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Screening for Drought Stress Tolerance in Wheat Genotypes Using Molecular Markers

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Wheat that is one of the most important staple food crops in the world is adversely affected by drought. Understanding its genetics and genome organization using molecular markers is of great value for plant breeding purposes. Several screening tests were evaluated in the present research for ability to estimate drought resistance in total 12 wheat genotypes including tolerant, semi-tolerant and non-tolerant. Randomly Amplified Polymorphic DNA primers (RAPDs) associated with drought tolerance were used initially to search genetic diversity in wheat plants. It was found out that primer P6 (TCGGCGGTTC) produced respectively a 920-bp band present mainly in drought tolerant and semi-tolerant (absent in sensitive) genotypes. Primer P7 (TCGGCGGTTC) produced a 750-bp band that is not absolutely universal for our genotypes. Genome-wide investigation was also conducted using *Dreb 1* genes as an example. Five pairs of genome-specific primers designed for the wheat *Dreb 1* genes were used for DNA amplification. Two primers, P21F/P21R and P25F/PR, amplified 596- and 1113-bp fragments, respectively, from the A genome. The P18F/P18R primer amplified a 717-bp fragment from the B genome. It was found out that *Dreb 1* gene was located on chromosome 3A in all genotypes, including drought-tolerant and drought-sensitive ones, excepting semi-tolerant genotype Tale-38. Contrary to other genotypes, a 717-bp PCR product of *Dreb-B1* gene was located on B genome from drought-tolerant variety Barakatli-95. Primers P22F/PR and P20F/P20R that amplify 596- and 1193-bp fragments, respectively, from D genome, that is common for hexaploid *Triticum aestivum* L. genotypes, did not reveal positive results.

Keywords: *wheat genotypes, RAPD primers, functional markers, Dreb genes, PCR analysis*

INTRODUCTION

Plant growth and productivity are greatly affected by environmental stresses such as drought, high salinity, and low temperature (Zheng et al., 2010). Upon exposure to abiotic stress conditions, plants undergo a variety of changes from physiological adaptation to gene expression (Shinozaki and Yamaguchi-Shinozaki, 2007). Drought is a major abiotic stress that adversely affects wheat production and quality in many regions of the world, the loss of which is the total for other natural disasters, with increasing global climate change making the situation more serious (Shao et el., 2005; Kirigwi et al., 2007). Currently, drought study has been one of the main directions in global plant biology and biological breeding. Many advances in relation to this hot topic, including molecular mechanism of anti-drought and corresponding molecular breeding have taken place (Patnaikt and Khurana, 2001; Rellegrineschi et al., 2002; Chen and Gallie, 2004; Rampino et al., 2006; Zhao et al., 2008; Wei et al., 2009; Ashraf, 2010).

The expression of many genes is induced by drought, and their gene products function directly in stress tolerance and regulation of gene expression and signal transduction in stress responses (Zhou et al., 2010). Among the products of many stress-inducible genes are those that directly protect against environmental stress: osmoprotectants, chaperones, and detoxification enzymes. Others include transcription factors and protein kinases that regulate gene expression and signal transduction during the stress responses (Seki et al., 2003). Thus, the timely expression of stress-responsive genes is crucial for the plants' ability to survive under different environmental stress conditions (Chinnusamy et al., 2007; Shinozaki and Yamaguchi-Shinozaki, 2007).

The identification of downstream target genes of stress-relating transcription factors (TFs) is desirable in understanding the cellular responses to various environmental stimuli (Agarwal et al., 2006; Wang et al., 2008). Genes regulated by a given TFs is partially

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determined by the DNA binding domain (DBD) of a protein (Pabo and Sauer, 1992).

The DBD in TFs binds to a specific DNA motif at the regulatory region of the target genes. Availability of genome sequences made it possible to discover the target genes of a specific TFs by looking for the locations of the specific recognition motifs in genome (Wang et al., 2009).

