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IMPLEMENTATION OF MOLECULAR SYSTEMS FOR IDENTIFICATION OF GENETIC POLYMORPHISM IN WINTER WHEAT TO OBTAIN HIGH-PERFORMANCE SPECIAL VARIETIES

Molecular genetic systems for polymorphism detection to screen the presence of alleles in 100 winter wheat varieties have been designed. Polymerase chain reactions (PCR) have been carried out to identify the relevant genes. The level of spread of alleles related to low and medium activity of polyphenoloxidase (PPO) enzymes has been defined and the validation has been performed. The wheat varieties carrying 1AL.1RS, 1BL.1RS rye translocations and those containing the Tamyb10 gene recessive allele and the Stb4 gene responsible for resistance to Septoria linked to Xgwm111 polymorphic locus have been identified. The waxy wheat and other varieties carrying atypical functional Wx-B1e allele have been determined. One hundred elite and promising wheat varieties have been characterized depending on the presence of alleles of genes determining the grain qualities (PPO, Tamyb10-A1, and Wx genes) and resistance to biotic and abiotic stresses (rye translocative material, Tamyb10-A1, Stb4).

Keywords: *wheat, allele, primer, grain quality characteristics, and polymerase chain reactions.*

Among the global problems of nowadays is to improve the wheat grain quality, since wheat (*Triticum aestivum* L.) is one of the most important food crops having the largest cultivation area and a basic nutrient in many world countries, including Ukraine. The development of agricultural and food industries needs stress-resistant, fungi-proof, high-productive crop varieties. This is especially important for Ukraine showing a stable trend towards escalating grain exports in the global market.

The key factor intensifying the development of new wheat varieties is the search of stability genes in both new and old wheat varieties, as well as in the remote varieties and hybrids. The use of molecular genetic approaches for identifying the valuable genotypes and screening the gene transplantation during the crossings is important as well. Therefore, this research is aimed at implementing the system for molecular determination of wheat genetic polymorphism degree; at making the molecular genetic analysis of breeding and certified winter wheat seeds from various domestic and foreign breeding centers (see Table 1) with respect to the presence of genetic polymorphism of sequences determining the grain quality parameters and resistance to biotic stress factors; at designing effective marker systems for the genes determining the grain quality parameters and resistance to biotic stress factors; and at analyzing 100 varieties of soft wheat, mainly, of domestic breeding, by molecular genetic methods.

1. WHEAT GENES DETERMINING THE GRAIN QUALITY AND RESISTANCE TO STRESS FACTORS

The breeding value of soft wheat varieties with wheat-rye translocation 1BL.1RS and translocation 1AL.1RS is determined by resistance to abiotic and biotic factors [1-4]. Usually, the wheat varieties with translocation 1BL.1RS have gene controlling resistance to such pathogenic fungi as brown (leaf) rust (*Lr26*), stem rust (*Sr31*), yellow rust (*Yr9*), mildew (*Pm8*) [5, 2, 6] and other genes of resistance to diseases and pests [7]. 1AL.1RS translocation has been widespread among commercial varieties due to the presence of genes of resistance to aphid biotypes (*Gb2, Gb6*), mildew (*Pm17*), and mite [8]. Wheat plants with rye translocation are more resistant to drought, have raised adaptability, yield capacity and protein content in grain [9, 10]. The manifestation of genes localized in the short shoulder 1RS of rye

Table 1

List of Wheat Varieties Given for Analysis

Origin	Varieties
Institute of Plant Physiology and Genetics, NAS of Ukraine Kyiv	Nyva Kyivschyny, Podolyanka, Favoritka, Volodarka, Novokyivska, Yatran 60, Kyivska ostysta, Solokha, Chorniava, Sotnytsia, Polianka, Boria, Hileya, Gold of Ukraine, Zdobutok, Grezdivlitsia, Dobroslava, Novosmuglianka, Solomia, Slavna, Kyivska
Institute of Plant Physiology and Genetics, NAS of Ukraine Kyiv, and the Remeslo Myronivka Institute of Wheat, NAAS of Ukraine, Myronivka	Pereiaslavka, Sonechko, Natalka, Pyvna, Lasunia, Kryzhynka, Vesnianka, Bogdan, Columbia, Zolotokolosa, Smuhlianka, Slavna, Spasivka
The Remeslo Myronivka Institute of Wheat, NAAS of Ukraine, Myronivka	Myronivska 808, Myronivska 30, Myronivska 61, Myronivska 65, Ukrainska 246
Institute of Crop Farming, NAAS of Ukraine, Kyiv	Poliska-90
Yuriev Institute of Crop Research, NAAS of Ukraine, Kharkiv	Doskonala, Statna
Institute of Plant Breeding and Genetics, National Center for Seed and Varieties Studies, NAAS of Ukraine, Odesa	Antonivka, Hospodynia, Dalnitska, Yednist, Zahrava, Zvytiaha, Istina, Knyahynia Olga, Kosovytsia, Lytanivka, Misia, Nyva Odeska, Sluzhnytsia, Chornobrova, Lebidka od., Lastivka od., Pysanka, Poshana, Hoduvalnytsia, Uzhynok, Biliava, Kuialnyk, Odeska 51, Albatross od., Odeska 267, Nikonia, Selianka, Zorepad, Lanovyi, Kiria, Diuk, Nebokrai, Liona, Dobrochyn, Hurt, Krasen, Turunchuk, Bunchuk, Poliovyk, Otaman
	Vatazhok, Zmina, Podiaka, Borvii, Zhaivir, Suputnytsia, Zaporuka, Zakhyst, Pylypivka, Sonata, Harmonia, Sofiika, Dobrochynna, Lira
Breeding and Research Station of Kolomiets Institute of Sugar Beets, NAAS of Ukraine, Bila Tserkva	Bilotserkivska
Institute for Agro-Industrial Production, NAAS of Ukraine, Donetsk	Donetska 46, Donetska 48
Institute of Plant Physiology and Genetics, NAS of Ukraine Kyiv, and Theosoph Farm Holding, Roznoshenske village, Ulianovski District, Kirovohrad Oblast	Trypilska
Lukianenko R&D Institute of Agriculture, Krasnodar, RF	Bezosta 1

depends on the genotype of wheat varieties [11, 12]. The secaline proteins have explicit adverse effect on flour quality [13]. To identify the rye 1R-chromosome or its short shoulder 1RS in wheat-rye translocations for the wheat varieties and lines the biochemical, molecular, biological, cytogenetic and molecular cytogenetic methods are used [11, 14-16].

