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RELATION BETWEEN FREEZING TOLERANCE AND SEED STORAGE PROTEINS IN WINTER BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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ABSTRACT

Low temperature is one of the most important abiotic stresses limiting wheat growth and productivity. Selection for complex genetic traits, such as freezing resistance, can simplify the plant breeding programs when related markers are identified and present in the breeding lines. In the present study, ten improved bread wheat lines were assessed for freezing resistance based on LT_{50} measurement. Randomized complete block design with three replications were used. Four types of seed proteins: high molecular weight glutenin, low molecular weight glutenin, gliadin and some water-salt soluble proteins were evaluated by SDS-PAG..Our results showed significant differences between studied lines in respect with LT_{50}. Some relationships were also seen between protein subunits and freezing resistance. In addition, significant increase in proline accumulation in leaf tissue of LT-tolerant cultivars was seen during cold acclimation. This may be used as a biochemical marker to identify resistant cultivars.

Key words: Freezing tolerance, LT_{50}, Seed storage proteins, Proline, Wheat

INTRODUCTION

Extreme winter climatic condition is one of the basic limiting factors for winter cereal production (Veisz et al., 2001). Ability of plants to increase their freezing resistance in response to a period of low nonfreezing temperatures prior to the onset of winter is known as cold acclimation (Browse and Xin, 2001). LT (Low temperature) tolerance in cereals is dependent upon a highly integrated system of inducible genes. It was showed that full expression of LT tolerance genes only occurs in the vegetative stage. Plants in the reproductive phase have a limited and reduced ability to cold acclimation (Mahfoozi et al., 2006; Galiba et al., 2009). It has also been shown that LT tolerance gene expression depends on level and duration of gene expression, determined the degree of LT tolerance (Ganeshan et al., 2008) Factors that delay the transition from the vegetative to the reproductive stage increases the duration of expression of LT tolerance genes in cereals (Fowler et al., 1999). Use of LT_{50} (the temperature at which 50% of plants are killed in a controlled-freezing test condition) as a measure of freezing resistance in different wheat varieties, provides more accurate results (Fowler et al., 1999; Limin and Fowler, 2000). Cold acclimation resulted in protection and stabilization of the integrity of cellular membranes, enhancement of the antioxidative mechanisms, increased intercellular sugar levels as well as accumulation of other cryoprotectants including polyamines that protect the intracellular proteins by inducing the genes encoding molecular chaperones (Mahajan and Tuteja, 2005).

Molecular and genomic analyses have revealed many QTLs associated with LT tolerance (Baga et al., 2007). Some of the genes associated with these QTL are induced by LT exposure, lead to stress tolerance. Many stress-inducible genes include signaling molecules, such as mitogen-activated protein (MAP) kinase proteins might contribute to freezing resistance as well as tolerance by controlling or regulating the expression and activity of the major stress genes as well as their proteins (Shinozaki et al., 2003). LEA (Late Embryogenesis Abundant) proteins and the polypeptides are synthesized during the late embryogenesis phase. Many of these proteins are predicted to contain regions capable of forming amphipathic α helices. The examples of cold responsive genes include: *COR15a*, alfalfa *Cas15* and wheat *WCS120* families. The expression of *COR* genes has been shown to be critical for both frost tolerance and cold acclimation in plants (Mahajan and Tuteja, 2005). Genetic experiments were demonstrated that winter hardiness of wheat depends both on the frost resistance genes (*Fr1* and *Fr2*) and genes related to storage proteins and another genes (*Win1*), which control resistance to oxygen deficit in the vicinity of roots in the cold season (Netsvetaev and Netsvetaeva, 2004). Several genes involved in the response to low temperature and other stresses were localized on the long arm of chromosome 5A in wheat which has a major effect on freezing resistance (Sutka, 1981; Baga et al., 2007; Kocsy et al., 2008). Negative correlation was observed between the abundance of VRN_1 transcripts and the ability of *COR* transcripts and proteins to accumulate in response to cold. Houde et al. (1992) identified several cold-induced proteins named *FTMs* for freezing resistance markers could be used as biochemical marker for freezing resistance. This protein family was found to be coordinately regulated specifically by low temperature (Ganeshan et al., 2008).

Accumulation of the proteins was higher in the freezing-tolerant genotype than in the less tolerant variety. Marker assisted selection (MAS) has emerged as a strategy for increasing selection gain, especially in the case of Quantitative traits (Knapp, 1998).