Dehydration responsive element binding proteins (DREBs) constitute a large family of TFs that induce the expression of a large number of functional genes and impart stress endurance to plants (Riechmann et al., 2000; Agarwal et al., 2006). The dehydration-responsive element (DRE) as a cis-acting element was found in the promoter regions of many drought- and low-temperature-inducible genes (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2007). All DREB genes feature three conserved regions, a EREBP/AP2 DNA binding domain, an N-terminal nuclear localization signal, and conserved Ser/Thr-rich region adjacent to the EREBP/AP2 domain. DREB TFs play key roles in plant stress signaling transduction pathway, they can specifically bind to DRE/CRT element (G/ACCGAC) and activate the expression of many stress inducible genes.

Although the obtained transgenic crops, mainly wheat, by different types of gene transfer technology all exhibit drought resistance to some extent, they have many shortfalls related to agronomical performance and/or development (Wang et al., 2003; Kern, 2002). These results imply that systemic, deeper and comprehensive understanding of physiological mechanism in crops under drought stress is not enough to manipulate the physiological regulatory mechanism and take advantage of all this potential for productivity, study of which is the bridge between molecular machinery of drought and anti-drought agriculture because the performance of genetic potential of crops is expressed by physiological realization in fields (Shao et al., 2005).

Wheat is a staple food crop for more than 35% of the world population and also one of the widely cultivated crops in Azerbaijan, where drought is the main abiotic stress limiting its grain yields. So wheat anti-drought mechanism study is of great importance to wheat production and biological breeding for the sake of coping with abiotic and biotic conditions. Much research is involved in this hot topic, but the pace of progress is not so large because of drought resistance being a multiple-gene-control quantitative character and wheat genome being larger (16,000 Mb). However, despite all the recent technological breakthroughs, the overall contribution of genomics-assisted breeding to the release of drought-resilient wheat cultivars has so far been marginal (Zhao et al., 2008). The elucidation of genomic regions associated with the expression of traits involved in drought adaptation, the novel genes discovery or the determination of their expression patterns in response to drought stress will provide the basis of effective engineering strategies leading to enhanced wheat germplast for specific agroecological niches. For any molecular assessment to be performed, it is paramount to firstly establish the plant adaptation strategy to overcome drought (Zhao, 2008).

Marker-assisted selection (MAS) provides a strategy for accelerating the process of wheat breeding (Wei et al., 2009). Through marker-assisted breeding (MAB) it is now possible to examine the usefulness of thousands of genomic regions of a crop germplasm under water limited regimes, which was, in fact, previously not possible (Ashraf, 2010). However, conventional markers, such as restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs) and simple sequence repeats (SSRs), used in common wheat, are usually not developed from the genes themselves because the cloning of genes in wheat is complicated by its allohexaploid nature and large genome size. In contrast, functional markers (FMs) are usually designed from polymorphisms within transcribed regions of functional genes. Such markers are completely correlated with gene function (Anderson and Lübberstedt, 2003). Therefore, FMs can dramatically facilitate accurate selection of target genes (Wei et al., 2009).

In the present research screening for drought stress tolerance was conducted in 12 wheat (*Triticum* L.) genotypes using RAPD primers and functional markers based on genome-specific primers for each of the ortologous *Dreb 1* loci on chromosomes 3A, 3B and 3D.

MATERIALS AND METHODS

Plant Materials. A total of 12 wheat genotypes including drought tolerant, semi-tolerant and non-tolerant were used (Table 1). Six of them are known as drought tolerant and extensively are planted by local farmers under drought conditions. Three genotypes were tetraploid (*Triticum durum* L., AABB, 2n = 4x = 28) and nine - hexaploid (*Triticum aestivum* L., AABBDD, 2n = 6x = 42). Different sensitivities of these genotypes to drought have been determined during few years in different regions of Azerbaijan based on grain yield (Aliev, 1998; Aliev, 2001). The plants were provided by Experimental Station of the Research Institute of Crop Husbandry. Leaf samples of all plant materials were harvested from 7 day-old seedlings.