The wheat grain quality is also determined by activity of polyphenoloxidase (PPO) enzymes which lead to undesirable darkening of products made of wheat flour [17-21]. Wheat contains many paralogical PPO genes represented by multigene family, located on different sites, and generated by duplication and further mutations [22-24, 5]. The PPO activity depends, mainly, on the PPO alleles in chromosomes of the 2nd homoeologous group of A and D wheat genomes [25-28]. In addition, the gene family consisting of *Ppo-A2, Ppo-B2* and *Ppo-D2*, and influencing PPO activity has been identified [29]. The STS (sequence tagged site)-markers have proved themselves to be an effective mean for determining the activity of PPO-genes [30, 31]. The allele polymorphism of *Ppo-A2, Ppo-B2* and *Ppo-D2* genes is identified with the help of molecular marker systems based on polymerase chain reactions (PCR) [32].

Resistance to germination of grains in spike (GGS) is another important qualitative indicator of variety. The red-grain wheat varieties, as a rule, are more resistant to GGS as compared with the white-grain ones. This correlation between resistance to GGS and red pigmentation is probably explained by pleotropic effects of the genes responsible for the grain color [33-35]. The color of wheat grain is controlled by R-1 (*Tamyb10*), genes that are transcription factors controlling the biosynthesis of flavonoids. One or more dominant alleles *R-A1a*, *R-B1a*, and/or *R-D1a* give red color to the grain [36, 37]. The control of allele condition of *Tamyb10* genes in red-grain varieties enables forecasting their resistance to GGS.

In terms of saving the wheat harvest its resistance to fungi-driven diseases, including, Septoria is a factor of paramount importance. Genes of resistance to Septoria are named *Stb* [38]. In 2004, the *Stb4*. gene mapping started. Seven *Stb* loci have been mapped at different chromosomes [39-41]. The molecular systems for analyzing the *Stb* genes, including the *Stb4* gene will be useful to select valuable genotypes resistant to Septoria.

Starch restructuring is among the promising directions of crop breeding. There are two different approaches to it: *increase in amylose* and increase in *amylopectin* [42]. The key enzyme of amylose biosynthesis is granule-associated starch synthase GBSS I having a molecular weight of about 60 000 daltons and named *Wx-protein*. In the bread wheat genome, three homoeologous genes *Wx-A1, Wx-B1,* and *Wx-D1* encode the isoforms of GBSS I of enzyme located in the 7AS, 4AL, and 7DS chromosome shoulders, respectively [43]. In the wheat, each gene *Wx* has several alleles. The wheat in which the combination of three inactive alleles leads to complete termination of the synthesis of GBSS I enzyme and amylose is named *Waxy (Wx)*. The varieties with several zero-alleles having reduced synthesis of amylose are called *partial Waxy* [44]. The zero-alleles of *Wx-A1*, *Wx-B1*, and *Wx-D1* genes cause inhomogeneous effect on amylose synthesis. The most important reduction has been reported for zero-allele gene *Wx-B1* [45]. Amylose/amylopectin ratio in wheat starch is of profound importance for the starch and wheat flour properties: Waxy-flour is notable for stimulation of gassiness and dough fermentation [13, 46]. The Waxy flour added to the common bread flour materially betters the bread quality [13]. The *Wx*-crop grains are an effective raw material for producing ethanol due to the presence of enzymes, reduction of power consumption, and acceleration of fermentation [47].

2. PROTOCOL OF DETECTION OF GENES DETERMINING THE WHEAT GRAIN QUALITIES AND RESISTANCE

To develop molecular genetic approaches for breeding purposes, a set of primers has been chosen to determine the selective detection of target allele genes in PCR (Table 2).

Table 2

List of Primers Used in Research

No.	Sequence	Size of amplified fragment, b.p.	Notes
	Reference wheat genes		
1	5′-GAGGGATACACGCTTCCTCA-3′ 5′-GAAAGTGCTAAGAGAGGCCAAA-3′ [36]	547	*actin* — encodes wheat protein actin
2	5′-AAGGGTTGCTCCTCTTCGCGATCT-TG-3′ 5′-GTACATGCCAGCACCGTATGGATTG-3′ [10]	934	*TaTM20* — protein transporting metallic ions (wheat)
	Rye-wheat translocations		
3	5′-TGACAACCCCCTTTCCCTCGT-3′ 5′-TCATCGACGCTAAGGAGGACCC-3′ [52]	226 — *1AL.1RS* 202 — *1BL.1RS*	*SCM9* — determination of translocation type
4	5′-AACGAGGGGTTCGAGGCC-3′ 5′-GAGTGTCAAACCCAACGA-3′ [51]	233, 338 — *1AL.1RS*	Rye, specific repeats *RT73*, determination *1AL.1RS*
	Ppo-A1 and *Ppo-D1*		
5	5′-CCAGATACACAACTGCTGGC-3′ 5′-TGATCTTGAGGTTCTCGTCG-3′ [31]	290 — *Ppo-A1a* 481 — *Ppo-A1b*	*Ppo-A1* gene allele determination
6	5′-TGAAGCTGCCGGTCATCTAC-3′ 5′-AAGTTGCCCATGTCCTCGCC-3′ [31]	490 — *Ppo-D1b*	*Ppo-D1* gene allele determination
	Tamyb10-A1		
7	5′-CTATGTGGATGGCCTTGGAT-3′ 5′-CTACCAGCTCGTTTGGGAAG-3′ [36]	665 — *R-A1b*	*R-A1b* (set 1) allele determination
8	5′-TTTCAATCGAGTGGGCATAA-3′ 5′-CCTGACGATGAGCTCCTCTT-3′ [36]	536 — *R-A1a*	*R-A1a* (set 1) allele determination
	Stb4		
9	5′-TCTGTAGGCTCTCTCCGACTG-3′ 5′-ACCTGATCAGATCCCACTCG-3′ [39]	210 — stable forms	Locus *Xgwm111*
	Wx		
10	5′-CCCCAAAGCAAAGCAGGAAAC-3′ 5′-CGGCGTCGGG TCCATAGATC-3′ [53]	After hydrolysis by restrictase HindIII 495+176 — *Wx-A1a* 652 — *Wx-A1b*	*Wx-A1* gene allele determination
11	5′-CTGGCCTGCTACCTCAAGAGCAACT-3′ 5′-GGTTGCGGTTGGGGTCGATGAC-3′ [54; 55]	778 — *Wx-B1a* 668 — *Wx-B1b* 804 — *Wx-B1e*	*Wx-B1a* and *Wx-B1e* (set 1) allele determination
12	5′-GTAGTAAGGTGCAAAAAAGTGCCACG-3′ 5′-CAGCCTTATTGTACCAAGACCCATGTGTG-3′ [54; 55]		*Wx-B1b* (set 2) allele determination
13	5′-GCCGACGTGA AGAAGGTGGTG-3′ 5′-CCCCTTGCGT CATTTGTTGTGT-3′ [56]	930 — *Wx-D1a* 342 — *Wx-D1b*	*Wx-D1* gene allele determination

