Allelic variants for *Waxy* genes in common wheat lines bred at the Lukyanenko National Grain Center

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This article presents the results of a molecular marker-assisted study of allelic variants of *Wx* genes in common wheat (*Triticum aestivum* L.) lines. The study was carried out as part of the work on the transfer of null alleles of the genes *Wx-A1*, *Wx-B1*, and *Wx-D1* to the varieties of soft wheat and creation of breeding material with modified activities of the main enzymes involved in amylase biosynthesis. The lines were obtained at the Department of Breeding and Seed Production of Wheat and Triticale, National Center of Grain named after P.P. Lukyanenko, by crossing mutant forms carrying inactive (null) alleles of genes *Wx-A1*, *Wx-B1*, and *Wx-D1* with bread wheat cultivars. The molecular markers selected for the study allowed identification of valuable breeding material carrying both single null alleles of *Wx* genes and their combinations in its genome. A combination of two null alleles (*Wx-A1b* + *Wx-D1b*) was detected in 30 lines. The presence of three null alleles (*Wx-A1b* + *Wx-B1b* + *Wx-D1b*), which corresponded to fully Wx wheat, was found in one line. We selected 37 lines that combined the presence of the *Wx-B1e* allele with the *Wx-A1b* and *Wx-D1b* null alleles. The *Wx-A1b* + *Wx-B1e* combination was identified in 26 lines, and 24 lines carried the combination of alleles *Wx-B1e* + *Wx-D1b*. The mutant forms PI619381, PI619384, and PI619386 were identified as carriers of the functional *Wx-B1e* allele. The *Wx-A1b* and *Wx-B1e* alleles could have been transferred to the studied lines from the donors used or from the Starshina and Korotyshka varieties, respectively. The mutant forms used in the crosses are donors of the *Wx-B1b* and *Wx-D1b* alleles. The use of molecular markers chosen by us for identification of the allelic state of the *Wx-A1*, *Wx-B1*, and *Wx-D1* genes can provide grounds for marker-assisted selection for this trait. Selected lines found to possess null alleles of the *Wx* genes are applicable in breeding programs aimed at the improvement of technological qualities of grain and raise of bread wheat varieties with modified starch properties.

Key words: common wheat; molecular markers; *Wx* genes.

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Изучение линий мягкой пшеницы
селекции Национального центра зерна им. П.П. Лукьяенко
по аллельным вариантам генов *Waxy*

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В статье представлены результаты изучения с помощью молекулярных маркеров линий мягкой пшеницы (*Triticum aestivum* L.) по аллельным вариантам генов *Wx*. Исследование проводили в рамках работ по передаче нуль-аллелей генов *Wx-A1*, *Wx-B1*, *Wx-D1* в сорта мягкой пшеницы и созданию селекционного материала с измененной активностью основных ферментов, участвующих в биосинтезе амилозы. Линии получены в отделе селекции и семеноводства пшеницы и тriticале Национального центра зерна им. П.П. Лукьяенко в результате скрещивания мутантных форм – носителей неактивных (нуль-аллелей) генов *Wx-A1*, *Wx-B1*, *Wx-D1*, с коммерческими сортами мягкой пшеницы. Отобранные для работы молекулярные маркеры позволили выявить ценный селекционный материал, имеющий в своем геноме как единичные нуль-аллели генов *Wx*, так и их комбинации. Сочетание двух нуль-аллелей (*Wx-A1b* + *Wx-D1b*) обнаружено у 30 линий. На линии трех нуль-аллелей (*Wx-A1b* + *Wx-B1b* + *Wx-D1b*), что соответствует полностью *Wx*-пшеницам, обнаружено у одной линии. Отобрано 37 линий, сочетающих присутствие аллелей *Wx-B1e* с нуль-аллелями *Wx-A1b* и *Wx-D1b*. У 26 линий идентифицирована комбинация (*Wx-A1b* + *Wx-B1e*), 24 линии имели сочетание аллелей (*Wx-B1e* + *Wx-D1b*). Идентифицировано, что мутантные формы PI619381, PI619384, PI619386 – носители функционального аллеля *Wx-B1e*. Аллели *Wx-A1b* и *Wx-B1e* могли быть переданы в изучаемые линии как от используемых доноров, так и от сортов Старшина и Коротышка соответственно. Донорами привнесения в линии аллелей *Wx-B1b* и *Wx-D1b* служат использованные в скрещиваниях мутантные формы. Применение
Introduction