Table 1. Wheat genotypes and their drought tolerance status

No	Genotype name	Ploidity level and genomes	Reaction to drought
		Triticum durum L.	
1	Barakatli-95	Tetraploid (AABB)	Tolerant
2	Garagylchyg-2		Sensitive
3	Gyrmyzy bugda		Tolerant
		Triticum aestivum L.	
4	Azamatli-95	Hexaploid (AABBDD)	Tolerant
5	Giymatli-2/17		Sensitive
6	Gobustan		Tolerant
7	Gyrmyzy gul		Semi-tolerant
8	Tale-38		Semi-tolerant
9	Ruzi-84		Tolerant
10	12nd FAWWON No 97 (130/21)		Sensitive
11	4^{tn} FEFWSN No 50 (130/32)		Semi-tolerant
12	Saratovskaya		Tolerant

DNA extraction. Total genomic DNA was extracted from leaves using CTAB method (Murray and Thompson, 1980) with some modifications. In the growth room 5-7 cm long piece of fresh leaf material was cut from the plants and the leaf tissues were ground in a preheated 2×CTAB extraction buffer (100 mM Tris, pH 8, 1.4 M sodium chloride, and 20 mM EDTA, pH 8.0). Liquid nitrogen ground samples were also processed with CTAB buffer. The samples were incubated for 60 minutes in 60°C water bath with occasional vigorous shaking. The samples were mixed gently after adding 400 µl of chloroform and placed on an orbit shaker for 20 minutes at room temperature. After centrifugation at 5000 rpm, an equal volume of cold absolute isopropanol was added to the supernatant. The solution was well mixed and incubated for 60 minutes at 20°C. The sample was centrifuged for 5 minutes at 5000 rpm to pellet the DNA was followed by washing with 70% alcohol and then dried at 56°C for 5 minutes. DNA was resuspended by adding 300 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

DNA Quantification. After diluting the DNA was quantified by taking the optical density (OD) at λ = 260 with a spectrophotometer ULTROSPEC 3300 PRO (“AMERSHAM”, USA). The purity of genomic DNA was determined by the A260/A280 absorbance ratio. The quality was also examined by running the extracted DNA samples on 0.8% agarose gel stained with 10 mg/ml ethidium bromide in 1×TBE (Tris base, Boric acid, EDTA) buffer. The gel was visualized and photographed under UV light.

Polymerase chain reaction conditions. RAPD-PCR was carried out essentially as described by Williams et al. (1990). Two 10-mer oligonucleotide primers (Eurogentec S.A., Belgique) were used for DNA amplification (Table 2). Amplifications were performed in “Applied Biosystems 2720 Thermal Cycler” first 4 min at 94°C followed by 10 cycles of: 1 min at 94°C, 1 min at 36°C and 1 min at 72°C. After that, for next 35 cycles, 0.2°C was added to annealing temperature. After the final cycle, samples were incubated at 72°C for 15 min and then hold at 4°C prior to analysis.

Table 2. Primer nucleotide sequence used to amplify DNA

Primer designation	Sequence 5′→ 3′
P6	TCGGCGGTTC
P7	CTGCATCGTG

The RAPD fragments were analyzed by electrophoresis on 1.2% agarose gels and detected using ethidium bromide (10 ng/100 mL of agarose solution in Tris-Borate-EDTA buffer). The bands were counted by starting from the top of the lanes to the bottom. All visible and unambiguously scorable fragments amplified by the primers were scored under the heading of total scorable fragments. Amplification profiles of the twelve genotypes were compared with each other, and bands of DNA fragments were scored as present or absent.

