

DOI: 10.5586/aa.1746

Publication history

Received: 2018-07-13

Accepted: 2018-10-03

Published: 2018-12-14

Handling editor

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Authors' contributions

RV, IR, DanA, DamA, NV: idea of the study; RV, IR, DamA, NV: conducting the experiment; RV, DanA, DamA: data analysis and drafting the manuscript; RV, IR, NV: revising the manuscript

Funding

This research was supported by FASO Russia project AAAA-A18-118031390148-1.

Competing interests

No competing interests have been declared.

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ORIGINAL RESEARCH PAPER

Screening of the genotypes of bread wheat (*Triticum aestivum* L.) by the allelic variants of *Waxy* genes and HMW glutenin subunits

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Screening genotypes of wheat by allelic variants of *Waxy* and *HMW-GS* genes is a constitutional unit of marker-assisted selection of varieties with high values of desirable properties for flour-baking and technological attributes of the grain. The aim of the study was to produce a molecular screening of samples of spring soft wheat of local selection lines for the detection of genotypes of *Triticum aestivum* L. with valuable alleles of *Waxy* and *HMW-GS* genes. Seventy samples of wheat were subjected to DNA testing for the identification of genotypes with alleles of *Waxy* and *HMW-GS* genes. Molecular screening of wheat samples with the selected systems of molecular marking of the allelic variants of the analyzed genes made it possible to detect three partially *Waxy* lines with a combination of two null-alleles (*Wx-A1b* and *Wx-B1b*) in the study collection, as well as 33 plants with an economically-valuable combination of Ax2*/5+10 subunits of *HMW-GS*, the genotypes of which are considered as starting material for further selection work on the creation of spring soft wheat cultivars with high quality indicators of grain. The approaches applied in the study to identify *Waxy* and *HMW-GS* alleles are effective methods of evaluating their allelic polymorphism.

Keywords

grain; starch; gluten; genotype; identification; PCR; RFLP

Introduction

Screening wheat genotypes for allelic variants of *Waxy* [1–3] and HMW glutenin subunits (*HMW-GS*) [4–6] is an integral part for marker-assisted selection of varieties with high value as indicators for flour-baking and technological properties of the grain. It is known that combinations of nonfunctional null alleles of *Waxy* genes in the genomes of wheat have a direct influence on the development of amylopectin-type starch with a reduced or complete absence of amylose, which, in turn, considerably affects the technological properties of starch and wheat flour [7–9]. Taking into account the positive influence of HMW glutenin subunits Dx5, Dy10 (5+10), and Ax2* on the increase of flour-baking qualities, as well as the negative influence of subunits Dx2, Dy12 (2+12), and Axnull causing a reduction in baking properties of wheat, the combination of Ax2*/5+10 subunits is one of the most economically-valuable and desirable for the selection associated with high quality properties of the grain [4,5,10].

Currently, molecular genetic methods are the most accurate tools for assessing allelic polymorphism of *Waxy* [1,8,11] and *HMW-GS* [5,6,10] genes. The systems of molecular marking of allelic variants of *Waxy* [1,12,13] and *HMW-GS* [6,10,14] of wheat using DNA technologies selected for allelic polymorphism of genes analyzed make it possible to identify *Triticum aestivum* L. genotypes and so expand the range of donors and sources of economically-valuable alleles. In general, DNA technologies are integrated into fundamental and applied research aimed at the search, mobilization, and preservation of genetic resources of cultivated plants and their wild types [15–17]. The purpose here is to study, preserve, and apply to the biodiversity of cultivated plant forms, as well as managing selection of new genotypes with high economical productivity and resistance against bio- and abiotic stressors.

The main aim of the present study was a molecular screening of spring soft wheat samples from the collection of the Tatar Scientific Research Institute of Agriculture in order to detect potentially useful *T. aestivum* genotypes bearing allelic variants of *Waxy* and *HMW-GS* genes, valuable for selection and associated with high values of indicators for flour-baking and technological properties of the grain.

Material and methods

Seventy samples of wheat [68 samples of spring soft wheat and two varieties of winter soft wheat (cultivars ‘Sila’ and ‘Starshina’)] from the collection of the Tatar Scientific Research Institute of Agriculture (TatSRIA) were subjected to DNA testing to identify *T. aestivum* genotypes with allelic variants of *Waxy* and *HMW-GS* genes.