Although some researchers outlined that HMW and LMW glutenin subunits play a major role in the rheological properties of wheat flour dough (Payne, 1987; Ikeda et al., 2003), the effect of this proteins in respect with frost resistance was also shown (Prasil et al., 2002; Witkowski et al., 2008). These proteins could also be associated with important agronomic traits such as frost tolerance, total protein content and the SDS-sedimentation value. The SDS-sedimentation value, resistance to leaf blotch and frost tolerance showed statistically significant associations with the status of the *Glu-A1* locus. It appears that chromosome 1A with the null allele at *Glu-A1* carries a closely linked locus responsible for frost tolerance. The possible relationship between gliadin storage proteins and frost resistance in French wheat were studied and it was concluded that cultivars with allele composition *GLI-*D_{2g}, on average were later maturing and more freeze resistant. Alternatively, cultivars with the allele *GLI-*D_{2m} were earlier maturity and cold sensitive, and are grown in the south of France (Metakowsky and Branlard, 1998). Thus, this study was designed to detect the possible relation between seed protein banding pattern and freezing resistance, in order to introduce these biochemical markers for any selection strategies.

MATERIAL AND METHODS

Plant material and freezing test

The plant material comprised ten winter wheat lines that were obtained from dry land agricultural research institute of Maragheh, and three spring cultivars including Zagros, Iniya and Anza. Freezing resistance was assessed using LT50 and FSI measurements after cold acclimation according to Mahfoozi et al. (2001). These freezing test comprised four test temperatures (-4, -7, -10, -13) and three replications were used based on randomized complete block design (Figure 1). The plant numbers in each row were 10 and there were 3 rows of each studding genotype in one repeat. Seeds were first imbibed in filter paper-lined petri dishes at 4 °C to ensure uniform germination. Germinated seeds were transplanted in a controlled greenhouse under 20 °C and a 14/10 h (D:N) photoperiod. When the growing seedlings reached the 3-4-leaf stage (Zadoks et al., 1974), the plants were transferred to a growth chamber at 4 °C with a 14/10 h (D:N) photoperiod for cold acclimation. After 30 days of acclimation, the crowns were detached from the plants and covered with moist sand in aluminum cans and placed in a programmable walking freeze chamber. Five crowns were removed for each of the tested temperatures. Samples were thawed overnight at 4 °C and replanted in controlled chambers at 20 °C with a 14/10 h (D:N) photoperiod. Plant recovery was rated after 3 weeks of re-growth and LT50 was calculated for each generation. In order to estimate FSI the similar experiment was conducted and the whole plants were used for freezing test.

Electrophoresis of proteins

Seed proteins from ten studied lines and check varieties including Zagros, Iniya and Anza including gliadins, total proteins (salt and water soluble proteins), LMW and HMW glutenins were extracted according to Singh et al. (1991). Extracted proteins were fractionated by SDS-PAGE technique using 10 % polyacrylamid gel electrophoresis (Figure 3).

Figure 1. Stages of freezing test based on Mahfoozi et al. (2001).

Proline Evaluation

Proline assessment was carried out for all lines and varieties as described before (Bietz and Wall, 1973).

Statistical analyses

Scoring the gels was based on binary code and cluster analysis using simple matching coefficient. Furthermore, mean comparison was carried out for LT_{50}. The cluster values analysis for proteins was performed based on average distance (Figure 2). Data was analyzed using SPSS ver. 14 statistical package and NTSYS pc ver. 2.1.

RESULTS AND DISCUSSION

Analysis of variance showed that there were significant differences among all studied genotypes ($p \leq 0.01$) for LT_{50} (Table 1).

Table 1. Analysis of variance for studied traits

SOV	df	LT_{50}	MS FSI	Accumulation Proline	Total proline	Acclimation proline	After frost proline
Repeat	2	0.623^{ns}	0.140^{ns}	11.85^{ns}	0.022^{ns}	0.013^{ns}	0.001^{ns}
Genotype	12	24.131^{**}	20.141^{**}	33.37^{**}	0.715^{**}	0.784^{**}	0.515^{**}
Error	24	0.616	1.018	6.07	0.30	0.022	0.001

*: significance at p≤0.01.