To identify polymorphisms in DNA sequences of the *Dreb* gene in each genome, five pairs of genome-specific primers were used for DNA amplification (Wei et al., 2009; http://www.premierbiosoft.com) (Table 3). Genome-specific PCR was performed in a total volume of 20 µl containing 80 ng of genomic DNA, 1 × PCR reaction buffer, 0.25µM of each primer, 0.45 mM of each deoxyribonucleotide, 4.0 mM $MgCl_2$ and 1.6 U of *Taq* DNA polymerase (Sigma, USA).

Table 3. Genome-specific primers used for chromosome assignment of the wheat *Dreb 1* genes

Primers	Sequences (5′→3′)	Chromosome location	Expected size (bp)	Ann. temp. (°C)
P18F	CCCAACCCAAGTGATAATAATCT	3B	717	50
P18R	TTGTGCTCCTCATGGGTACTT			
P20F	TCGTCCCTCTTCTCGCTCCAT	3D	1193	63
P20R	GCGGTTGCCCCATTAGACATAG			
P21F	CGGAACCACTCCCTCCATCTC	3A	1113	63
P21R	CGGTTGCCCCATTAGACGTAA			
P22F	CTGGCACCTCCATTGCCGCT	3D	596	63
P25F	CTGGCACCTCCATTGCTGCC	3A	596	57
PRa	AGTACATGAACTCAACGCACAGGACAAC			

a PR is a public primer matched with P22F and P25F, respectively

The PCR was carried out as follows: initial denaturation at 94°C for 3 min; 34 cycles of 94°C for 1 min, an annealing step at variable annealing temperatures depending on the primer pairs for 1 min, 72°C for 1.5 min; and a final extension at 72°C for 10 min and then held at 4°C prior to analysis. The PCR products were electrophoresized on 2.5% agarose gels, stained with ethidium bromide and visualized under UV light by "Gel Documentation System *UVITEK*".

RESULTS AND DISCUSSION

RAPD-PCR analysis was performed with a subset (12 genotypes) of wheat (*Triticum* L.) genotypes with different levels of drought tolerance (Table 1). P6 and P7 primers associated with drought tolerance were used (Pakniyat and Tavakol, 2007). According to the literature data in tolerant genotypes these primers should produce appropriate fragments.

Figure 1 shows the electrophoretic pattern generated by RAPD primer P6 (TCGGCGGTTC). This primer produced a 920-bp band present in drought tolerant and semi-tolerant genotypes and absent in sensitive durum wheat genotype Garagylchyg-2 and bread wheat genotypes Giymatli-2/17 and Gyrmyzy gul.

This band may be associated with drought stress tolerance in wheat (*Triticum* L.) and may be used in selection of tolerant genotypes in breeding programs.