Nucleic acid extraction from wheat grains at milky-wax ripeness of a 2017 generation was performed using a commercial set of DNA-sorb C (Central Scientific Research Institute of Epidemiology, Russia), in accordance with the manufacturer’s instructions. The principle of the method used for the isolation of nucleic acids consists of treating a sample (one ground wheat grain) with a lysis solution with proteinase K, which results in the destruction of cell membranes and the release of nucleic acids and cellular components. Dissolved nucleic acids bind to sorbed particles while other components of the lysed test material remain in the solution and are removed after the sorbent’s precipitation by centrifugation and subsequent washing. When the buffer is added to the sorbent for elution, the nucleic acid transfers from the surface of the silicate into the solution, which is then separated from the sorbent particles by centrifugation. This procedure results in a highly purified nucleic acid preparation free of inhibitors of the amplification reaction, which provides a high analytical sensitivity in the PCR study. The screening of wheat lines and varieties was performed with the application of the selected systems of molecular marking of the alleles of the gene loci analyzed (Tab. 1).

The selected 4F+4R [1,12] and 4Fc+4R [18] primer sets were intended for complex identification of allelic variants of *T. aestivum* *Waxy* genes, whereas AFC+AR2 [13] and 4Fc+Wx-B2R [18,19] were intended to identify allelic variants *Wx-A1* and *Wx-B1* loci with diagnostically significant discrimination of *Wx-A1b* and *Wx-B1b* alleles, respectively.

Other selected primer sets of the UMN series [10]: UMN19F+UMN19R (*Ax1/Axnull* and *Ax2** alleles of the *Glu-A1* locus), UMN25F+UMN25R (*Dx2* and *Dx5* alleles of the *Glu-D1* locus), UMN26F+UMN26R (*Dy10* and *Dy12* alleles of the *Glu-D1* locus) were intended for the identification of allelic variants of *HMW-GS T. aestivum*, whereas Axnull-F+Axnull-R [20] was used for the discrimination of the *Axnull* allele of the *Glu-A1* locus, respectively.

The amplification of the genomic DNA was conducted on thermocyclers Tertsik (DNA-technology, Russia), PTC-200 (MJ Research, Canada) and MyCycler with a gradient (Bio-Rad, USA) in 20 µL volume containing buffer [60 mM Tris-HCl (pH 8.5), 1.5 mM MgCl₂, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1 mM triton X-100], 0.25 mM dNTP, 1 unit Taq DNA polymerase (SibEnzyme, Russia), and 0.5 µM corresponding pairs of synthesized primers (DNA-synthesis, Russia) (Tab. 1). As a confirmatory test [18], after the stage of PCR with primers UMN19F+UMN19R, UMN25F+UMN25R, and UMN26F+UMN26R, a RFLP analysis with endonuclease cleavage of amplicons by *HaeIII* restriction enzyme was performed in buffer G (SibEnzyme, Russia) at 37°C for 3 h.

Tab. 1 The primers used, regimes of PCR amplification, and RFLP analysis for the identification of alleles of *Waxy* and *HMW-GS* genes of *T. aestivum*.