For the wheat varieties studied, the following processes have been done: DNA purification, spectrophotometric measurements of extracted DNA concentration, DNA concentration normalization to 30 ng/μl, and electrophoresis of total extracted DNA in agarose gel. The quality of extracted DNA was controlled in parallel with reaction for target genes in multiplex PCRs.

The total DNA was extracted and purified from sprouts and dried material of wheat by the conventional method [47]. For identifying the presence of total plant DNA, the nucleic acid electrophoresis method in agarose gel was used after the extraction. In order to measure the size of DNA fragments in agarose gel they were compared with the marker containing linear DNA fragments of known length and quantity. λ/HindIII (Thermo Scientific) with amplicon size of 23130, 9416, 6557, 4361, 2322, 2027, 564, 125 b.p. was added at a dose of 400 ng and used as molecular weight marker.

The total DNA concentration was measured using BioPhotometer Plus (Eppendorf) spectrophotometer at a wavelength of 260 nm. The purity of extracted DNA was assessed by absorption ratios at wavelengths of 230, 260, 280, and 320 nm. The samples were standardized pursuant to measured concentrations by diluting the DNA samples (30 ng/μl).

To optimize some reactions, the touchdown and gradient PCR techniques were used.

In order to detect in a fast and reliable way the studied genes or genetic sequences determining the grain qualities and resistance to biotic and abiotic stresses, the multiplex and uniplex PCR methods have been designed and optimized (Table 3). The multiplex method foresees synthesizing several DNA fragments limited with two or more different primer pairs in one reaction. For the gene or target DNA fragment the primer pairs are selected to be used for the amplification of necessary fragments of specific length in order to identify the gene alleles after separation in agarose gel.

The reaction mixes consisted of specific primers (Table 2), 2 μl buffer for PCR 10xDreamTaq™ Green Buffer (Thermo Scientific), 0.2 mM of each desoxyribonucleotide-3-phosphate (Thermo Scientific), 0.5 units of DreamTaq™ DNA Polymerase (Thermo Scientific), 30 ng of total DNA, deionized water Milli-Q (up to a volume of 20 μl).

Table 3

Multiplex and Uniplex Reactions with Ultimate Concentration of Primers in Reaction

No.	Purpose of reaction	Genes detected (primer concentration in the brackets)
1	Determination of translocations 1AL.1RS and 1BL.1RS	*SCM9* (0.5 μM) + *TaTM20* (0.4 μM)
2	Determination of translocation 1AL.1RS	*RT73* (0.5 μM) + *actin* (0.25 μM)
3	*PPO-A1* gene allele detection	*PPO-A1* (0.5 μM) + *TaTM20* (0.25 μM)
4	*PPO-D1* gene allele detection	*PPO-D1* (0.5 μM) + *TaTM20* (0.3 μM)
5	*Tamyb10-A1* gene allele detection (primer set 1)	*Tamyb10-A1* (0.5 μM) + *TaTM20* (0.3 μM)
6	*Tamyb10-A1* gene allele detection (primer set 2)	*Tamyb10-A1* (0.5 μM) + *TaTM20* (0.15 μM)
7	Analysis of locus *Xgwm111* tagged with gene of resistance to septospirose *Stb4*	*Xgwm111* (0.5 μM)
8	*Wx-A1* gene allele detection	*Wx-A1* (0.5 μM) + hydrolysis with endonuclease HindIII
9	*Wx-B1* gene allele detection	*Wx-B1* (set 1, 0.75 μM) + *Wx-B1* (set 2, 0.75 μM) + *TaTM20* (0.5 μM)
10	*Wx-D1* gene allele detection	*Wx-D1* (0.5 μM)

The amplification programs were as follows:

1. Multiplex PCR for identifying type of rye-wheat translocation with the use of *TaTM20* gene as reference one: initial denaturation at 94 °C for 3 min; 34 cycles: denaturation at 94 °C for 30 s, renaturation at 60 °C for 30 s, elongation at 72 °C for 1 min, final elongation at 72 °C for 5 min.

2. Multiplex PCR for identifying type of rye-wheat translocation 1AL.1RS with the use of *actin* gene as reference one: initial denaturation at 94 °C for 3 min; 34 cycles: denaturation at 94 °C for 30 s, renaturation at 61 °C for 30 s, elongation at 72 °C for 24 s, final elongation at 72 °C for 5 min.

3. Multiplex PCR for identifying *PPO-A1* gene alleles: initial denaturation at 94 °C for 3 min; 34 cycles: denaturation at 94 °C for 30 s, renaturation at 59 °C for 30 s, elongation at 72 °C for 1 min, final elongation at 72 °C for 5 min.

4. Multiplex Touchdown PCR for identifying *PPO-D1* gene alleles: initial denaturation at 94 °C for 3 min; 8 cycles: denaturation at 94 °C for 30 s, renaturation at 68 °C for 30 s (with each cycle, temperature decreases by 1 °C), elongation at 72 °C for 1 min, and 24 cycles: denaturation at 94 °C for 30 s, renaturation at 61 °C for 30 s, elongation at 72 °C for 1 min, final elongation at 72 °C for 5 min.

5. Multiplex Touchdown PCR for identifying the dominant allele of *Tamyb10-A1* gene (primer set 1): initial denaturation at 94 °C for 3 min; 5 cycles: denaturation at 94 °C for 30 s, renaturation at 67 °C for 30 s (with each cycle, temperature decreases by 1 °C), elongation at 72 °C for 43 s, and 27 cycles: denaturation at 94 °C for 30 s, renaturation at 61 °C for 30 s, elongation at 72 °C for 43 s, final elongation at 72 °C for 5 min.

