quick method to identify frost tolerant cereal genotypes.

In the paper, we describe a comparative study of the existence of the genes induced by cold in barley, wheat, and rye genotypes and determine the relationship between the gene appearance and the evaluation of genotype frost tolerance by artificial freeze tests. From a plant breeders' point of view, detection of cold-induced genes by PCR could be a useful method in a marker-assisted genotype selection in plant breeding program.

Materials and Methods

The experiments were performed on 15 wheat (Norstar, Bezostaja 1, Doğu 88, Karasu 90, Alpaslan, Hawk, Lancer, Nenehatun, Daphan, Yıldırım, Gerek 79, Tir, Palandöken 97, Kıraç 66 and Kırik), 8 barley (Dicktoo, Krusewak 1, Hudson, ABVD-6, ABVD-2, ABVD-4, Bülbül 89 and Tokak 157/37) and 4 rye (diploid *S. cereale*, 2n = 2x = 14; tetraploid *S .cereale*, 2n = 4x = 28; diploid *S. montanum*, 2n = 2x = 14 and tetraploid *S. montanum*, 2n = 4x = 28) genotypes.

Imbibed seeds were held for 2 days at 4°C and then transferred to an incubator and held for 1 day at 22°C. Thirty germinating seeds were transferred to trays and the trays were placed over aerated, full strength Hoagland's solution (9), which was changed at two-week interval. The seedlings were then grown at 20°C and a 16-h photoperiod and a light intensity of 300 m mol m^{-2} until three-four leaf stage.

Cold acclimation and freezing tests

Seedlings were transferred to a 4°C chamber with a 16-h photoperiod with light intensity. Cold acclimation periods were 35 and 49 days. At the end of each treatment period, the LT_{50} (temperature at which 50% of the plants are killed) for whole crowns was determined, using the method outlined by Limin and Fowler (1985) (18). For each replicate in each of

the treatment, five plants were frozen at test temperatures and separated at 2° C increments. Plant survival was estimated from regrowth after 3 weeks.

PCR Analysis

Genomic DNA was extracted from barley, wheat and rye genotypes using the extraction procedure of Dellaporta et al. (4). The PCR primers were designed according to the nucleotide sequence of cold-induced genes in barley as shown in **Table 1**. Primer pairs were used separately in each PCR application. Cycling parameters were: an initial template denaturation at 94°C for 5 minutes (1 cycle) followed by 30 cycles of 1) 94°C for 1 min, 2) 55°C for 1 min, and 3) 72°C for 2 min. The thirty cycles were followed by a final elongation step at 72°C for 5 min. Following PCR amplification, 10 µl PCR product was loaded into a 1% agarose gel and subjected to electrophoresis for one hour at 90V followed by visualization of ethidium bromide staining under UV light.

Statistical Analysis

Analysis of variance was used to compare the means. The means were separated using Fisher's least significant difference. Relationship between the existence of the cold-induced genes and the LT_{50} values by artificial freezing tests was evaluated for barley, wheat and rye genotypes by Spearman correlation coefficients analysis. The SAS/PC statistical program was used for all computations (25).

TABLE 1

Forward and reverse primer sequences, product size and references of 6 cold-induced genes in barley

Gene	Forward	Reverse	Product Size	References
pAF 93	GAGGATGAGAGGAGCACCCA	TCTTCTCCTCCTCGGGCACT	504	Grossi et al.1995
pAO 29	GGGAGGGAGCTGAACCAGAA	TGAGCAGGGATCCCAGGAA	166	Grossi et al.1998
pT 59	ATCCATCGGCGCCAGACTA	TGCAGGTTCAATGTTCAGACG	524	Cattivelli and Bartels 1990
PA 986	GGATCCAAGTCCGCTCGTCT	AATCATGGGTGAGCAGGGAC	429	Cattivelli and Bartels 1990
Cor 14b	ACTGGGTGGTGGCCAACAT	AGAAGACGGCCGTGAGCTCT	290	Cattivelli 2000
Blt 14	ATGGCAAAGAGTCTCGCCG	GCTTCTTGTAGCGCCAGCAC	263	Dunn et al.1990