In addition, Duncan's multiple range test showed that (Table 3) lines 1, 2 and 3 had higher LT50 and FSI and Zagros as spring varieties considered with the lowest resistance for these two traits. Sutka (1981) and the Veisz et al. (2001) also indicated the existence of differences between winter-survival in the case of different wheat varieties. Gray et al. (1997) were also showed that cold acclimation could increases level of freezing resistance. According to our

Table 2. Correlation between traits in studied lines

SOV	LT_{50}	FSI	Total proline	Accumulation proline	Acclimation proline	After frost proline
LT_{50}	1	0.95^{**}	0.17	0.91^{**}	0.55^{*}	0.025
FSI		1	0.40	0.65^{*}	0.56^{*}	0.49
Total proline			1	0.41	0.084	0.21
Accumulation proline				1	0.56^{*}	0.06
Acclimation proline					1	0.13
After frost proline						1

* and **: significance at p≤0.05 and significant at p≤0.01 respectively.

results, proline accumulation after acclimation was higher in resistant lines (1, 2 and 3). Dorffling et al. (2009) indicate similar deductions in respect to acclimation conditions. According to the Pearson Correlation coefficient (Table 2), there was a strong significant correlation (r= 0.95) between LT50 and FSI (p≤0.001). This correlation between the field survival index and LT50 was prerecorded by Bridger et al. (1996). Also, these two traits had a good correlation with proline accumulation (r = 0.91** and r = 0.65**). Cluster analysis using the average distance (UPGMA) resulted in three groups including: first encompass the most resistant

Table 4. Correlation between LT_{50}, FSI and total protein markers

	M1	M3	M8	M9	M10	Lt_{50}
Lt_{50}	$^{*}0.569$	$^{**}0.694$	$^{*}0.670$	$^{*}0.670$	$^{*}0.670$	1
	M3	M8	M9	M10	FSI	
FSI	$^{*}0.639$	$^{*}0.555$	$^{*}0.555$	$^{*}0.555$	1	

*,**: significance at p≤0.05 and significance at p≤0.01 level.

lines 1, 2 and 3, whereas another group comprised Zagros and the rest of the studied lines fall in the last group (Figure 2). Banding pattern of total proteins (Fig. 3c) revealed the correlation between some protein markers (band) including 1, 3, 8 and 9 and 10 and LT50, markers including 3, 8, 9 and 10 and FSI (Table 4). Regression analysis showed that some

Table 5. Regression analysis for total protein

markers	Lt_{50}	FSI
M3	β=0/69	β=0.35
R^2	0.43	0.35

part of this trait variance could be justified by these markers (Table 5). Similar results with respect to high molecular weight in Fig. 3b and Tab. 6, 7 were also observed. In our studied lines 25% of subunits encoded by *$GluA_1$* locus was subunit 1 and 53% of subunits related to *$GluB_1$* locus were 8+7 and 33% for GluD1 locus were 10+5. According to

Table 3. Means comparison table using Duncan's multiple range test at 0.05.

genotype	LT_{50}	FSI	Accumulation proline	Total proline	Acclimation proline	After frost proline
1	-11.18a	-12.00a	56.40a	0.654ed	1.50a	0.149d
2	-10.41a	-12.94a	55.60a	0.666d	1.50a	0.177d
3	-10.42a	-11.07a	55.60a	0.568e	1.28b	0.453b
4	-8.09b	-7.98bc	47.34b	0.516e	0.98c	0.448b
5	-8.84b	-8.68b	47.6b	0.528e	0.98c	0.324bc
6	-7.44b	-6.97c	31.85c	0.661cd	0.97c	0.190e
7	-4.62ed	-6.54c	18.86cd	0.714c	0.88cd	0.243e
8	-6.34c	-8.64b	8.19df	1.128a	1.22b	0.197cd
9	-6.44c	-8.67b	10.31d	1.135a	1.26b	0.565a
10	-5.97cd	-8.78b	5.16f	0.930b	0.98c	0.141d
zagros	-1.26f	-2.96d	5.10f	0.474e	0.22e	0.251c
iniya	-4.83ed	-6.65c	5.60f	0.497e	0.42d	0.287c
anza	-4.54e	-6.61c	10.16d	0.669d	0.46d	0.296c

Genotypes that display with same letter had not significant difference.

Witkowsky et al. (2008) in the polish wheat cultivars *GluA$_1$* encoded subunits were related with freezing resistance. Similarly in this study lines 1 and 2 contained subunit1 in glu-A1 and it is proposed that this subunit play important role in freezing resistance. Recently, there were some evidence about potential QTL of frost resistance in chromosome 1A, and that the location of some important loci in correspondence with *Glu$_1$* loci (Sofalian et al., 2010).