6. Multiplex PCR for identifying the recessive allele of *Tamyb10-A1* gene (primer set 2): initial denaturation at 94 °C for 3 min; 34 cycles: denaturation at 94 °C for 30 s, renaturation at 56 °C for 30 s, elongation at 72 °C for 35 s, final elongation at 72 °C for 5 min.

7. PCR for the analysis of microsatellite locus *Xgwm111* tagged with gene of resistance to Septoria *Stb4*: initial denaturation at 94 °C for 3 min; 34 cycles: denaturation at 94 °C for 30 s, renaturation at 52 °C for 30 s, elongation at 72 °C for 30 s, final elongation at 72 °C for 5 min.

8. PCR for identifying the *Wx-A1* gene alleles: initial denaturation at 94 °C for 3 min; 34 cycles: denaturation at 94 °C for 30 s, renaturation at 58 °C for 30 s, elongation at 72 °C for 40 s, final elongation at 72 °C for 5 min. After amplification, the products of reaction are hydrolyzed by endonuclease HindIII.

9. Multiplex Touchdown PCR for identifying the alleles of *Wx-B1* gene: initial denaturation at 94 °C for 3 min; 6 cycles: denaturation at 94 °C for 30 s, renaturation at 69 °C for 1 min (with each cycle, temperature decreases by 1 °C), elongation at 72 °C for 2 min, and 24 cycles: denaturation at 94 °C for 30 s, renaturation at 62 °C for 30 s, elongation at 72 °C for 2 min, final elongation at 72 °C for 5 min.

10. Touchdown PCR for identifying the alleles of *Wx-D1* gene: initial denaturation at 94 °C for 3 min; 7 cycles: denaturation at 94 °C for 30 s, renaturation at 67 °C for 30 s (with each cycle, temperature decreases by 1 °C), elongation at 72 °C for 1 min, and 25 cycles: denaturation at 94 °C for 30 s, renaturation at 60 °C for 30 s, elongation at 72 °C for 1 min, final elongation at 72 °C for 5 min.

The amplification reactions were made in Arctic Thermal Cycler (Thermo Scientific) and Mastercycler gradient (Eppendorf). After PCR, the amplification products of *Wx-A1* gene sequence were restricted: the reaction mix was prepared of amplification products (10 μl), restriction endonuclease HindIII (7.5 units) and normal strength R buffer (Thermo Scientific, 10 mM Tris-HCl (pH 8.5), 10 mM magnesium chloride, 100 mM potassium chloride, 0.1 mg/ml BSA). The digestion at 37 °C lasted 1.5 hours. The obtained restricted fragments were separated by the horizontal gel electrophoresis method in agarose gel with ethidium bromide in SB-buffer, at

an electric field voltage of 5 V/cm. To measure the size of amplification products, GeneRuler™ DNA Ladder Mix molecular weight marker was used (amplicon size, b.p.: 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100). The electrophoresis results were visualized with the help of UV light trans-illuminator. Ethidium bromide intercalates among the duplex nitrogenous bases, with the complex fluorescing in UV-rays. The images were processed using GIMP editor.

The activity of PPO enzymes was determined by dyeing with 1% phenol solution. The 0.1 g samples of ground wheat grain cakes were submerged in 700 μl 1% phenol solution, mixed and kept during 16 hours at an ambient temperature. In addition, the sample was carefully analyzed by multiplex PCR for identifying the *Ppo-A1* and *Ppo-D1* gene alleles. The centrifugation at a rate of 6000 rpm lasted 3 minutes. The wavelength in colored solution was measured at 405 nm; 1% phenol solution was used as reference. While measuring, the cuvette was washed with 70% ethanol solution and with distilled water. For each 5 samples, 1% phenol measurements were done.

To assess the effectiveness of molecular genetic approaches for determining *Wx* genes, the seeds were dyed with iodine solution: grinded seeds were put into a 1.5 ml vial with 700 μl iodine solution (1:10 iodine alcohol solution : distilled water) added, and mixed in the shaker for a short while. The results were determined visually by changing color of the solution.

The gliadine fraction of wheat reserve proteins was analyzed by the electrophoresis method in polyacrylamide gel [48].

The statistical analysis of biochemical tests was done by standard methods [49] that included the calculation of average variance S2 and standard deviation S, gross errors with the use of 3*S*-, β-, and the Romanovski criteria, and the calculation of confidence interval for direct measurements.

3. IDENTIFICATION OF RYE-WHEAT TRANSLOCATION MATERIAL

During the screening of wheat DNA collection on the presence of rye introgressive material with the help of selected marker systems, 100 wheat varieties have been studied using the primers to microsatellite locus *SCM9* and rye specific repeat R173. All varieties contained a 934 b.p. amplicon that testified to adequate reaction behaviour. For the varieties bearing 1AL.1RS translocation, 226 b.p. amplicones were reported, whereas those with 1BL.1RS translocation had 206 b.p. amplicones.

Rye-wheat translocations of various types have been found in 29 varieties. Among them, 19 varieties have 1AL.1RS translocation, and 10 ones have 1BL.1RS. 27 varieties belong to Kyiv and Myronivka breeding centers, and 2 ones to the Odesa center. This means that the varieties originating from the northern part of Ukraine are more stable. The translocations were found in the varieties that have been bred in Ukraine for more than several decades and among the ones newly bred in northern and central regions. However, in the new varieties bred in Odesa, no rye translocation has been found. This can be explained by a significant degradation of bread-making quality for the wheat varieties with translocations in steppe climatic zone of Ukraine [13]. Increasing frequency of varieties with translocation 1AL.1RS is explained by its insignificant influence on wheat bread-making qualities [50].

The further research of the gliadine fraction of wheat proteins by the separation method using electrophoresis has confirmed the reliability of rye translocation identification and use the molecular systems for the analysis of varieties and plant breeding material.

4. ANALYSIS OF WHEAT VARIETIES DIVERSITY WITH RESPECT TO THE PRESENCE OF PPO ACTIVITY GENE ALLELES

While identifying the *Ppo-A1* and *Ppo-D1* gene alleles, PPO33 (co-dominant type, the Ppo-A1 gene) and PPO29 (dominant type, Ppo-D1

gene) markers were selected. To obtain effective marker systems for the applied breeding, the primers to reference gene *TaTM20* were proposed to be introduced into reaction. It was proposed to use a gradient PCR for PPO33 marker (Fig. 1) and a touchdown PCR for PPO29 (Fig. 2).