Names and sequences of oligonucleotide primers	Loci of genes (alleles)	Regimes of PCR amplification	RFLP analysis
4F: 5'-AAGAGCAACTACCAGT-3' 4R: 5'-TCGTACCCGTCGATGAAGTCGA-3' [1,12]	<i>Wx-A1, Wx-B1,</i> <i>Wx-D1</i>	×1: 94°C – 4 min; ×40: 94°C – 30 s, 58°C – 30 s, 72°C – 30 s; ×1: 72°C – 7 min	
4F-c: 5'-CCCCAAGAGCAACTACCAGT-3' 4R: 5'-TCGTACCCGTCGATGAAGTCGA-3' [18]	<i>Wx-A1, Wx-B1,</i> <i>Wx-D1</i>	×1: 94°C – 4 min; ×40: 94°C – 30 s, 64°C – 30 s, 72°C – 30 s; ×1: 72°C – 7 min	
4F-c: 5'-CCCCAAGAGCAACTACCAGT-3' Wx-B2R: 5'-CGTTGACGATGCCGGTGTG-3' [18,19]	<i>Wx-B1 (B1b)</i>	×1: 94°C – 4 min; ×40: 94°C – 15 s, 65°C – 15 s, 72°C – 15 s; ×1: 72°C – 7 min	
AFC: 5'-TCGTGTTTCGTCGGCGCCGAGATGG-3' AR2: 5'-CCGCGCTTGTAGCAGTGGAAGTACC-3' [13]	<i>Wx-A1 (A1b)</i>	×1: 94°C – 4 min; ×40: 94°C – 30 s, 65°C – 30 s, 72°C – 1 min; ×1: 72°C – 7 min	
Axnull-F: 5'-ACGTTCCCCTACAGGTACTA-3' Axnull-R: 5'-TATCACTGGCTAGCCGACAA-3' [20]	<i>Glu-A1 (Axnull)</i>	×1: 94°C – 4 min; ×40: 94°C – 1 min, 58°C – 1 min, 72°C – 1 min; ×1: 72°C – 7 min	
UMN19F: 5'-CGAGACAATATGAGCAGCAAG-3' UMN19R: 5'-CTGCCATGGAGAAGTTGGA-3' [10,18]	<i>Glu-A1 (Ax1/ Axnull, Ax2*)</i>	×1: 94°C – 4 min; ×40: 94°C – 30 s, 60°C – 30 s, 72°C – 30 s; ×1: 72°C – 5 min	<i>HaeIII</i> 37°C, 3 h
UMN25F: 5'-GGGACAATACGAGCAGCAAA-3' UMN25R: 5'-CTTGTTCGGTTGTTGCCA-3' [10,18]	<i>Glu-D1 (Dx2, Dx5)</i>	×1: 94°C – 4 min; ×40: 94°C – 30 s, 60°C – 30 s, 72°C – 30 s; ×1: 72°C – 5 min	<i>HaeIII</i> 37°C, 3 h
UMN26F: 5'-CGCAAGACAATATGAGCAAAC-3' UMN26R: 5'-TTGCCTTTGTCTGTGTGC-3' [10,18]	<i>Glu-D1 (Dy10, Dy12)</i>	×1: 94°C – 4 min; ×40: 94°C – 30 s, 60°C – 30 s, 72°C – 30 s; ×1: 72°C – 7 min	<i>HaeIII</i> 37°C, 3 h

An electrophoretic detection of PCR products and RFLP fragments was conducted in 2–3% agarose gels in TBE buffer containing ethidium bromide followed by visualization of amplicons in UV-transilluminator ($\lambda = 310$ nm), the dimensions of which were evaluated by mobility by comparison with standard DNA markers (SibEnzyme, Russia).

Results

As a result of the DNA testing of the 70 samples in order to identify *T. aestivum* genotypes by allelic variants of *Waxy* genes, it was established that 46 samples (65.7%) had a combination of active alleles *Wx-A1a/B1a/D1a* (first type), two samples: lines K-139-08-1 and K-311-08-1 (2.9%) – combination *Wx-A1g/B1a/D1a* (first type), by the classification of wheat types with various content of *Waxy* genes, 10 samples (14.3%) belonged to the second type (*Wx-A1b/B1a/D1a*), eight samples (11.4%) – to the third type (*Wx-A1a/B1b/D1a*), three samples: lines K-243-13Wx-2, K-243-13Wx-4, and K-243-13Wx-6 (4.3%) – to the seventh type (*Wx-A1b/B1b/D1a*). One line, K-242-13Wx-10 (1.4%), manifested a heterozygous state of the *Wx-A1* locus (*Wx-A1a-b/B1a/D1a*) (Fig. 1, Tab. 2).

Among the analyzed wheat samples, three lines (K-243-13Wx-2, K-243-13Wx-4, and K-243-13Wx-6), simultaneously carrying a combination of two null alleles *Wx-A1b* and *Wx-B1b* (Tab. 2) in their genomes, are potentially the most promising genotypes considered as a starting material for further selection work on the creation of spring soft wheat with amylopectin-type starch. These three lines are the result of the crossing of cv. 'Starshina' winter soft wheat carrying the *Wx-A1b* allele with the O-192-03-5 line of spring soft wheat which is a carrier of the *Wx-B1b* allele.