Table 6. Significant correlation between LT_{50}, FSI and HMW protein markers

	M4	M6	M14	M19	M31	Lt50
Lt50	0.627*	0.570*	0.579*	0.570*	0.579*	1
	M4	M19	M20	M30	FSI	
FSI	0.603*	0.607*	0.616*	0.556*	1	

* means: significance in ($p \leq 0.05$) percent.

Table 7. Regressing analysis for HMW protein

markers	Lt_{50}	FSI
M31	β=0.57	β=0.59
M20	β=0.50	β=0.52
M14	β=0.47	--------
M19	β=0.39	--------
M30	β=0.21	β=0.39
M4	----------	β=0.44
R^2	0.93	0.90

Our result did not show any correlation between low molecular weight glutenin and freezing resistance in studied lines. Finally gliadin storage proteins (Table. 8) had weak correlation with freezing resistance.

Table 8. Regression analysis for gliadin protein

Markers	Lt_{50}
M7	B=0.59
R^2	0.35

Witkowski et al. (2008) on the study the relationship between HMW subunits and freezing resistance of winter wheat of 116 Polish lines, showed correlation between the presence of *Glu-A1* subunits with SDS sedimentation rate and freezing resistance. Thus null allele frequencies between resistant genotypes and allele frequencies of *Glu-A$_1$-$_1$* among susceptible genotypes were high. Sarhan and Perras (1987) studied protein variation in the three varieties including Norstar, Frederick and Glnliya under reduced temperatures and showed that an increased amount of expressed HMW proteins. Amount of proteins in two cultivars Frederick and Norstar had increased more than spring varieties Glnliya. Other research determined that in reducing temperature, amount of HMW protein with 200 kD molecular weight increased in the resistant cultivars (Perras and Sarhan, 1989).

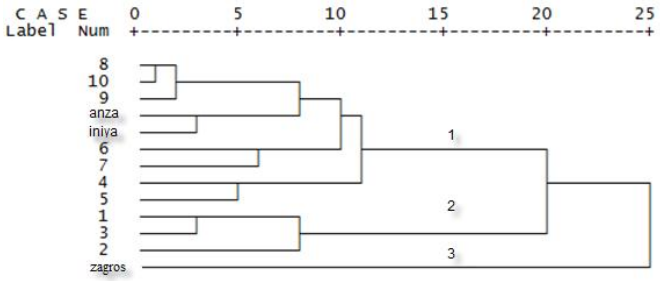

Figure 2. Cluster analysis for all quantitative traits using the average distance

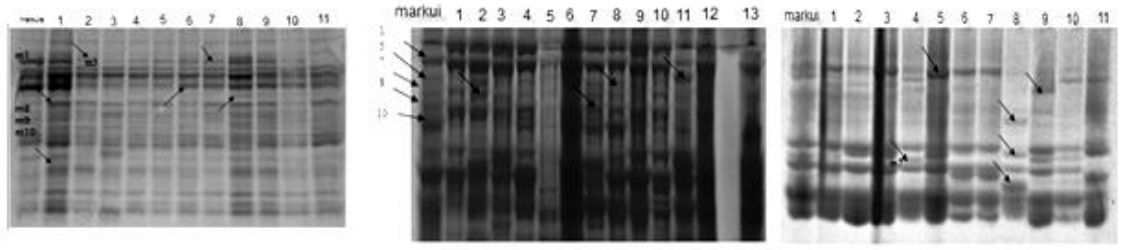

Figure 3. SDS-PAGE a) gliadin banding pattern for studded genotypes b) HMW banding pattern for studded genotypes c) total protein banding pattern.

In another research (Tasgin et al., 2006) it was shown that storage proteins correlated with increased levels of freezing resistance in cereals. Liatukas et al. (2008) investigate the relationship between alleles *GLU-A$_1$* and *GLU-B$_1$* of HMW glutenins and certain characters including high correlation

between winter survival and *GLU-B1* alleles and moderate correlation for winter growth habit.

CONCLUSION

Our results showed that molecular approaches could help breeders in the quantitative complex traits selection and the seed proteins could be mentioned as important biochemical markers in freezing resistance breeding perspectives. The results showed that relation between freezing resistance and seed storage protein in winter type bread wheat in total protein and high molecular weight subunit glutenin rather than other two types were greater.

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