Amplicons of 391 and 582 b.p. have been reported and confirmed by the analysis of respective sequence of *Ppo-A1* gene from the genetic bank of the National Center for Biotechnology Information. For some varieties (Hurt, Zahrava, Hileya), both amplicons were observed that testifies to heterogeneity of grains of these varieties.

For the PPO-29 marker, an 490 b.p. expected amplicon was reported. It means the presence of *b* allele of *Ppo-D1* gene. All varieties contained amplicon of 934 b.p. testifying to the adequacy of reaction behavior.

The sample of 100 domestically bred wheat varieties was screened using designed multiplex PCRs. The *Ppo-A1b* allele was found only in the *Yednist* and *Biliava* varieties (Ukrainian-bred white-grain wheat) that represented 2% of the sample. The grain heterogeneity by *Ppo-A1* gene was reported for 12 varieties, both old (Myronivska 808) and newly bred ones. For other varieties, *Ppo-A1a* allele was identified. It testifies to a high level of PPO activity. In 36 varieties (36%), *Ppo-D1a* allele causing a low PPO activity was revealed, while the rest contained *Ppo-D1b* allele.

To confirm the results of molecular genetic systems with the help of which the wheat varieties were divided into groups depending on allele composition of *PPO* genes, a series of biochemical tests was performed for determining the PPO activity using 1% phenol solution as incubation medium. For this purpose, contrast varieties and white-grain lines (including, the foreign ones) were selected. The color intensity was measured using a spectrophotometer, at a wavelength of 405 nm (Table. 4).

The obtained data testify to a high PPO activity of *Kuialnyk*, *Yatran 60*, and *Nedra* varieties bearing *Ppo-A1a* allele. However, the *Nedra* variety (*Ppo-D1a*) cannot be reliably distinguished from the *Kuialnyk* and *Yatran 60* (*Ppo-D1b*). The *Biliava* variety and the white-grain wheat line 3162 showed a low PPO activity that correlates with their genotype. The varieties that are heterogeneous by *Ppo-A1* gene have a medium PPO activity. Hence, the obtained data have showed the effectiveness of molecular genetic approaches for characterizing the varieties by PPO enzyme activity.

5. IDENTIFICATION OF *Tamyb10-A1* GENE ALLELE VARIANTS

To identify *Tamyb10-A1* gene allele, multiplex PCRs for two primer sets (Figs. 3, 4) have been designed. To control the adequacy of reaction behavior, the primers to reference *TaTM20* gene were used.

In the case of set 1, a 665 b.p. amplicon was observed for the varieties bearing *R-A1b* dominant allele; for all samples, a 934 b.p. amplicon testifying to the adequacy of reaction behavior was expected.

In the case of set 2, a 536 b.p. amplicon was found for the varieties bearing *R-A1a* recessive allele; for all samples, a 934 b.p. amplicon testifying to the adequacy of reaction behavior was expected.

Upon the results of analyses, 100 varieties were characterized by *Tamyb10-A1* gene. The varieties with *R-A1b* allele potentially are more resistant to GGS.

Having analyzed 100 wheat varieties, we have revealed 26 ones bearing the *R-A1a* recessive allele responsible for white color of seeds and having adverse effect on resistance to GGS. Seventy four varieties have been established to have the *R-A1b* dominant allele that potentially favorably effects the resistance. The frequency of recessive allele is quite high despite the fact that there have been only a few Ukraine-bred white-grain varieties. Therefore, the proposed molecular approaches to assessing the varieties are effective for designing the white-grain and GGS resistant varieties.

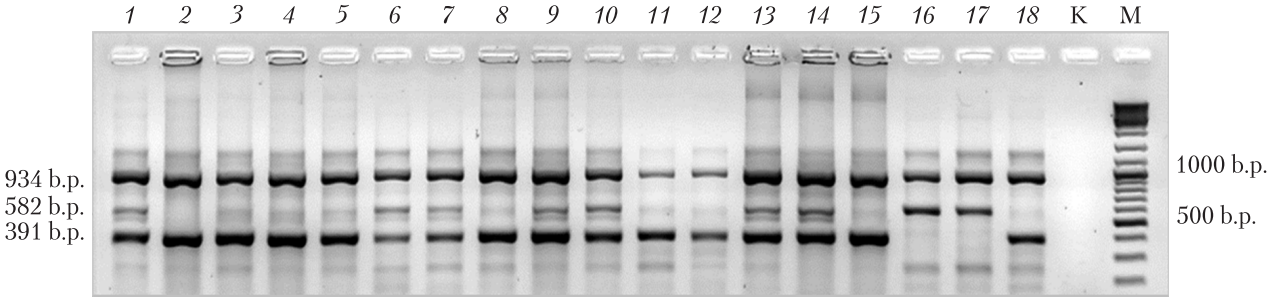

Fig. 1. Electrophoregram of the products of multiplex amplification for identifying the *Ppo-A1a* and *Ppo-A1b* alleles. Tracks *1—16* — varieties studied; *17* — positive control (*Ppo-A1b*); *18* — positive control (*Ppo-A1a*); K — negative control (TE buffer); M —DNA Ladder Mix molecular weight marker

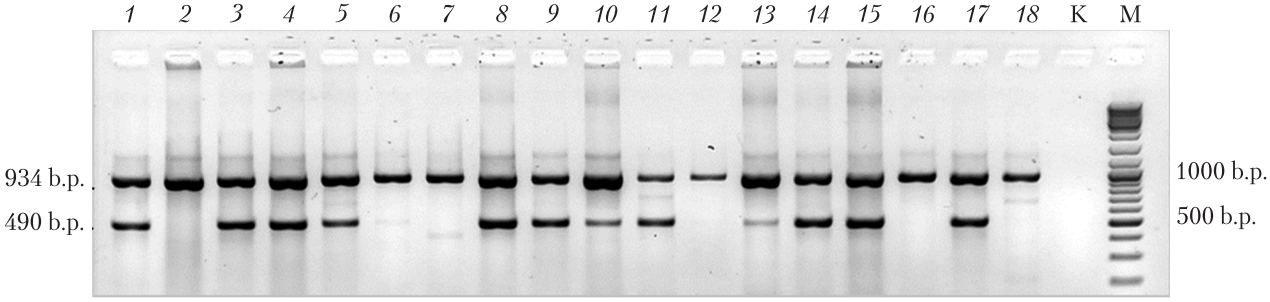

Fig. 2. Electrophoregram of the multiplex PCR products for identifying the *Ppo-D1b* allele. Tracks *1—16* — varieties studied; i — positive control (*Ppo-A1b*); *18* — positive control (*Ppo-A1a*); K — negative control (TE buffer); M —DNA Ladder Mix molecular weight marker