Molecular genetic evaluation for the identification of genotypes by allelic variants of *Glu-A1* locus of *HMW-GS* established that 12 (17.1%) from the 70 wheat samples

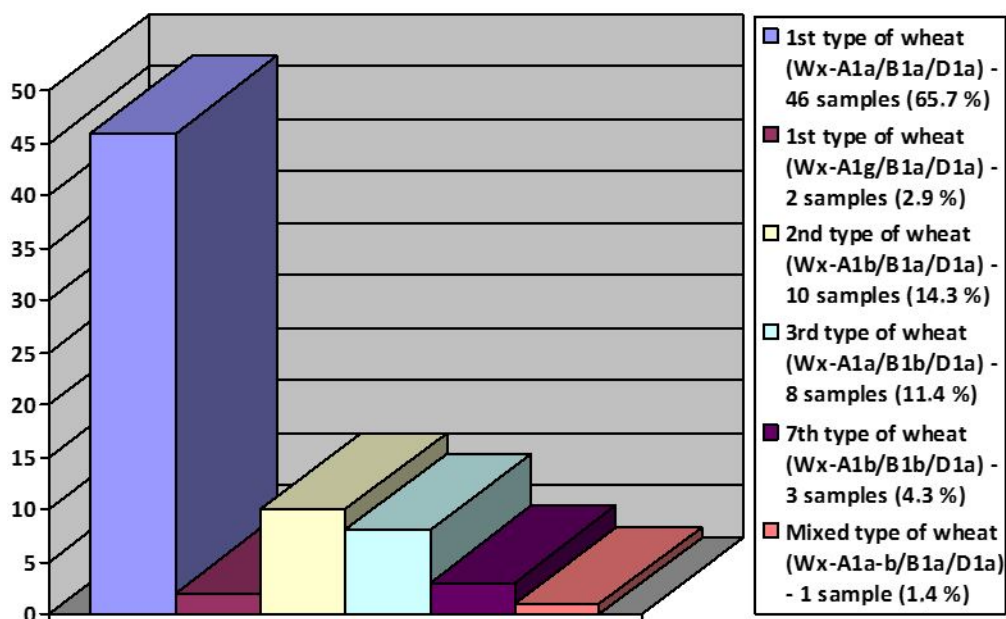


Fig. 1 Distribution of genotypes of *T. aestivum* identified by allelic variants of *Waxy* genes from wheat types with corresponding combinations of alleles of *Waxy* genes. Note: the y axis indicates the number of genotypes; the x axis indicates the types of wheat (combinations of alleles).

analyzed had only subunit Ax1 encoded by the allelic variant *Glu-A1a* (Ax1 allele); 57 samples (81.4%) had only subunit Ax2* encoded by the allelic variant *Glu-A1b* (Ax2* allele). One line, K-214-13Wx-5 (1.4%), manifested a heterozygous state of the *Glu-A1* locus (Glu-A1a-b) (Tab. 2).

An illustrative example of PCR identification of *T. aestivum* genotypes by the range of allelic variants of *HMW-GS* and *Waxy* loci is presented in Fig. 2.

The reliability of the results of PCR identification of *T. aestivum* genotypes by allelic variants of loci *Glu-A1* and *Glu-D1* of *HMW-GS* with the use of UMN series sets of primers is also confirmed by the RFLP analysis with endonuclease cleavage of amplicons by *HaeIII* restriction enzyme.

During the assessment these samples of wheat by the *Glu-D1* locus of *HMW-GS*, it was established that 44 of them (62.9%) were characterized by the presence of a combination of subunits Dx5 and Dy10 (5+10) encoded by allelic variant *Glu-D1d* (Dx5 and Dy10 alleles), and 26 samples (37.1%) had a combination of subunits Dx2 and Dy12 (2+12) encoded by allelic variant *Glu-D1a* (Dx2 and Dy12 alleles) (Tab. 2).

The distribution of the genotypes identified by the combination of subunits of *Glu-A1/D1* loci of *HMW-GS* was as follows: Ax2*/5+10 = 33 (47.1%); Ax2*/2+12 = 24 (34.3%); Ax1/5+10 = 10 (14.3%); Ax1/2+12 = 2 (2.9%); Ax1+Ax2*/5+10 = 1 (1.4%) (Fig. 3).