TABLE 2

LT_{50} (°C) values for wheat, barley and rye genotypes acclimated at different cold durations

Wheat				**Barley**			
	Acclimation duration (d)				**Acclimation duration (d)**		
Genotypes	**35**	**49**	**Mean**	**Genotypes**	**35**	**49**	**Mean**
Norstar	-13.7	-18.3	-16.0 a[1/]	Dicktoo	-7.0	-6.3	-6.7 a
Bezostaya 1	-11.0	-14.3	-12.7 b	Krusewak 1	-6.3	-5.7	-6.0 ab
Doğu 88	-11.7	-13.7	-12.7 b	Hudson	-6.3	-5.0	-5.7 abc
Karasu 90	-11.0	-14.3	-12.7 b	ABVD-6	-5.0	-6.3	-5.7 abc
Alpaslan	-11.0	-11.7	-11.3 bc	ABVD-2	-5.7	-5.0	-5.3 abc
Hawk	-9.0	-11.7	-10.3 cd	ABVD-4	-4.3	-6.3	-5.3 abc
Lancer	-9.0	-11.0	-10.0 cd	Bülbül 89	-5.0	-4.3	-4.7 bc
Nenehatun	-8.3	-10.3	-9.3 de	Tokak 157/37	-4.3	-4.3	-4.3 c
Daphan	-9.0	-9.0	-9.0 def	**Mean**	**-5.5**	**-5.4**	**-5.5**
Yıldırım	-9.0	-9.0	-9.0 def	LSD	1.33	1.00	1.17
Gerek 79	-7.7	-8.3	-8.0 ef	**Rye**			
Tir	-7.0	-9.0	-8.0 ef	*S.cereale* (D)*	-18.3	-22.7	-20.5 a
Palandöken 97	-7.7	-7.7	-7.7 f	*S.cereale* (T)	-17.7	-21.3	-19.5 a
Kıraç 66	-5.7	-6.3	-6.0 g	*S.montanum* (D)	-14.3	-21.7	-18.0 b
Kırik	-5.0	-7.0	-6.0 g	*S.montanum* (T)	-14.3	-19.0	-16.7 c
Mean	**-9.0 b**	**-10.8 a**	**-9.9**	**Mean**	**-16.2 b**	**-21.2 a**	**-18.7**
LSD	1.07	1.69	1.38		1.33	0.50	0.92

[1/] Values with the same letter in columns are not significantly different at the 0.05 level according to Fisher`s Least Significant Difference (LSD) Test.

* D= Diploid form (2n = 14), T= Tetraploid form (2n = 28)

Results and Discussion

Cold acclimation significantly influenced the frost tolerance in wheat genotypes (**Table 2**). While in thirty five days cold acclimated wheat genotypes LT_{50} decreased to -9 °C, forty nine days of acclimation increased frost tolerance down to -10.8 °C. Significant differences ($P<0.001$) in frost tolerance were found between wheat genotypes. Frost tolerance of *cv*. Norstar was the highest (-13.7 and -18.3 °C when acclimated for 35 and 49 days respectively whereas the LT_{50} in Bezostaya 1, Doğu 88 and Karasu 90 cultivars was -12.7°C, independently of the acclimation period. The most susceptible genotype was Kırik with LT_{50} = -6°C. Hard red wheat genotypes: cvs. Doğu 88, Hawk, Lancer, Karasu 90, Bezostaya 1, Norstar and Alparslan showed higher frost tolerance than white wheat genotypes: cvs. Palandöken 97, Kırik, Tir, Nenehatun, Daphan, Yıldırım, Gerek 79 and Kıraç 66 (**Table 2**).

