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BRIEF COMMUNICATION

Frost tolerance in winter wheat cultivars: different effects of chromosome 5A and association with microsatellite alleles

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Abstract

Frost tolerance of ten Bulgarian winter wheat (*Triticum aestivum* L.) cultivars (Milena, Pobeda, Sadovo-1, Enola, Kristal, Laska, Svilena, Russalka, No301, and Lozen) and five foreign cultivars (Mironovskaya 808, Bezostaya-1, Rannaya-12, Skorospelka-35, and Chinese Spring) was studied in two experimental seasons following natural cold acclimation and in one experiment carried out in controlled acclimation conditions. Considerable intercultivar variability in plant survival was observed after freezing at -21 ºC following sufficient cold acclimation, or at -18 ºC following insufficient or controlled acclimation. In seven cultivars, the effects of chromosome 5A on frost tolerance were investigated in their F_2 hybrids with chromosome 5A monosomic lines of cultivars with high, intermediate, and low frost tolerance. The effects of chromosome 5A depended on the stress severity and the genetic background of the hybrids and varied even in cultivars of similar frost tolerance and vernalization requirements. Effects of other chromosomes besides 5A on frost tolerance were assumed. The analysis of six microsatellite loci located in the interval from centromere to *Vrn-1* on chromosomes 5AL, 5BL, and 5DL showed that the major loci determining frost tolerance in Bulgarian winter wheats were *Fr-A2* on chromosome 5AL and, to a lesser extent, *Fr-B1* on chromosome 5BL. A strong association of the 176 bp allele at locus *wmc327* tightly linked to *Fr-A2* with the elevated frost tolerance of cvs. Milena, Pobeda, Sadovo-1, Mironovskaya-808, and Bezostaya-1 was revealed. Relatively weaker association between frost tolerance and the presence of the 172 bp allele at locus *Xgwm639* tightly linked to *Fr-B1* was also observed.

Additional key words: cold acclimation, genetic variability, PCR, QTL, *Triticum aestivum*.

In hexaploid wheat, almost all chromosomes contribute to plant response to low temperature stress (Sutka 2001). Frost tolerance is determined by major loci (*Fr)* and genes controlling plant response to acclimation and vernalization (*Vrn*), both located on the long arms of homoeologous group 5 of chromosomes (Sutka 2001, Tóth *et al.* 2003). The most potent loci, *Fr-A1* and *Fr-A2*, were mapped on chromosome 5AL in close proximity (2 and 30 cM, respectively) to the gene *Vrn-A1* (Galiba *et al.* 1995, Vágújfalvi *et al.* 2003). Orthologous frost tolerance loci were identified on chromosomes 5B and 5D (Tóth *et al*. 2003). The evaluation of plant genetic resources for variation in frost tolerance and the examination of the association between phenotypic variation and variation at frost tolerance loci could accelerate modern wheat breeding. The presented study aimed at *1*) assessment of frost tolerance of Bulgarian bread wheat cultivars under different cold acclimation conditions and freezing temperatures; *2*) estimation of the effects of chromosome 5A on frost tolerance, and

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Abbreviations: CBF - C-repeat binding factor; PCR - polymerase chain reaction; QTL - quantitative trait locus.
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3) investigation of the allele polymorphism at microsatellite loci within the region from centromere to *Vrn-1* genes on homoelologous group 5 of chromosomes in association with frost tolerance of wheat cultivars.

Ten Bulgarian winter wheat cultivars which are of interest for grain production and breeding were included in frost tolerance studies and microsatellite analysis (Table 1). Five foreign cultivars of known frost tolerance were used for comparison: Bezostaya-1 (Bez1) and Mironovskaya-808 (Mir808) – highly tolerant; Skorospelka-35 (Sk35) and Chinese Spring – susceptible, and Rannaya-12 (R12) – intermediate. Chromosome 5A monosomic lines of Mir808, R12, and Sk35 were crossed with seven of the Bulgarian cultivars and Bez1. Their euploid and monosomic F_2 hybrids were compared to elucidate the effects of chromosome 5A on frost tolerance. Effects of chromosome 5A on frost tolerance were affirmed when the differences in the plant survival percentage between the 5A monosomic and euploid hybrids were significant according to the *t*-tests.