Thus, there is an established predominance of the combination of subunits Ax2*/5+10 desirable for the selection on flour-baking qualities of grain over other less economically-valuable combinations (Fig. 3).

Discussion

Molecular genetic study identified genotypes of *T. aestivum* from the collection of the Tatar Scientific Research Institute of Agriculture by allelic variants of *Waxy* and *HMW-GS* genes, as well as revealing more potentially useful samples of wheat, the screening of which was performed by the use of selected PCR systems for molecular marking of the gene loci analyzed (Tab. 1, Fig. 2).

Inactive null alleles of loci *Wx-A1*, *Wx-B1*, and *Wx-D1* of *Waxy* genes of *T. aestivum* have a direct influence on the formation of amylopectin-type starch (with a low content of amylose) [7,8,11].

Tab. 1 Continued

No.	Variety/line	Waxy										HMW-GS								
		AI			BI			DI			AI		Ax2*		5+10		2+12			
		a	b	g	a	b	e	a	a	b	a	b	Ax1	Ax2*	a	b	a	b		
31	K-90-08-4	+	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-
32	2004R-177-4-7-2	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-
33	Ecada 231 milr	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-
34	Ksh-359-11-12	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-
35	Ksh-358-11-10	+	-	-	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-

Note: “+” – presence of the corresponding allele; “-” – absence of the corresponding allele.

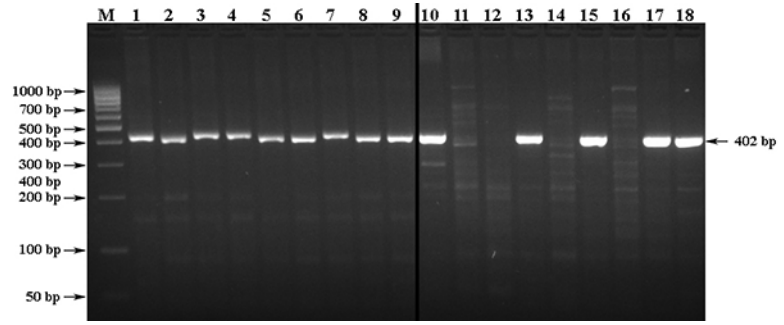


Fig. 2 Electrophoregram of PCR identification of *T. aestivum* genotypes by alleles *Dy10* and *Dy12* *Glu-D1* locus of *HMW-GS* (primers UMN26F and UMN26R) and alleles *Wx-B1* locus of *Waxy* gene (primers 4F-c and *Wx-B2R*).

It is noteworthy that out of the 70 samples analyzed, the great majority of plants (65.7%) were characterized by a combination of active alleles *Wx-A1a/B1a/D1a* traditional for the first type of wheat and the rare combination *Wx-A1g/B1a/D1a* of the same type in two lines (2.9%) (Fig. 1, Tab. 2).

The principle of allelic discrimination of *Wx-A1g* from *Wx-A1a* was based on the absence of PCR product of 262 bp size in our modified method of genotyping with primers 4F-c and 4R, as well as the presence of amplified fragment *Wx-A1g* of 257 bp size in the setting up of PCR reactions with primers 4F and 4R [18].

In total, there were 18 wheat samples that carried single null-alleles of *Waxy* genes, 10 of which (14.3%) belonged to the second type of wheat (*Wx-A1b/B1a/D1a*), eight samples (11.4%) to the third type (*Wx-A1a/B1b/D1a*). Moreover, by the classification of wheat types by various contents of *Waxy* genes, three lines belonged to the seventh type (*Wx-A1b/B1b/D1a*) with the simultaneous combination of two null alleles – *Wx-A1b* and *Wx-B1b* (Fig. 1, Tab. 2).

The three lines developed as a result of marker-assisted selection by the crossing of winter soft wheat ‘Starchina’ (carrier of the null-allele *Wx-A1b*), courtesy of the National Center of Grain named after P. P. Lukyanenko (Russia, Krasnodar) [2], with the O-192-03-5 line of spring soft wheat (carrier of the null-allele *Wx-B1b*) [19] are assessed as more useful genotypes followed by the insertion of null alleles of the *Wx-D1* locus (*Wx-D1b*, *Wx-D1d*, or *Wx-D1e*) [21,22] into their genomes in order to obtain cultivars of *Waxy* wheat. The allelic discrimination of *Wx-B1b* from *Wx-B1a* and *Wx-B1e* was achieved using the PCR method developed by us with primers 4F-c and *Wx-B2R* [18,19], generating in comparison to the prototype [12], PCR products reduced by 61 bp with a length of 402 bp (*Wx-B1a* allele) and 436 bp (*Wx-B1e* allele), providing a better separation of amplified fragments in agarose gel and, accordingly, increased accuracy of the interpretation of the genotyping results.