Frost tolerance of barley genotypes was not significantly affected by the length of cold acclimation period (**Table 2**). However, significant differences ($P<0.05$) in frost tolerance were observed between the genotypes: the highest tolerance was observed for Dicktoo average LT_{50} = -6.7°C whereas Tokak 157/37 tolerance was only -4.3°C (**Table 2**).

There was a significant ($P<0.01$) effect of different cold acclimation periods on cold tolerance of rye genotypes (**Table 2**). LT_{50} for plants acclimated to low temperature for 35 and 49 days was -16.2°C and -21.2°C, respectively. Also there were significant differences among rye genotypes ($P<0.01$): both diploid and tetraploid forms of *Secale cereale* were more frost tolerant than diploid and tetraploid forms of *Secale montanum*. There was no significant difference between diploid and tetraploid forms of *Secale cerale* regarding the duration of cold acclimation. However, significant difference in low temperature tolerance was detected between diploid and tetraploid forms of *Secale montanum* after 49 days of cold acclimation. In general, diploid forms were more cold tolerant than tetraploid forms.

There was significant relationship correlation between frost tolerance of genotypes and existence of cold-induced genes in barley (**Table 3**). After 35 days of cold acclimation, frost cold tolerance of barley was positively correlated with the number of cold-induced genes ($p<0.001$). For wheat and rye genotypes, no such correlation was found (**Table 3)**. Therefore, only barley genotypes can be screened for frost tolerance through PCR-detected presence of cold-induced genes.

Table 4 shows band patterns of 6 cold-induced genes in 8 barley genotypes. Cor14b, PAF93 and PAO29 genes were found in all barley genotypes. Seven out of these 8 genotypes showed a 263 bp band for blt14 gene (**Fig. 1**). In three genotypes, Dictoo, Krusevac and Hudson, having the highest frost tolerance were assented by PCR analysis indicating the presence of all the detected cold-induced genes. This point out that PCR analysis can replace the field test to asses the frost tolerance in barley. On the other hand, there was no significant relationship between cold induced genes and LT_{50} values for

TABLE 3

Spearman correlation coefficients of existence between cold-induced genes and LT_{50} (°C) values of barley, wheat and rye genotypes acclimated at different cold durations

	LT_{50} (°C) values based on acclimation duration (d)		
	35	**49**	**Mean**
Existence of cold induced genes in **barley**	-0.956**	-0.260	-0.849*
Existence of cold induced genes in **rye**	-0.211	-0.6	0.01
Existence of cold induced genes in **wheat**	0.062	0.227	0.156

* .** . Significantly different at $P<0.05$ and $P<0.01$, respectively

TABLE 4

Band patterns of 6-cold induced genes in 8 barley genotypes

Genotypes	**pAF93**	**pT59**	**pA986**	**pAO29**	**Blt14**	**Cor14b**	**Total number of bands**
Hudson	+	+	+	+	+	+	6
Krusevak	+	+	+	+	+	+	6
Dictoo	+	+	+	+	+	+	6
ABVD-6	+	-	+	+	+	+	5
Bülbül 89	+	+	-	+	+	+	5
ABVD-2	+	+	-	+	+	+	5
ABVD-4	+	-	-	+	+	+	4
Tokak 157/37	+	-	-	+	-	+	3
Total	**8**	**5**	**4**	**8**	**7**	**8**	

+ Band seen on the gel.

wheat and rye genotypes (**Table 3**). However, in wheat and rye genotypes, no cold induced genes seem to be associated with LT_{50} values. In *Secale cereale* genotypes of different ploidity levels, the tetraploid form, which carries all the 6 cold-induced genes, achieved LT_{50} of -21.3°C when acclimated in cold for 49 days. However, diploid form which carries only 4 of the cold induced genes, could acclimate to somewhat higher level (LT_{50} = -22.7°C).