Screening for frost tolerance was performed at the Dobrudzha Agricultural Institute, General Toshevo, Bulgaria and at the Agricultural Institute, Martonvásár, Hungary using different approaches as described below. In Bulgaria, two experiments (each of three replicates) were conducted in 2005/06 and 2006/07 following a field-laboratory method. For each genotype, 40 to 50 plants were analysed in each replicate. Following acclimation under natural conditions in the open air, low temperature treatment was applied using freezing chambers *KTK 3000* (*ILKA*, *VEB Maschinenfabrik Nema*, Netzschkau, Germany) for 7 d, of which 2 d at -4 ºC followed by a temperature decrease with 2 to 4 ºC per day at a rate of 1 ºC/h to reach the critical temperature. After 30 h at the critical temperature, temperature was gradually raised up to 0 ºC. Thawing was allowed outside the chambers at 5 ºC after which the plants were transferred to a greenhouse with day/night temperature of 20 - 22/10 - 15 ºC. The percentage of survived plants was recorded after a 20-d recovery. The critical temperatures were different during the two seasons because of the different hardening: -18 and -21 ºC in 2005/06, and -13, -16 and -18 ºC in 2006/07. For each temperature, a separate chamber was used. In Hungary, the experiment was performed in 2008 following a laboratory method. The caryopses were germinated and the seedlings were planted in wooden boxes. The plants were treated for 6 weeks with a regime of decreasing day/night temperature (1 week at 15/10 ºC, 2 weeks at 10/5 ºC, 2 weeks at 5/0 ºC, and 1 week at 2/-2 ºC, respectively) and 8-h photoperiod (irradiance of 260 μmol m^{-2} s^{-1}). After hardening, the temperature was decreased gradually with 2 to 6 ºC per day to reach the critical ones (-16 and -18 ºC). After 24 h at freezing temperature (at darkness), the temperature was raised by 2 ºC/h steps to +1 ºC and the plants were kept at this temperature for 24 h. After thawing, the boxes were placed at day/night temperature 17/16 ºC and 14-h photoperiod (180 μmol m^{-2} s^{-1}) for 3 weeks. After recovery, the plants were scored based on their regeneration/re-growth ability (0 - dead plants, 5 - well-developed tillering plants). The genotypes were tested in 5 replications for each freezing temperature with 5 individuals per replication. The data were analyzed using *Excel* based *ANOVA* (two-way factor analysis with replications).

For each genotype, genomic DNA was isolated from bulked samples each consisting of 5 two-week old plants following the method of Murray and Tompson (1980) with minor modifications. Four microsatellite markers (Röder *et al.* 1998) previously mapped on chromosome 5AL were used. The markers were chosen based on two assumptions: first, uniform distribution along the region centromere *Vrn-A1* on chromosome 5AL, and second, close location to the *Fr-A2* locus in wheat (Vágújfalvi *et al*. 2003, Båga *et al*. 2007, Knox *et al.* 2008). The four primer pairs used for amplification resulted in total of six microsatellite loci: four on 5AL (*Xgwm186*, *wmc327*, *Xgwm639*, and *Xgwm666*), one on 5BL (*Xgwm639*), and one on 5DL (*Xgwm639*). Polymerase chain reaction (PCR) amplifications were performed following Röder *et al.* (1998) on *Gene Amp* PCR system *9700* thermal cycler (*Applied Biosystems*, Carlsbad, CA, USA). PCR products were separated on standard sequencing gels (*Reprogel*™, *Amersham Biosciences*, Piscataway, NJ, USA) using automated laser fluorescence (*ALF Express II*) sequencer (*Amersham Biosciences*). Fragment sizes were calculated using the computer program *Allele Locator v. 1.03* (*Amersham Biosciences*) by comparison with the internal size standards.