Table 4

Values After Phenol Test and Genotypes of Wheat Varieties Studied

Variety	A_{405}	PPO activity	Genotype
Biliava	0.424±0.1	Low	*Ppo-A1b, Ppo-D1a*
Kuialnyk	2.237±0.263	High	*Ppo-A1a, Ppo-D1b*
Favorytka	0.848±0.3	Medium	*Ppo-A1a/b, Ppo-D1b*
Hrenni	0.588±0.215	Low	*Ppo-A1a/b, Ppo-Da*
Yatran 60	2.105±0.39	High	*Ppo-A1a, Ppo-D1b*
Nedra	2.349±0.286	High	*Ppo-A1a, Ppo-D1a*
Khutorianka	0.426±0.105	Low	*Ppo-A1a/b, Ppo-D1a*
Torchynska	1.196±0.36	Medium	*Ppo-A1a/b, Ppo-D1b*
3162, white-grain	0.462±0.162	Low	*Ppo-A1b, Ppo-D1a*

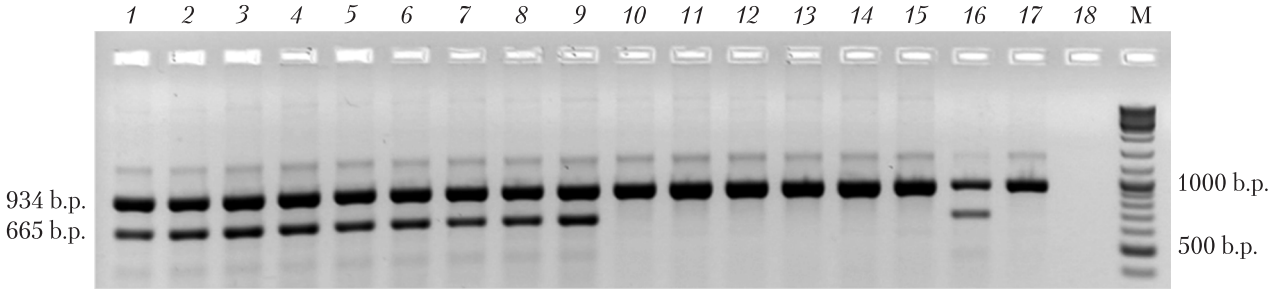

Fig. 3. Electrophoregram of the *Tamyb10-A1* gene multiplex amplification with primers of the set 1. Tracks *1–9* — wheat samples bearing the *R-A1b* allele; *10–15* — wheat samples bearing the *R-A1a* allele; *16* — positive control (wheat); *17* — negative control (wheat); *18* — negative control (TE buffer); M — GeneRuler™ DNA Ladder Mix molecular weight marker

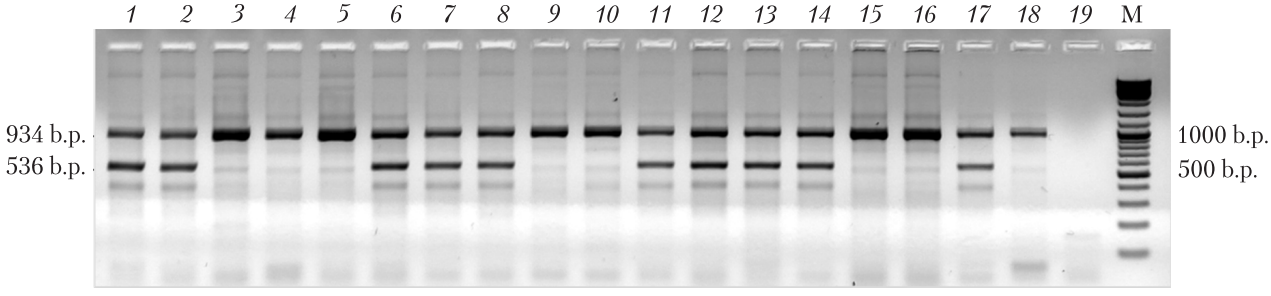

Fig. 4. Electrophoregram of the *Tamyb10-A1* gene multiplex amplification with primers of the set 2. Tracks *1, 2, 6–8, 11–14* — wheat samples bearing the *R-A1a* allele; *3–5, 9–11, 15, 16* — wheat samples bearing the *R-A1b* allele; *17* — positive control (wheat); *18* — negative control (wheat); *19* — negative control (TE buffer); M — GeneRuler™ DNA Ladder Mix molecular weight marker

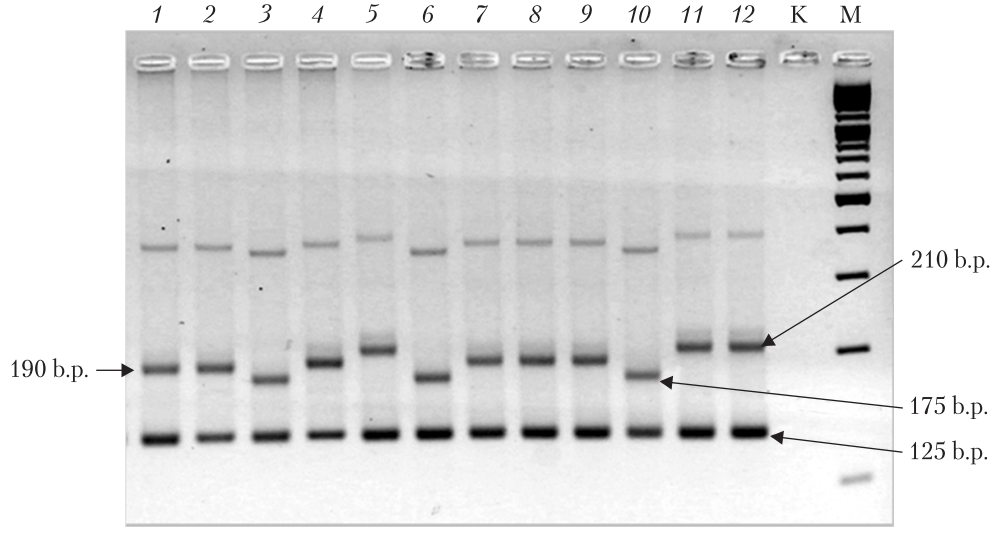

Fig. 5. Electrophoregram of the *Xgwm111* locus amplification products. tracks: *1–12* — wheat varieties studied; K – negative control, TE buffer; M – GeneRuler™ DNA Ladder Mix molecular weight marker