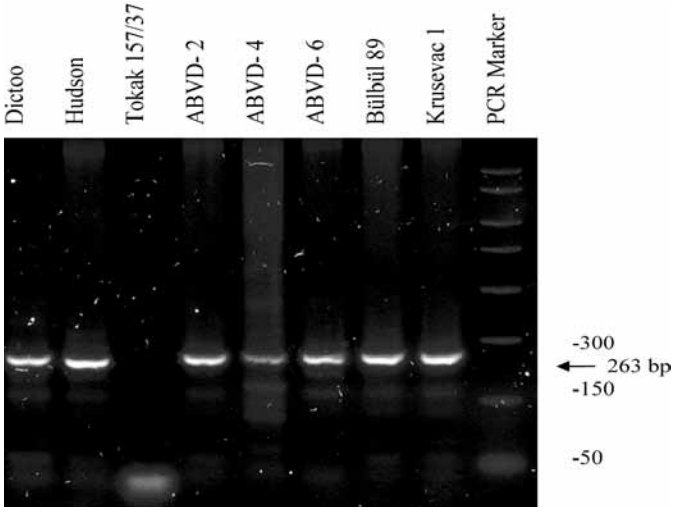

Fig. 1. Band pattern of cold induced gene, *Blt14* in 8 barley genotypes.

Based on these results, existence and detection of barley cold induced genes by PCR can be used to evaluate frost tolerance of barley genotypes and can replace the methods of field and laboratory tests for frost tolerance studies in barley. However, this cannot be utilized, at least, to evaluate wheat and rye genotypes for frost tolerance based on the existence of barley cold induced genes.

Winter wheat and rye genotypes reached maximum low temperature tolerance after 49 days of acclimation in cold whereas barley genotypes reached such a maximum after 35 days at 4°C. Similarly, Fowler et al. (1995) (8) reported that in wheat and rye genotypes cold tolerance increased until completion of vernalization. Maximum frost tolerance was achieved by 49 days of acclimation and then, frost tolerance development gradually slowed and eventually started to decline. It is well known that changes in gene expression occur during cold acclimation (14). There have been considerable efforts to determine the nature of cold-inducible genes and establish whether they have roles in frost tolerance. This has resulted in the identification of many genes that are induced during cold acclimation. A large number of these genes encode proteins with known enzyme activities that potentially contribute to frost tolerance. For instance, the Arabidopsis *FAD8* gene (10) encodes a fatty acid desaturase that might contribute to frost tolerance by altering lipid composition. Cold-responsive genes encoding molecular chaperones including a spinach *hsp70* gene (1) and a *Brassica napus hsp90* gene (16) might contribute to frost tolerance by stabilizing proteins against freeze-induced denaturation. Also, cold-responsive genes encoding various signal transduction and regulatory proteins have been identified including a mitogen-activated protein (MAP) kinase (20), a MAP kinase kinase kinase (21), calmodulin-related proteins (23), and 14-3-3 proteins (15). These proteins might contribute to frost tolerance by controlling the expression of frost tolerance genes or by regulating the activity of proteins involved in frost tolerance.

Significant differences in frost tolerance were found among wheat, barley and rye genotypes used in our study. The observation is in accordance with results of other researchers (8, 13, 22, 26). Frost tolerance was the highest in rye (LT_{50} -18.2°C) then in red wheat (LT_{50} -12.3°C) and white wheat (LT_{50} -7.9°C); barley frost tolerance was the lowest (LT_{50} -5.5°C). Based on the classification made by Lewitt (1980), rye could be considered as extremely hardy, red wheat as moderately and very hardy, white wheat as moderately hardy, and barley as slightly hardy.

Conclusions

In the present study we report that existence and detection of cold induced genes by PCR can be used for evaluation of frost tolerance barley genotypes. The method enables breeders to screen barley genotypes very quickly and saves time, resources and labor. It also does not depend upon environmental factors that restrict screening procedure in the field. However, this method cannot be used for wheat and rye genotypes.

Acknowledgements

We are grateful for the financial support from the Scientific Research Projects Fund (BAP-2003/343 and BAP-2003/230) of Ataturk University, Erzurum, Turkey.

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