The critical temperature of plant frost survival is not constant and changes according to the temperature regime during cold acclimation (Fowler *et al.* 1999). The two experimental winter seasons differed considerably by the recorded number of days with sub-zero average daily temperatures (46 d in 2005/06 and 14 d in 2006/07) and the total sum of sub-zero temperatures (-172.1 ºC in 2005/06 and -78.0 ºC in 2006/07). Moreover, during the 2005/06 season there were fewer days with temperatures above 0 ºC (55) compared to 74 d in 2006/07 and the total sum of maximum temperatures above 10 ºC was 369 ºC compared to 762 ºC in 2006/07. In both seasons, the two factors (freezing temperature and genotype) and their interaction had significant effects on plant survival ($P < 0.001$). The difference in the plant cold acclimation during the two seasons resulted in differences in plant frost susceptibility. After sufficient acclimation in 2005/06, all cultivars showed high plant survival after freezing at -18 ºC (64.4 - 97.7 %). After insufficient acclimation in 2006/07, similar survival rates were recorded at -13 ºC (72.4 - 100 %). Larger genotypic variation was observed at -21 ºC in 2005/06 (1.3 - 98.8 %) and at -18 ºC in 2006/07 (0 - 85.7 %) in experiments performed in Bulgaria and at -18 ºC in the experiments performed in Hungary after controlled acclimation conditions (Table 1). Based on these results, the cultivars were ranked and classified into three groups: tolerant (Mir808, Milena, and Pobeda), intermediate (Bez1,

Sadovo-1, Enola, Kristal, Laska, and R12), and susceptible (Russalka, Svilena, Lozen, No301, Sk35, and Chinese Spring). Cultivars Mir808, Milena, and Pobeda were highly tolerant to all freezing temperatures after both sufficient and insufficient acclimations, whereas cultivars Bez1 and Sadovo-1 were tolerant (77 - 79 % plant survival) only after sufficient acclimation in 2005/06. The observed wide genetic variability for plant survival after freezing and the differentiation between more tolerant and less tolerant cultivars with respect to plant survival is in agreement with the reported variation in the content of free phenols after freezing (Petrova *et al.* 2007). In that study, the tolerant (Milena and Bez1) and less tolerant (Enola and Russalka) cultivars had different content of phenols after freezing. Low temperature treatment generally triggers oxidative stress (Kocsy *et al.* 2011) which in turn causes tissue damage. This damage is supposed to be compensated by changes in activities of antioxidative enzymes (Dai *et al.* 2009) and phenolic compounds. The higher frost tolerance of cv. Milena is probably inherited from its ancestor, cv. Odesskaya-16, which is a donor of winter resistance genes (Rabinovich 1972).

According to Veisz *et al.* (1997), genes determining plant survival after low temperature stress are dominant or recessive depending on the conditions. Skinner and Garland-Campbell (2008) reported on recessive genes for frost susceptibility. The high frost tolerance of cvs. Milena and Pobeda was inherited as a recessive trait as revealed by the analysis of euploid F_2 hybrids with cultivars of low (Sk35), intermediate (R12), and high (Mir808) frost resistance (data not shown). In the hybrids of cv. Bez1, frost tolerance was inherited as a recessive or dominant trait, or had an intermediate mode of

Table 1. Frost tolerance of 10 Bulgarian and 5 foreign bread wheat cultivars based on three tests at different acclimation conditions and freezing temperatures (*above*) and the allele variants [bp] at six microsatellite loci located in the interval from centromere to *Vrn1* of chromosomes 5AL, 5BL, and 5DL (*below*). The frost tolerance is presented as plant survival rate (in % in 2005/06, sufficient acclimation, and 2006/07, insufficient acclimation) or frost scores (in 2008, controlled acclimation). The frost tolerance rank was determined according to the observed survival rate/frost score, the genotypes of lowest value ranked first.

Cultivars	2005/06 -21 °C	rank	2006/07 -18 °C	rank	2008 -18 °C	rank	mean rank
Chinese Spring	1.3	1	0.0	1	0.00	1	1.00
Lozen	3.4	2	2.6	3	0.00	1	2.00
No301	12.6	4	2.0	2	0.00	1	2.33
Russalka	34.5	7	4.1	4	0.39	3	4.67
Svilena	23.7	6	15.4	8	0.04	2	5.33
Skorospelka-35	14.0	5	7.7	6	-	-	5.50
Laska	37.0	8	6.0	5	0.60	4	5.67
Kristal	5.0	3	48.0	12	0.85	5	6.67
Enola	53.0	9	14.8	7	1.84	8	8.00
Bezostaya-1	76.6	11	20.2	9	1.76	7	9.00
Sadovo-1	79.0	12	21.3	10	1.55	6	9.33
Rannaya-12	54.7	10	47.4	11	-	-	10.50
Pobeda	85.8	14	70.3	14	2.40	9	12.33
Milena	84.9	13	85.7	15	3.77	10	12.67
Mir808	98.8	15	62.9	13	-	-	14.00