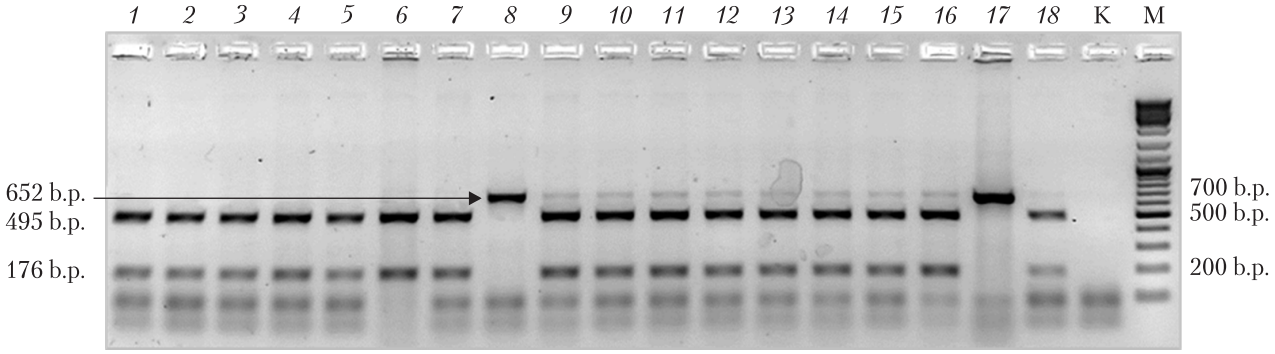

Fig. 6. Electrophoregram of the products of PCR with restriction analysis for the *Wx-A1* gene. Tracks: *1—16* — wheat varieties studied; *17* — reference sample bearing the zero-allele of the *Wx-A1b* gene; *18* — reference sample bearing the wild-type allele (*Wx-A1a*); K — negative control; M — GeneRuler™ DNA Ladder Mix molecular weight marker

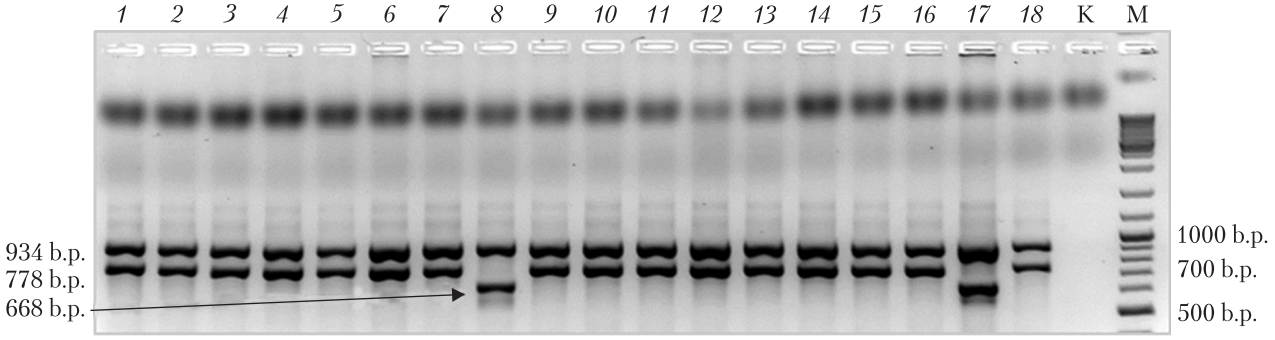

Fig. 7. Electrophoregram of the *Wx-B1* gene multiplex amplification. Tracks: *1—16* — wheat varieties studied; *17* — reference sample bearing the zero-allele of the *Wx-B1b* gene; *18* — reference sample bearing the wild-type allele (*Wx-B1a*); K — negative control; M — GeneRuler™ DNA Ladder Mix molecular weight marker

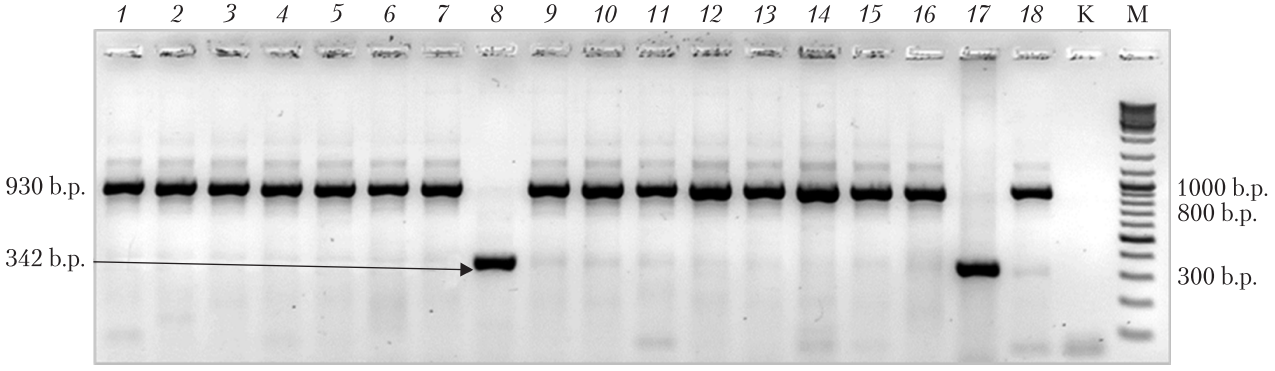

Fig. 8. Electrophoregram of the *Wx-D1* gene amplification products. Tracks *1—16* — wheat varieties studied; *17* — reference sample bearing the zero-allele of the *Wx-D1b* gene; *18* — reference sample bearing the wild-type allele (*Wx-D1a*); K — negative control; M — GeneRuler™ DNA Ladder Mix molecular weight marker

6. *Xgwm111* LOCUS ANALYSIS OF WHEAT VARIETIES

The identification of *Xgwm111* locus situated on 7D chromosome at a distance of 0.7 sM from *Stb4*. PCR with specific pair of primers was carried out with further separation by the horizontal electrophoresis method in 3% agarose gel. The amplicon size was determined using GelAnalyzer software, ver. 2010a. It was proposed to extract 3 amplicons having an approximate size of 175, 190, and 210 b.p., with the amplicon of 210 b.p. observed in the varieties resistant to Septoria, according to literature data. In addition, the one more group of amplicons having a size of 125 and 135 b.p. has been identified. This group does not correlate with resistance, but is very important for variety genotyping purposes. The results of typical amplification are showed in Fig. 5. The obtained quality of separation of amplification products in agarose gel is acceptable for clear identification of the results.