An urgent task of common wheat breeding programs is the creation of varieties with improved technological qualities of grain.

A starch fraction of about 70 % of the total dry matter in wheat grain can significantly affect the quality of the final use of common wheat flour (Zeng et al., 1997). The broad use of wheat starch in the chemical and food industries is due to its properties. These include: hygroscopicity, neutrality of taste, good tolerance of heat treatment, moderate viscosity, and emulsion stabilization (Maningat, Seib, 1997). One of the interesting properties is the ability of grains to swell in warm liquid. Another distinctive feature is the ability to form pastes stable under thermal stress or long-term storage (Maningat et al., 2009).

Starch quality is closely related to the ratio between amylose and amyllopectin, the two major constituents of starch. Granule-bound starch synthase (GBSSI) is the enzyme responsible for amylose synthesis in wheat grain. As some important technological properties of starch, such as gelatinization, bonding, and gelation, depend on the amylose/amyllopectin ratio (Zeng et al., 1997). The GBSSI enzyme, or Waxy protein, has been the subject of many studies in recent years. In common wheat, this enzyme is encoded by three homologous Waxy (Wx) genes located on chromosomes 7A (locus Wx-A1), 7D (Wx-D1), and 4A (Wx-B1), (Shure et al., 1983; Chao et al., 1989; Yamamori et al., 1994). Each of the Wx genes has several allelic variants. The most common wild-type alleles are called Wx-A1a, Wx-B1a, and Wx-D1a (Yamamori et al., 1994; Nakamura et al., 1995). These alleles carry no mutations, and they intensely express GBSSI protein. Another type of allele (null allele) is nonfunctional and less common. It leads to a decrease in amylose content in starch. It is known that the Wx-B1 gene has the greatest effect on amylose content in common wheat starch, followed by Wx-D1 and Wx-A1 (Yamamori et al., 1994). Functional alleles different from those of the wild type were also isolated, but their effects remain poorly understood. The presence of three null alleles for the Wx-A1, Wx-B1, and Wx-D1 genes leads to complete elimination of GBSSI, absence of amylose synthesis, and the formation of amyllopectin-type starch (Nakamura et al., 2002).

At present, molecular marking methods are in broad use to identify the allelic state of Wx genes. They make it possible to detect various alleles of Wx genes, including null alleles, and can be used as the basis for breeding programs aimed at producing common wheat with a modified amylose/amyllopectin ratio (Nakamura et al., 1995; Kiribuchi-Otobe et al., 1997).

Earlier, M.V. Klimushina et al. (2012) applied molecular markers to a study of the allelic composition of Wx genes in 99 varieties and lines of common wheat bred at the Lukyanenko National Grain Center (NGC). They found that most accessions had wild-type alleles (which do not reduce amylose content in starch). The data obtained motivated the work on the transfer of null alleles of the Wx-A1, Wx-B1, and Wx-D1 genes to common wheat varieties of NGC and the creation of breeding material with altered activities of the main enzymes involved in amylose biosynthesis.

This article presents the results of molecular marker-assisted analysis of F1 lines of common wheat for allelic variants of Wx genes. The purpose of the work was to select valuable genotypes carrying both individual null alleles and their combinations for their subsequent involvement in the breeding process aimed at obtaining varieties with improved technological qualities of grain.