It is well known that winter soft varieties are widely used in the local selection of spring soft wheat in order to increase the genetic potential of productivity [23]. Given the positive influence of allelic variants *Glu-D1d* (5+10) and *Glu-A1b* (Ax2*) *HMW-GS* on the increase of flour-baking qualities, the competitive advantage of *Glu-A1b* over *Glu-A1a* (Ax1), and the negative influence of alleles *Glu-D1a* (2+12) and *Glu-A1c* (Axnull) causing a decrease in flour-baking properties of wheat, the combination Ax2*/5+10 [18] desirable for the selection is one of the most economically-valuable combinations of *HMW* glutenin subunits.

The analysis of the distribution of *T. aestivum* genotypes identified by the combination of subunits of *Glu-A1/D1* loci of *HMW-GS* showed the predominance of this very desirable combination (Ax2*/5+10 = 47.1%) over the other ones (Ax2*/2+12 = 34.3%; Ax1/5+10 = 14.3%; Ax1/2+12 = 2.9%; Ax1+Ax2*/5+10 = 1.4%) (Fig. 3, Tab. 2), which is, to a certain

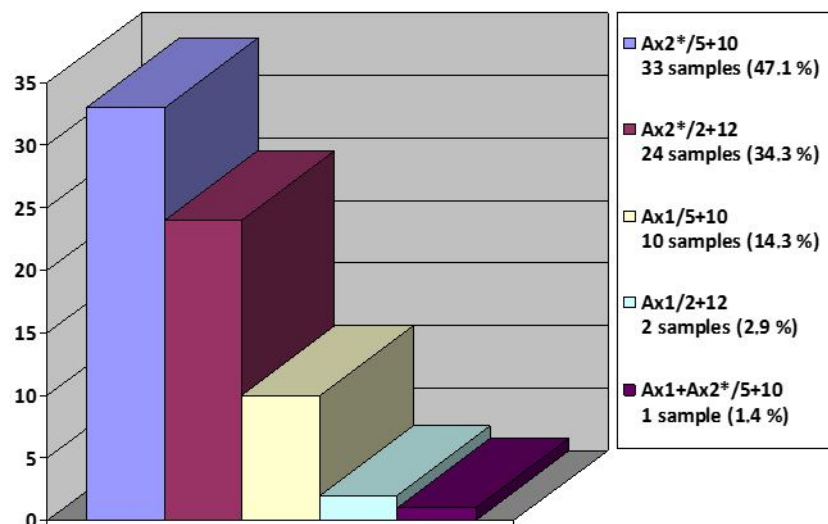


Fig. 3 The distribution of the genotypes of *T. aestivum* identified by the combination of subunits of *Glu-A1/D1* loci of *HMW-GS*. Note: the y axis indicates the number of genotypes; the x axis indicates the combinations of subunits.

degree, typical of most of the lines and cultivars of spring soft wheat in this Russian selection [18,24].

The well-known primers used in the present work: UMN19F+UMN19R (*Ax1/Axnull* and *Ax2** alleles), UMN25F+UMN25R (*Dx2* and *Dx5* alleles) and UMN26F+UMN26R (*Dy10* and *Dy12* alleles) [10], were constructed for PCR identification of allelic variants of *HMW-GS* of *T. aestivum*. This was mainly by the capillary and vertical gel-electrophoresis detection methods in PAGE, which showed the effectiveness of their use in the application of PCR with the detection of the results by the method of horizontal electrophoresis in agarose gel [24].

The reliability of our results of PCR identification of the wheat genotypes studied by the use of primer sets of the series UMN [10] was further confirmed by our proposed procedure for RFLP analysis by restriction endonuclease *HaeIII*, providing a correct interpretation of generating PCR-RFLP fragments at their electrophoretic detection in agarose gel [18].