Cultivars	*Xgwm186-5A*	*wmc327-5A*	*Xgwm639-5A*	*Xgwm666-5A*	*Xgwm639-5B*	*Xgwm639-5D*
Chinese Spring	135	186	0	0	166	134
Lozen	121	200	142/150	110	170	134
No301	130	174	144	110/114	0	134
Russalka	97	170	152	0	0	134
Svilena	97	174/186	144	112	0	134
Skorospelka-35	-	-	-	-	-	-
Laska	97	174	154	112	172	134
Kristal	97	192	0	112	172	134
Enola	97	174	152	114	0	134
Bezostaya-1	97	176	0	98	172	134
Sadovo-1	97	176	0	112	172	134
Rannaya-12	-	-	-	-	-	-
Pobeda	121/138	176	144	110/114	174	136
Milena	125	176	154	112	172	134
Mir808	138	176	0	114	172	134

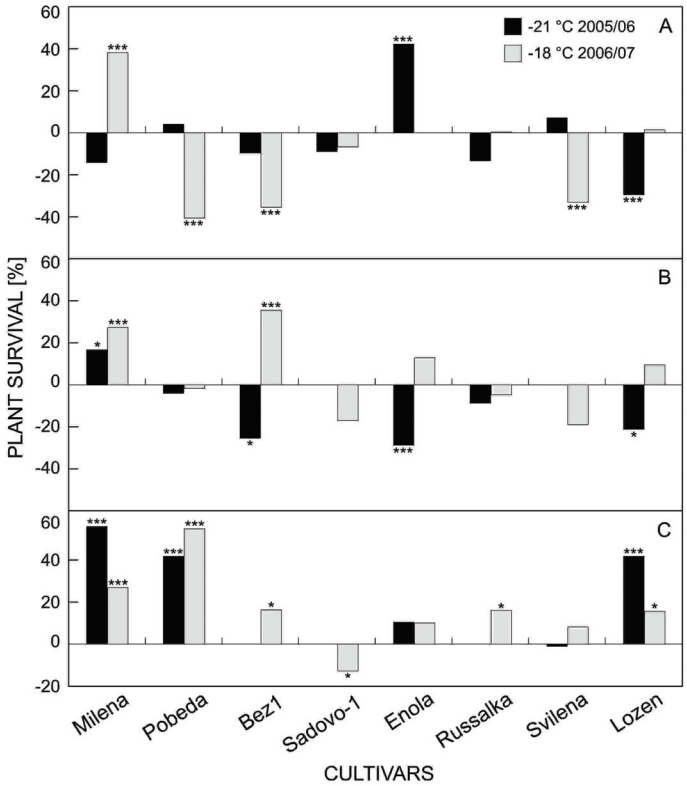

Fig. 1. Plant survival after freezing following sufficient (2005/06) or insufficient cold acclimations (2006/07) of F_2 monosomic hybrids of bread wheat cultivars with chromosome 5A monosomic lines of cultivars with high (Mironovskaya 808) (*A*), intermediate (Rannaya 12) (*B*), and low (Skorospelka 35) (*C*) frost resistance, expressed as deviation from the corresponding euploid F_2 hybrids.

inheritance. The differences in the plant survival between 5A monosomic and euploid F_2 hybrids (Fig. 1) illustrate the differences in the effects of chromosome 5A between the corresponding parental cultivars. Chromosome 5A of the tolerant cv. Milena had tolerance increasing effect in hybrids with Mir808 at the critical freezing temperature only after insufficient acclimation in 2006/07 (Fig. 1*A*) whereas in hybrids with R12 (Fig. 1*B*) and Sk35 (Fig. 1*C*), this chromosome had tolerance increasing effect in both seasons. Chromosome 5A of the other tolerant cultivar, Pobeda, had tolerance increasing effect in both seasons in hybrids with Sk35 (Fig. 1*C*) or tolerance decreasing effect in hybrids with Mir808 (Fig. 1*A*) after insufficient acclimation in 2006/07. Chromosome 5A of the cultivars with intermediate frost tolerance (Bez1, Sadovo-1, and Enola) had different effects at the various acclimation conditions.