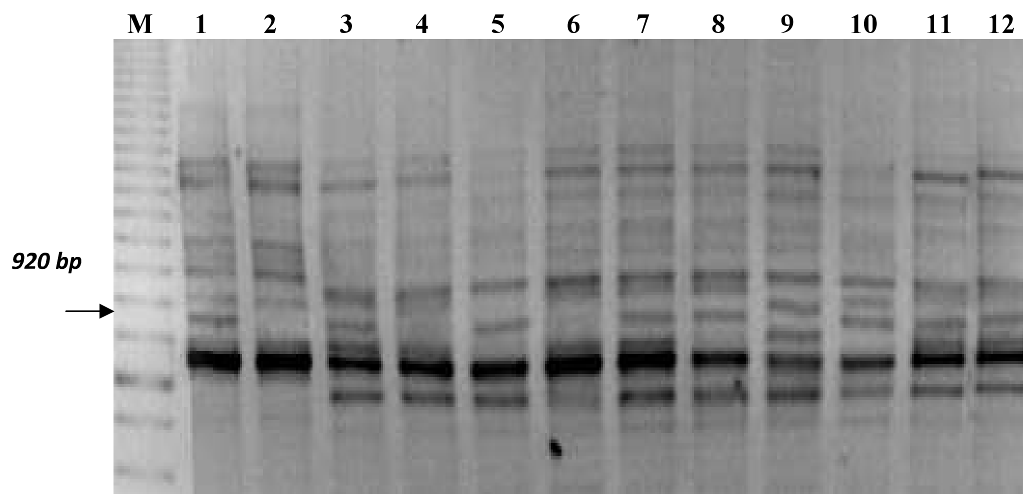

Figure 1. PCR amplification profiles of *Triticum* L. wheat genotypes using P6 primer (5′ TCGGCGGTTC 3′). Arrow shows 920-bp DNA fragment, present in drought tolerant and semi-tolerant wheat genotypes and absent in sensitive ones. M - DNA ladder 100-bp. 1 - Barakatli-95; 2 - Garagylchyg-2; 3 - Azamatli-95; 4 - Giymatli-2/17; 5 - Gyrmyzy bugda; 6 - Gyrmyzy gul; 7 - Tale-38; 8 - Ruzi-84; 9 - 12 nd FAWWON No 97 (130/21); 10 - 4 tn FEFWSN No 50 (130/32); 11 - Nurlu-99; 12 - Gobustan.

It is important to note that 12nd FAWWON No 97 (130/21) is considered to be non-tolerant genotype. However, a 920-bp band is also present in 12nd FAWWON No 97 (130/21) genotype. Probably, it may have a potential for tolerance, but for some reasons expression of these genes are not realized.

It should be noted that P7 primer (TCGGCGGTTC) produces a 750-bp band. Electrophoretic pattern generated by RAPD primer P7 (Figure 2) demonstrates that this band has occurred neither in tolerant genotype Barakatli-95, nor in non-tolerant genotypes Garagylchyg-2 and Giymatli-2/17. Meanwhile, in drought-sensitive genotype Gyrmyzy gul this band is present. Therefore, it was conclude that P7 primer is not absolutely universal for drought tolerance.

Thus, RAPD technology is a powerful tool in quick identifying markers related to drought tolerance in wheat. Results obtained from RAPD-PCR analysis are promising beginning for further research of plant tolerance to water stress on molecular-genetic basis. Marker-assisted selection based on genotype will greatly increase breeding efficiency (Manavalan et al., 2009). Recent advances in wheat researches, ranging from breeding programs to genome sequencing and genomics technologies, provide unprecedented opportunities to understand global patterns of gene expression and their association with the development of specific phenotypes, as well as promising tools for the genetic improvement of plants cultivated in adverse environments by molecular breeding or transgenic approaches.

DREB proteins are also important in determining tolerance or resistance to water deficit. Therefore, we then conducted genome-wide investigation using *Dreb 1* genes as an example. Five pairs of genome-specific primer sets will be useful as FMs to trace each locus during MAS in search of more drought-tolerant wheat genotypes. Primer P25F/PR was designed to amplify a 596-bp DNA fragment downstream of *Dreb-A1* in the A genome. P21F/P21R was selected to amplify an upstream region (a 1113-bp DNA fragment) of the same gene. Primers P22F/PR and P20F/P20R were designed to amplify sequences from the D genome, with the amplifications resulting in 596 and 1193-bp DNA fragments, respectively. The P18F/P18R primer which amplify a 717-bp DNA fragment, were designed as a B genome-specific primer pair (Table 3). To confirm these genome-specific primers, the genomic DNAs of various wheat genotypes were amplified using the primer pairs.

PCR amplification profiles of DNA from *Triticum* L. genotypes with primer pair P21F/P21R are shown in Figure 3. Fragment amplified with this primer in 1113-bp band revealed in all genotypes, excepting semi-tolerant bread wheat genotype Tale-38. However, this band is seemingly indicated in drought-tolerant durum wheat genotypes Barakatli-95. These results indicate that *Dreb 1* gene responsive for the tolerance to drought located in the third chromosome of A genome in these genotypes. 596-bp fragments that amplify by P25F/PR primer were not observed in these samples. Absence of these fragments can be explained by some mutations that, probably, took place in *Dreb 1* gene region, complementary to this primer.