Materials and methods

The study was conducted with 502 lines of common wheat generation F6. The lines were obtained at NGC by crossing carriers of inactive (null) alleles Wx-A1, Wx-B1, Wx-D1 to commercial varieties of common wheat. The mutant forms PI619381, PI619384, PI619376, PI619386, PI619377, and PI619378, in which the functional Waxy protein was not synthesized, were used as null alleles donors. These wheat forms were obtained from CIMMYT Turkey as part of a collaboration on the exchange of breeding material. The mutant forms had been created at the National Center for the Study of Small-Grain Germplasm, USDA-ARS, United States, by crossbreeding of Bai Huo common wheat varieties from China, a null allele carrier of the Wx-D1 gene, to Kanto 107 and like varieties from the USA, carrying null alleles of the Wx-A1 and Wx-B1 genes. Varieties Starshina, Vassa, Utrish, Tabor, Esaul, Kuma, Grom, and Sila, raised at NGC, and variety Korotyshka, bred at the Belgorod Research Institute of Agriculture, were used as recipients. The crossing combinations of the lines under study are shown in Table 1.

DNA was isolated from 5 to 7-day-old etiolated wheat seedlings according to the method (Plaschke et al., 1995). The lines were genotyped for the allelic state of Wx genes by PCR. Primers were selected on the basis of literature data; their names and amplification conditions are presented in Table 2. The reaction mixture of the volume 25 μl contained 1× buffer for TaqDNA polymerase (50 mM KCl, 20 mM TrisHCl pH 8.4, 2–5 mM MgCl2, and 0.01 % tween-20), 2 mM MgCl2, 0.2 mM each dNTP, 12.5 pM each primer, 50 ng of DNA and 1 U of Taq polymerase.

Amplification was carried out according to the conditions recommended by the authors with minor modifications (see Table 2). PCR products were resolved by agarose gel elec-
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Table 1. Crossing combinations of the lines under study (year of crossing 2012).

| Cross combination No. | Crossbreeding combination |
|-----------------------|---------------------------|
| 54                    | (Pl 619381/Korotyshka) × Starshina |
| 55                    | (Pl 619384/Korotyshka) × Starshina |
| 56                    | (Pl 619376/Vassa) × Vassa |
| 58                    | (Pl 619384/Utrish) × Utrish |
| 59                    | (Pl 619384/Korotyshka) × Sila |
| 60                    | (Esaul/Pl 619386/Vassa) × Vassa |
| 61                    | Tabor × (Pl619381/Korotyshka/Starshina) |
| 62                    | Grom × (Pl619378/Korotyshka/Starshina) |
| 70                    | (Esaul/Pl 619381) × Esaul |
| 71                    | (Esaul/Pl 619377) × Kuma |

trophoresis in 0.5 × TBE buffer. The gel concentration ranged from 1.5 to 2.0 % depending on the size of the amplified fragment. The gels were stained with ethidium bromide and photographed under ultraviolet light using an Infiniti 1000 photo box. The 100 bp M 24 DNA marker SibEnzyme was used as a molecular weight ladder.

Results

A total of 502 soft wheat generation F1 lines were analyzed for the allelic state of the Wx-A1, Wx-B1, and Wx-D1 genes with the use of molecular markers. The numbers of samples studied for each crossing combination, as well as the numbers of identified alleles, are presented in Table 3.

The study of the allelic states of the Wx-A1 gene in the lines was carried out using the codominant marker designed by T. Nakamura et al. (2002). The null allele of the Wx-A1 gene was detected in all crossing combinations except for No. 59 and 60. Altogether, 122 lines carrying Wx-A1b were identified; in the rest, the Wx-A1a allele (wild type) was present. The presence of the Wx-A1b null allele was confirmed in all donors used (mutant for the Wx-A1, Wx-B1, and Wx-D1 genes).