Previous genetic studies pointed to the importance of the *Vrn-A1* region for the regulation of frost tolerance (Prášil *et al.* 2005, Kosová *et al.* 2008, Galiba *et al.* 2009). Using deletion mutants, Dhillon *et al.* (2011) provided more precise delimitation of the chromosome region responsible for the differences in frost tolerance. They showed that the frost tolerance QTLs mapped on this region are likely pleiotropic effects of *Vrn-A1* rather than effects of a separate closely linked locus (*Fr-A1*) as proposed in earlier studies. Our study showed that the chromosome 5A effects were different in cultivars with similar vernalization requirement and survival rate after freezing. For example, cvs. Pobeda (tolerant) and Bez1 (intermediate tolerance) have the same vernalization requirements but their chromosomes 5A exert different effects on the survival rate in the F_2-monosomic hybrids. Cultivars Bez1 and Sadovo-1 (intermediate tolerant) also demonstrated difference in their chromosome 5A effects. The higher frost tolerance of these cultivars compared to that of Sk35 and R12 cannot be associated solely with differences in the effects of chromosome 5A. According to our earlier study, chromosomes 5A, 2D, 4A, 5D, 6A, 1A, and 7A of cv. Bez1, a key cultivar in breeding programmes in Bulgaria, had effects on this trait (Ganeva *et al*. 2008).

The cultivars studied were highly polymorphic with respect to the allele composition of six microsatellite loci in the interval from centromere to *Vrn-1* on chromosomes 5AL, 5BL, and 5DL (Table 1). The number of alleles ranged from two (*Xgwm639-5A*) to six (*wmc327-5A*). At locus *Xgwm186-5A* (~84 cM apart from *Vrn-A1*), the 97 bp allele was of highest frequency and was present only in cultivars with intermediate to low frost tolerance (Table 1). The most tolerant cultivars (Milena, Pobeda, and Mir808) and those of intermediate tolerance (Sadovo-1 and Bez1) possessed a 176 bp allele at locus *wmc327-5A* (~10 cM apart from *Fr-A2*). Prevalence of 172 bp allele at locus *Xgwm639* tightly linked to the *Fr-B1* locus on chromosome 5B was observed in

cultivars with high and intermediate frost tolerance except for cvs. Pobeda (174 bp allele) and Enola (null allele) (Table 1).

Natural differences in frost tolerance in wheat were mapped to the *Fr-A2* locus and were related to the differences in threshold induction temperatures and/or transcript levels of several *CBF* (C-repeat binding factor) genes (Galiba *et al.* 2009). The locus *wmc327* on 5AL is about 10 cM apart from the major QTL for cold tolerance in wheat (Båga *et al.* 2007). The mapped QTL coincides with *Fr-2* locus on 5AL where *CBF* gene cluster is located (Knox *et al.* 2008). The involvement of *Fr-2* region in the development of higher frost tolerance of cv. Milena has been suggested earlier by Todorovska *et al*. (2011). The authors reported for higher steady state expression level before the onset of the low temperature treatment as well as higher transcription level of few *CBF* genes at 2 °C in cv. Milena as compared to the susceptible ones Russalka and Chinese Spring. The observed strong association of the 176 bp allele at locus *wmc327* with the higher frost tolerance of the studied wheat cultivars suggests that the main determinant of frost tolerance in Bulgarian cultivars is *Fr-A2* on chromosome 5A. The locus *Xgwm639* on 5BL was mapped near to the peak of *Fr-B1* QTL (Tóth *et al.* 2003). The observed weaker association of the marker 172 bp allele at locus *Xgwm639-5B* with high and intermediate frost tolerance suggests that a modifier with minor effect is located on chromosome 5BL.

In conclusion, this study demonstrated that the major loci determining frost tolerance in Bulgarian winter wheats were *Fr-A2* on 5AL and, to a lesser extent, *Fr-B1* on 5BL. Associations of marker alleles at loci *wmc327* (tightly linked to *Fr-A2*) and *Xgwm639* (tightly linked to *Fr-B1*) with increased frost tolerance of Bulgarian winter wheats were revealed. This might have implications in breeding programmes for improvement of wheat frost tolerance. Effects of other chromosomes besides 5A on frost tolerance were assumed.

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