Over the past 5 years, a total of 223 samples of *T. aestivum* have been studied from the collections of the Tatar Scientific Research Institute of Agriculture, using allelic variants of *Waxy* and *HMW-GS* genes [18,19,24]. The collection includes potentially useful *T. aestivum* genotypes – carriers of valuable null alleles *Wx-A1b* and *Wx-B1b*, as well as rare active alleles of *Wx-A1g* and *Wx-B1e* *Waxy* genes including the genotypes with an economically-valuable combination *Ax2*/5+10* *HMW-GS*, which is of potentially high interest not only to Russian, but also foreign breeders. However, unlike previous studies [18,19,24] performed in the course of marker-assisted selection, new wheat genotypes with a combination of two null alleles (*Wx-A1b* and *Wx-B1b*) of *Waxy* genes have been revealed by us for the first time. The new genotypes of *T. aestivum* presented in this study expand the circle of donors and sources of economically-valuable alleles of *Waxy* and *HMW-GS* genes which indicates the importance of the results of our research reported here.

The methods developed by us to identify allelic variants of *Waxy* and *HMW-GS* genes of wheat on the basis of PCR and RFLP analysis, which were used in the current work, are effective approaches for the assessment of their allelic polymorphism expanding the arsenal of systems of molecular marking of alleles of the genes analyzed.

In the course of any expanded screening of the world collection of *T. aestivum* genotypes from various geographic zones, the selection of systems of molecular marking of allelic variants of *Waxy* [8,21,25] and *HMW-GS* [6,14] should be carried out taking into the account our current knowledge of allelic polymorphism of the such genes.

Conclusion

Molecular screening of lines and cultivars of wheat in the local collection studied by selection realized with the use of the selected systems of molecular marking of allelic variants of the loci of genes analyzed made it possible to establish the presence of three partially *Waxy* lines (K-243-13Wx-2, K-243-13Wx-4, K-243-13Wx-6), with a simultaneous combination of two null alleles (*Wx-A1b* and *Wx-B1b*). Additionally, 33 plants with an economically-valuable combination of subunits Ax2*/5+10 of *HMW-GS* were identified whose genotypes are considered as a starting material for further breeding new wheat varieties with high quality indicators of grain quality. These potentially useful wheat genotypes add to the circle of donors and sources of economically-valuable alleles of *Waxy* and *HMW-GS* genes and have a high potential to meet the demands of national and foreign plant breeders.

Methods of identification of allelic variants of *Waxy* and *HMW-GS* genes of wheat used in this work, also developed by us, are effective tools for assessing their allelic polymorphism. However, given the new knowledge of allelic polymorphism of the genes analyzed, the arsenal of systems for molecular marking must be expanded further in the course of the screening of the world collection of *T. aestivum* genotypes in various geographic zones.

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Porównanie genotypów pszenicy pszennej (*Triticum aestivum* L.) pod względem występowania wariantów allelicznych genów *Waxy* i podjednostek gluteninowych HMW

Streszczenie

Analiza genotypów pszenicy pod względem występowania wariantów allelicznych genów *Waxy* i *HMW-GS* jest kluczowa dla selekcji odmian o pożądanych wartościach wypiekowych i parametrach technologicznych ziarna. Celem badań była analiza molekularna 70 lokalnych linii jarej pszenicy zwyczajnej w celu wykrycia genotypów *Triticum aestivum* L. z allelami genów *Waxy* i *HMW-GS*. Badanie prób pszenicy za pomocą systemu selekcji wspomaganej markerami molekularnymi dla wariantów allelicznych analizowanych genów pozwoliło na wykrycie trzech linii z kombinacją dwóch niemych alleli (*Wx-A1b* i *Wx-B1b*), a także 33 roślin o ekonomicznie istotnej kombinacji

podjednostek Ax2*/5+10 *HMW-GS*, których genotypy są uważane za materiał wyjściowy do dalszych prac selekcyjnych nad tworzeniem odmian jarej pszenicy zwyczajnej o wysokiej jakości parametrów ziarna. Zastosowane w badaniach podejście do identyfikacji alleli *Waxy* i *HMW-GS* może być skuteczną metodą do oceny ich polimorfizmu allelicznego.