Upon the analysis results, 18 out of 100 wheat varieties have been established to have an amplicon of 210 b.p., that testifies to the presence of *Stb4* gene determining resistance to Septoria in the mentioned varieties. The *Dobrochyn* and *Podiaka* varieties are heterogeneous by locus, i.e. there are the seed material with *Stb4* genotype and the one without it. The obtained data can be useful for breeding the wheat varieties resistant to Septoria and for genotyping new varieties.

7. IDENTIFICATION OF ALLELE COMPOSITION OF *Wx* WHEAT GENES

The allele condition of *Wx* genes was determined using pre-optimized programs with specific primers. Fig. 6 shows typical results of visualization of *Wx-A1* gene amplification after restriction analysis.

A 652 b.p. amplicon was found in the varieties bearing the zero allele of *Wx-A1* genes. For the varieties containing the wild-type allele, amplicons of 495 and 176 b.p. were reported. The negative control has confirmed the reliability of results. Fig. 7 gives typical results of visualization of *Wx-B1* gene amplification.

An amplicon of 668 b.p. was reported for the varieties with zero-allele of *Wx-B1* gene, while in the other samples an amplicon of 778 b.p. typical for the wild-type allele was detected. An amplicon of 804 b.p. typical for the *Wx-B1e* functional allele was present in some varieties. An amplicon of 934 b.p. corresponding to the *TaTM20* gene is expected to be detected in all samples, which testifies to adequate behavior of reaction. An amplicon of 342 b.p. is typical for the zero-allele of *Wx-D1* gene, whereas that of 930 b.p. is typical for wild-type allele of *Wx-D1* gene (Fig. 8).

Among the studied varieties, one waxy wheat variety was found. It is the *Sofiika* variety bred in Odesa. This result has been confirmed with the help of molecular genetic analysis by the presence of zero-alleles of all *Wx* genes. All samples by genes *Wx-A1* and *Wx-D1* were represented by wild-type alleles. For the first time, among the Ukrainian wheat varieties, the *Wx-B1* gene functional allele *e* has been detected. A 804 b.p. amplicon corresponding to the *Wx-B1e* functional allele was found in the *Kiria* and *Krasen* varieties while detecting the *Wx-B*1 gene. The *Selianka* variety bears simultaneously both wild-type and functional alleles. Hence, the polymorphism of *Wx* genes was insignificant among the studied samples of wheat varieties.

For confirming the reliability and effectiveness of molecular genetic tests, the wheat seeds were colored with iodine solution. For the further comparison, in addition to studied varieties, a set of lines with various allele composition of *Wx* genes was used. A negative reaction with iodine was reported in the waxy varieties that do not contained a regularly structured starch and iodine did not react with amylose, whereas for the waxy wheat and the *Yatran 60* variety a partial reaction with the formation of blue clathrate has been reported. Hence, this reaction is useful for determining the wheat waxy genotypes.

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ВПРОВАДЖЕННЯ МОЛЕКУЛЯРНИХ СИСТЕМ ВИЗНАЧЕННЯ ГЕНЕТИЧНОГО ПОЛІМОРФІЗМУ ОЗИМОЇ ПШЕНИЦІ ДЛЯ ОТРИМАННЯ ВИСОКОПРОДУКТИВНИХ СПЕЦІАЛІЗОВАНИХ СОРТІВ

Впроваджено молекулярні системи визначення генетичного поліморфізму для 100 сортів озимої пшениці: проведено скринінг наявності цінних алелів на основі полімеразних ланцюгових реакцій; з'ясовано рівень поширення алелів низької та середньої активності поліфенолоксидазних ферментів та проведене валідування. Виявлено сорти пшениці з житніми транслокаціями 1AL.1RS, 1BL.1RS, рецесивним алелем гена *Tamyb10*, геном стійкості до септоріозу *Stb4*, зчепленим з поліморфним локусом *Xgwm111*. Визначено сорт ваксі-пшениці та сорти-носії нетипового функціонального алелю *Wx-B1e*. Складено характеристику 100 елітних та перспективних сортів пшениці за наявністю цінних алелів генів, які детермінують якісні ознаки зерна (гени *PPO*, *Tamyb10-A1, Wx*) та стійкість до біотичних та абіотичних стресових факторів (житній транслокативний матеріал, *Tamyb10-A1, Stb4*).

Keywords: пшениця, алель, праймер, якісні характеристики зерна, полімеразна ланцюгова реакція.

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ВНЕДРЕНИЕ МОЛЕКУЛЯРНЫХ СИСТЕМ ОПРЕДЕЛЕНИЯ ГЕНЕТИЧЕСКОГО ПОЛИМОРФИЗМА ОЗИМОЙ ПШЕНИЦЫ ДЛЯ ПОЛУЧЕНИЯ ВЫСОКОПРОДУКТИВНЫХ СПЕЦИАЛИЗИРОВАННЫХ СОРТОВ

Внедрены молекулярные системы определения генетического полиморфизма для 100 сортов озимой пшеницы: проведен скрининг наличия ценных аллелей, на основе полимеразных цепных реакций; установлен уровень распространения аллелей низкой и средней активности полифенолоксидазных ферментов и проведена валидация. Определены сорта пшеницы с ржаными транслокациями 1AL.1RS, 1BL.1RS, рецессивным аллелем гена *Tamyb10,* геном устойчивости к септориозу *Stb4*, сцепленным с полиморфным локусом *Xgwm111*. Выявлен сорт ваксі-пшеницы и сорта-носители нетипичного функционального аллеля *Wx-B1e*. Составлена характеристика 100 элитных и перспективных сортов пшеницы по наличию ценных аллелей генов, детерминирующих качественные признаки зерна (гены *PPO*, *Tamyb*10-A1, *Wx*) и устойчивость к биотическим и абиотическим стрессовым факторам (ржаной транслокационный материал, *Tamyb*10-A1, *Stb*4).

Ключевые слова: пшеница, аллель, праймер, качественные характеристики зерна, полимеразная цепная реакция.

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