To identify the allelic state of the Wx-B1 gene, a marker developed by A. McLauchlan et al. (2001) was used at the first step. Samples with identified Wx-B1b null alleles were then rescreened with the marker proposed by L.S. Vanzetti et al. (2009) (Figure). L.S. Vanzetti et al. (2009) have shown that the use of a molecular marker developed by A. McLauchlan et al. (2001) does not discriminate Wx-B1e and the Wx-B1b null allele, whereas no amplification occurs in samples with null allele with the use of the other marker. This can cause errors stemming from poor DNA isolation and PCR inhibition in some samples (Klimushina et al., 2012).

The null allele Wx-B1b was detected in one line obtained from cross combination No. 56, in four lines of combination No. 62, and in five lines of combination No. 71. It was also found that out of six mutant forms used as donors, the Wx-B1b allele was present in three: Pl619376, Pl619377, and Pl619378. A functional allele of Wx-B1e, other than Wx-B1a, was detected in 108 lines. It was absent from combinations No. 56, 59, 62, and 71.

The nonfunctional allele Wx-D1b was detected in 100 lines, in all crossing combinations except for No. 56 and 59. The largest number of lines with this allele were identified in the combination of crossing No. 70. As a result of the analysis, lines carrying combinations of null alleles of the Wx genes were selected (Table 4).

The combination of two null alleles (Wx-A1b + Wx-D1b) was detected in 19 lines from cross combination No. 58, in three lines in combinations No. 61 and 62, in two lines in combination No. 70, and in one line in combination No. 71. The presence of three null alleles (Wx-A1b + Wx-B1b + Wx-D1b), which corresponds to fully Wx wheat, was found in lines 56-12Mc4, obtained from crossing the mutant form Pl 619376 to Vassa variety (crossing combination No. 56). Thirty-seven lines were selected combining the presence of the Wx-B1e null allele with the null alleles Wx-A1b and Wx-D1b. The combination (Wx-A1b + Wx-B1e) was identified in 26 lines, and 24 lines carried the combination of alleles (Wx-B1e + Wx-D1b).

Table 2. PCR conditions and names of primers used to identify the corresponding alleles of Wx genes.

| Gene | Primers | Annealing temperature, °C | Allele | Amplicon size, bp |
|------|---------|---------------------------|--------|------------------|
| Wx-A1 | AFC and AR2 (Nakamura et al., 2002) | 65 | Wx-A1a | 389 |
|       |         |                           |        | Wx-A1b | 370 |
| Wx-B1 | 4F and 4R (McLauchlan et al., 2001) | 65 | Wx-B1a | 3 fragments, 299, 257, 227 |
|       |         |                           |        | Wx-B1b | 2 fragments, 299, 257 |
|       | Wx-B1L and Wx-B1R (Vanzetti et al., 2009) | 65 | Wx-B1a | 461 |
|       |         |                           |        | Wx-B1b | Lack of fragment |
|       |         |                           |        | Wx-B1e | 495 |
| Wx-D1 | Wx-D1-2-F and Wx-D1-2-R (Shariflou et al., 2001) | 55 | Wx-D1a | 900 |
|       |         |                           |        | Wx-D1b | 279 |
Discussion

The chosen molecular markers revealed common wheat lines bearing single null alleles of \( Wx \) genes or their combinations. The use of molecular markers to identify the allelic states of the \( Wx-A1 \), \( Wx-B1 \), and \( Wx-D1 \) genes can provide grounds for marker-assisted selection for this trait. At the initial stages of selection to study the allelic state of the \( Wx-B1 \) gene, screening with the codominant marker designed by M. Saito et al. (2009), which allows the identification of heterozygous plants, is advisable. However, work with this marker requires high-resolution electrophoresis to more accurately discriminate PCR fragments corresponding to \( Wx-B1e \) and \( Wx-B1a \) alleles. The \( Wx-A1b \) null allele may have come to the crossing combination lines No. 54, 55, 61, 62 from the mutant forms PI619381, PI619384, and PI619378 used as donors, as well as from Starshina. According to M.V. Klimushina (2012), this variety carries \( Wx-A1b \). In case of lines obtained from the combination of crosses No. 56, 58, 70, and 71, the \( Wx-A1b \) allele was transferred from donors PI619376, PI619384, PI619381, and PI619377, respectively. M.G. Divashuk et al. (2011) showed that the variety Korotyshka, in which the null allele \( Wx-B1b \) was previously mistakenly identified, is a
Table 4. The ordinal numbers of the combination of crosses and the numbers of lines with the identified combination of alleles of Wx genes

