

Identification of *Oryza sativa*'s Awn Development Regulatory Gene Orthologs in Triticinae Accessions

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Abstract—Information on the genetic control of awn development in bread wheat is currently limited to the identification of three genes that suppress awnedness, *Hd*, *B1*, and *B2*, and no promoters have yet been identified. Another Gramineae species, *Oryza sativa*, has more than ten genes involved in awn morphogenesis. This article presents results of the wheat genome sequence analysis for the search of genes orthologous to the known awn development regulators in rice, *TOB1*, *ETT2*, and *DL*. Using bioinformatic methods, three genes, *TaTOB1*, *TaETT2*, and *TaDL*, are identified in the bread wheat genome; their location is defined on the chromosomes of the second, third, and fourth homoeologous groups, respectively. The polymorphisms between homoeoalleles of the genes located on subgenomes A, B, and D are described. Identified polymorphisms include variation in the length of exons and introns in all the three genes, variation in the number of exons and introns for the *TaETT2* gene homoeoalleles, inversion of *TaDL-B* homoeoallele relative to the *TaDL-A* and *TaDL-D*, and inversion of *TaETT2-B* and *TaETT2-D* relative to *TaETT2-A*. With the PCR method using primers designed for the *TaTOB1* gene sequence, the homoeoalleles of this gene were identified in the genomes A^u, A^b, B, G, D, S^{Sh}, M, U, and T in diploid, tetraploid, and hexaploid wheat species. The marker potential of two pairs of primers for the *TaTOB1* gene for the study into the genome structure of the introgressive wheat lines in relation to this gene is shown.

Keywords: bread wheat, awns, awn morphogenesis genes, *Aegilops* species, wheat genome sequence analysis

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INTRODUCTION

The presence of awns, needle-like extensions on the lemmas of Gramineae species, is a morphological trait that, along with several others, is used for differentiating cultivars of bread wheat (*Triticum aestivum* L.): among the 14 cultivars, six are awnless and eight are awned [1]. There is no natural awnedness polymorphism in durum wheat (*T. durum* Desf.), and all nine of its cultivars have awned ears. Existing cultivars of durum wheat without well-developed awns have bread wheat in their pedigree, from which they received genes inhibiting awn development. Genetic regulation of awnedness in wheat has been studied for a long time [2] and not least because of the apparent marker potential of this trait, especially when working with introgressive wheat genotypes [3, 4]. So far, awnedness in bread wheat is associated with the fact that its genotype lacks at least two of the three known dominant awnedness inhibitors, *Hd* (4AS), *B1* (5AL), and *B2* (6BL) [5–7]. Awns are determined structures that are considered to be modified leaves, therefore, regu-

lators of their development, among others, should include homeotic genes. Awns are formed by the division of cells in a plane perpendicular to the direction of the apical-basal axis [8]. There is no doubt that genes involved in the regulation of this trait should include cell division promoters whose activity leads to the formation of needle-like extensions. For *Oryza sativa*, there are approximately ten loci of quantitative traits involved in the formation of awns [9]. Most of the identified genes encode transcription factors and, therefore, have a pleiotropic effect: the *An-1* gene encodes a transcription factor from the bHLH family, the *DL* and *TOB1* genes encode transcription factors from the YABBY family, and the *OsETT2* gene encodes a transcription factor from the ARF family [9–11]. Other rice genes affecting the morphogenesis of awns, *SHL2*, *SHO1*, *SHO2*, and *WAF1*, encode enzymes in the pathway of small interfering RNA biogenesis, hence they also have a regulatory function [12–15]. It is likely that all known rice genes form a network that regulates the development of awns, but

there is currently no information on molecular mechanisms and interaction of their products within this network. It has only been found that there is activation interaction between *DL* and *OsETT2* (since *dl* mutants do not express *OsETT2*), and small RNA transcripts synthesized by the already mentioned *SHL2*, *SHO1*, *SHO2*, and *WAF1* genes suppress the expression of *OsETT2* [9, 15]. Regarding wheat, the promoter genes of awn development are not part of the current gene catalog [5]. Indeed, the *Hd* gene is associated with the development of awn-like elongations [6]; it has been shown that awnedness can dominate over awnlessness in diploid and tetraploid wheats [16] and the promoter of awnedness is localized on chromosome 6B in durum wheat [17] and chromosomes 6U and 6S^{sh} in *Aegilops umbellulata* L. and *Ae. sharonensis* Eig., respectively [3, 4].

It is known that molecular mechanisms for the regulation of ontogenesis of homologous organs, in particular leaves, are conservative in related groups of plants [18, 19]. It is logical to assume that the development of awns in wheat and rice is regulated by similar networks; that is, wheat has genes orthologous to the genes that regulate the development of awns in rice. Therefore, information on the rice genes involved in the development of awns can be used for *in silico* search of the orthologous sequences in the bread wheat genome. This paper presents the results of *in silico* exploration of the wheat genome for the *TaDL*, *TaTOB1*, and *TaETT2* bread wheat genes orthologous to the respective awn development regulators in rice. The information obtained for the *TaTOB1* gene was verified through a comparative study of different wheat genotypes DNA amplification spectra, which were obtained with primers designed for sequences of bread wheat.