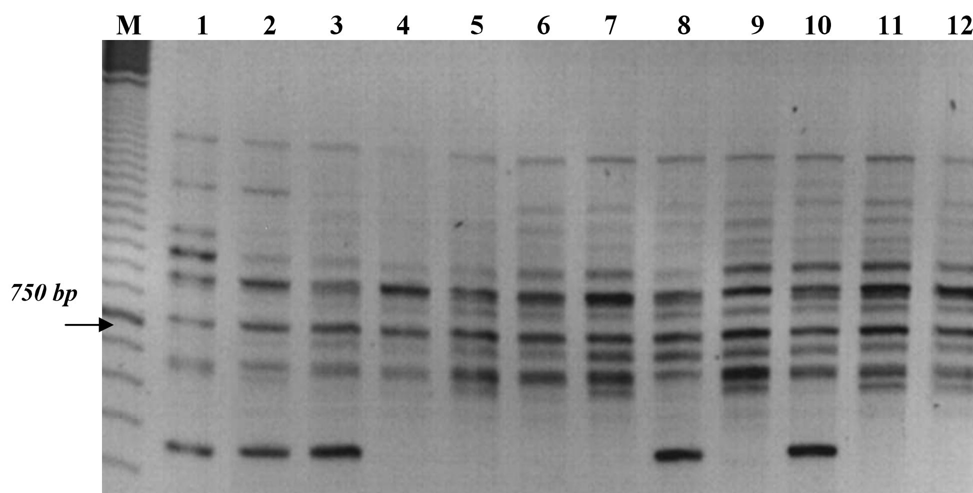

Figure 2. PCR amplification profiles of wheat genotypes *Triticum* L. using a primer P7 (5′ TCGGCGGTTC 3′). Arrow shows a 750-bp band. M - DNA ladder 100-bp. 1-Barakatli-95, 2 - Garagylchyg-2, 3 - Azamatli-95, 4 - Giymatli-2/17, 5 - Gyrmyzy bugda, 6 - Gyrmyzy gul, 7 - Tale-38, 8 – Ruzi-84, 9 - 12 [nd] FAWWON No 97 (130/21), 10 - 4 [tn] FEFWSN No 50 (130/32), 11 - Nurlu-99, 12 - Gobustan.