| Cross combination No. | Numbers of lines with an identified combination of alleles of Wx genes |
|-----------------------|------------------------------------------------------------------------|
|                       | Wx-A1b + Wx-B1e | Wx-A1b + Wx-D1b | Wx-B1e + Wx-D1b | Wx-A1b + Wx-B1e + Wx-D1b | Wx-A1b + Wx-B1b + Wx-D1b |
| 54                    | 9               | 15              |                |                           |                           |
| 55                    | 17              | 2               | 22             |                           |                           |
| 56                    | 1               |                 |                |                           |                           |
| 58                    | 19              |                 |                |                           |                           |
| 61                    | 5               | 3               | 1              |                           |                           |
| 62                    | 3               |                 |                |                           |                           |
| 70                    | 2               |                 |                |                           |                           |
| 71                    | 1               | 23              |                |                           |                           |
| Total number of lines | 26              | 30              | 24             | 37                        | 1                          |

carrier of the functional allele $Wx-B1e$. Therefore, this variety could be a donor of the $Wx-B1e$ allele for its descendant lines (crossing combinations No. 54, 55, 61). We have identified the functional allele $Wx-B1e$ in the mutant forms PI619381, PI619384, and PI619386. Thus, the $Wx-B1e$ allele could also have been transmitted from PI619381 and PI619384 and in the case of crossing combination No. 70, only from PI619381. Varieties participating in the crosses are not carriers of null alleles of the $Wx-B1$ or $Wx-D1$ genes. Therefore, the donors of the $Wx-B1b$ allele in 10 selected lines were the mutant forms PI619376, PI619377, and PI619378, and in case of the $Wx-D1b$ allele, PI619381, PI619384, PI619386, PI619377, and PI619378.

The selected lines with the $Wx$ gene alleles identified therein are of interest for breeding programs aimed at improving the technological qualities of grain and obtaining common wheat varieties with new starch properties. $Wx-B1b$ null allele lines are promising for the production of special types of noodles, such as udon or ramen. This is due to their high swelling volumes and high paste viscosity peak, which are observed in wheats with low amylose contents. For example, the suitability of Australian common wheat varieties for the production of Japanese udon noodles is partly due to the low level of amylose in these varieties (Oda et al., 1980; Toyokawa et al., 1989). It has also been found that most of them lack $Wx-B1$ protein (Yamamori et al., 1994). The properties of starch from fully $Wx$ wheats (bearing the null alleles $Wx-A1b$, $Wx-B1b$, and $Wx-D1b$) are not suitable for use in the production of noodles, but may be useful for industrial purposes. The use of fully $Wx$-wheats in regular flour mixtures increases the weight yield of the product and the volume of baked bread, and pure flour obtained from $Wx$ wheat varieties has a low specific volume and a sticky crumb structure and is not suitable for bakery products (Hayakawa et al., 2004). The maximum content of $Wx$ wheat flour without significant negative changes in the quality of bakery products is 30%. However, $Wx$ wheat flour can serve as improver, and it contributes to the long-term storage of finished products (Hayakawa et al., 2004).

Conclusion

The study of allelic variants of $Wx$ genes is an important step in the breeding of common wheat varieties with starch composition modified without chemical modification. As a result of the work, valuable source material was selected for breeding common wheat with improved technological qualities of grain.

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