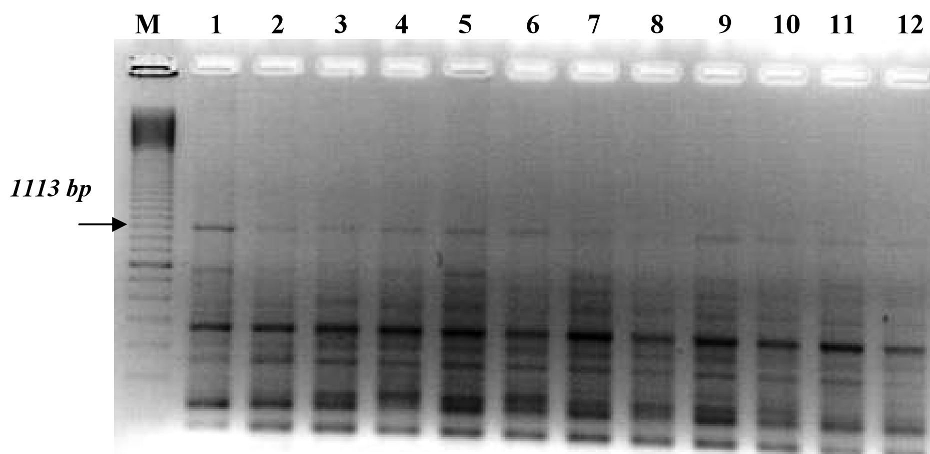

Figure 3. PCR-based chromosome assignments of the *Dreb 1* genes in wheat *Triticum* L. genotypes using an A genome-specific primer pair P21F/P21R. Arrow shows a 1113-bp DNA fragment. M – DNA ladder 100-bp. 1 - Barakatli-95, 2 - Garagylchyg-2, 3 - Gyrmyzy bugda, 4 - Azamatli-95, 5 - Giymatli-2/17, 6 - Gobustan, 7 - Gyrmyzy gul, 8 - Tale-38, 9 - Ruzi-84, 10 - 12nd FAWWON No 97 (130/21), 11- 4th FEFWSN No 50 (130/32), 12 - Saratovskaya.

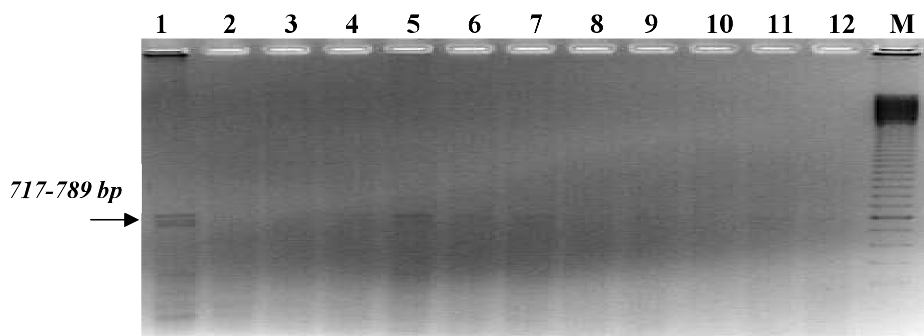

Figure 5. PCR amplification profiles of wheat genotypes *Triticum* L. using a B genom-specific primer pair P18F/P18R. Arrow shows a 717-789-bp DNA fragment. M – DNA ladder 100-bp. 1 - Barakatli-95, 2 - Garagylchyg-2, 3 - Gyrmyzy bugda, 4 - Azamatli-95, 5 - Giymatli-2/17, 6 - Gobustan, 7 - Gyrmyzy gul, 8 - Tale-38, 9 - Ruzi-84, 10 - 12nd FAWWON No 97 (130/21), 11 - 4th FEFWSN No 50 (130/32), 12 - Saratovskaya.

Results obtained from primer pair P18F/P18R, specific for *Dreb 1* gene in B genome, are shown in Figure 5. A 717-789-bp fragment present only in tolerant genotype Barakatli-95. It is interesting to note that this genotype also demonstrates high drought tolerance in other parameters (Aliev, 1998; Aliev, 2001). The absence of 717-789-bp fragments in other wheat genotypes can be explained by the fact that the DREB1 proteins showed the most specific variations in the B genome, including three single amino acid mutations (amino acids 46, 140 and 200) and a deletion of 24 amino acids in a region rich in *Ser* and *Thr* in the orthologous A and D genomes (Wei et al., 2009).

PCR analysis was also performed with P22F/PR and P20F/P20R primers specific for D genome. It is known that hexaploid wheat (*Triticum aestivum* L.) genotypes have D genome. However, in our experiments there were not any appropriate fragments amplified by these primers. This data indicates that hexaploid wheat genotypes, possibly, were nulliosomics, i.e. chromosome pair which has a *Dreb 1* gene is absent in their D genomes.

Hence, using functional markers was identified the presence of *Dreb 1* gene located in A genome for 3 tetraploid and 8 hexaploid wheat genotypes. Unlike other genotypes *Dreb 1* gene was also revealed in B genome in tetraploid genotype

Barakatli-95.

Thus, understanding the functions of these stress-inducible genes helps to unravel the possible mechanisms of stress tolerance. Marker-assisted selection was also employed to improve the staygreen trait involved in drought tolerance of wheat. The obtained results open an excellent opportunity to develop stress tolerant crops in future. These results may be helpful in wheat breeding programs aimed at improving drought tolerance.

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