MATERIALS AND METHODS

The bioinformatic search for orthologous sequences in the bread wheat genome was carried out using the BLAST tool in the sequences deposited in the GenBank and Plant Transcription Factor Database [20, 21]. The search was performed using the *DL* (AB106553), *TOB1* (AK070205), and *OsETT2* (AB071291) rice gene sequences. The corresponding encoding sequences for the two orthologs *TaDL* (AB470269) and *TaETT2* (AY376129) were found in GenBank and *TaTOB1* (Traes_2BL_8BEA9CE1B) in the Plant Transcription Factor Database.

To identify the chromosomal localization of genes, the coding sequences of the studied genes were subjected to the search in the sequence of the bread wheat genome at the Sequencing of the *Aegilops tauschii* Genome project site [22]. The exon-intron structure of the gene sequences was analyzed by comparing the nucleotide positions of the genome sequence for which the known coding sequences were aligned. The

length of exons was determined by the size of the aligned cDNA fragments, and the length of introns was determined as the difference between the position of the nucleotide that is the start of the next exon and the nucleotide position that is the end of the previous exon in the genome sequence.

Based on the gene structure found in the sequence, the primers for the *TaTOB1* gene were created using the Primer-BLAST [23] service so that each pair of primers was located in different exons and so that amplification products included one or more introns. The four pairs of primers created were named after the respective gene plus the sequence numbers: TaTOB1-1, TaTOB1-2, TaTOB1-3, and TaTOB1-4. Sequences of primers are provided upon request.

To identify the presence of the *TaTOB1* gene in the wheat genome by PCR, the following plant material was used for DNA extraction: genotypes of bread wheat ($2n = 6x = 42$, A^aA^aBBDD) cultivars Tira, Nikoniya, Odeska 267, Yuvileyna (awned), Aurora, and Lybid (awnless); genotypes of durum wheat ($2n = 4x = 28$, A^aA^aBB) cultivars Chernomor, Leucurum (awned), Candicans, and Rubrum (awn-like elongations); *Aegilops* species ($2n = 2x = 14$): *Ae. mutica* (TT, awnless), *Ae. sharonensis* (S^{sh}S^{sh} with awn-like elongations), *Ae. comosa* (MM, awned), *Ae. umbellulata* (UU, awned), and the hexaploid synthetic species *T. migushovae* ($2n = 6x = 42$, A^bA^bGGDD, awned) [24]. Genomic DNA was isolated from sprouts using the CTAB method [25]. Polymerase chain reaction was carried out by the touchdown method using primer hybridization temperatures for ten cycles starting with 60°C with decrements of 0.5°C each cycle down to 55°C [26]. The reaction mixture composition was as follows: 50 ng DNA, 0.2 mM each dNTP (Thermo Scientific, United States), 1.5 mM MgCl₂, 250 nM each primer, and 1 U Taq DNA polymerase (Solis Biodyne, Estonia). The amplification products were separated in 1.5% agarose gel on SB buffer and in 6% denaturing PAGE with 6M urea [25].

RESULTS AND DISCUSSION

Bioinformatic identification of wheat genes orthologous to rice awn development regulators *TOB1*, *DL*, and *OsETT2*. For the three known awn development regulatory genes *TOB1*, *DL*, and *OsETT2* in rice, the bread wheat genome sequence was found to have orthologs *TaTOB1*, *TaDL*, and *TaETT2*, for each of which homoeoalleles were localized on three subgenomes.

The *TaTOB1* gene was found on the second group of chromosomes in all the three bread wheat subgenomes. Its gene structure contains six exons and five introns, some of which are polymorphic for the length among homoeoalleles (Table 1). Length polymorphisms were found for exons 1, 2, and 5 and in all

Table 1. Structure of genes *TaTOB1*, *TaDL*, and *TaETT2*

Element of the structure	Element no.	Homoeoalleles		
		A	B	D
<i>TaTOB1</i>				
Exon, bp	1	-139	-139	-142
	2	-181	-186	-181
	3	-190	-190	-190
	4	-75	-75	-75
	5	-81	-81	-88
Intron, bp	6	-103	-102	-102
	1	-92	-112	-89
	2	-95	-90	-100
	3	-704	-720	-702
	4	-113	-119	-119
	5	-127	-125	-89
<i>TaDL</i>				
Exon, bp	1	79	-79	79
	2	148	-148	148
	3	95	-95	95
	4	77	-77	77
	5	86	-86	86
Intron, bp	6	73	-72	73
	1	582	-581	582
	2	721	-759	721
	3	1801	-1801	1801
	4	120	-119	120
	5	97	-97	97
<i>TaETT2</i>				
Exon, bp	1	84	Missing	Missing
	2	60	Missing	Missing
	3	194	-194	-194
	4	86	-86	-86
	5	92	-92	-92
	6	171	-171	-173
	7	124	-124	-124
	8	76	-76	-76
	9	162	-162	-162
	10	870	-858	-873
Intron, bp	11	409	-395	-409
	1	330	Missing	Missing
	2	104	Missing	Missing
	3	85	85	86
	4	313	339	313
	5	76	76	75
	6	860	860	857
	7	66	65	67
	8	1503	1455	4789
	9	79	80	79
	10	68	68	68

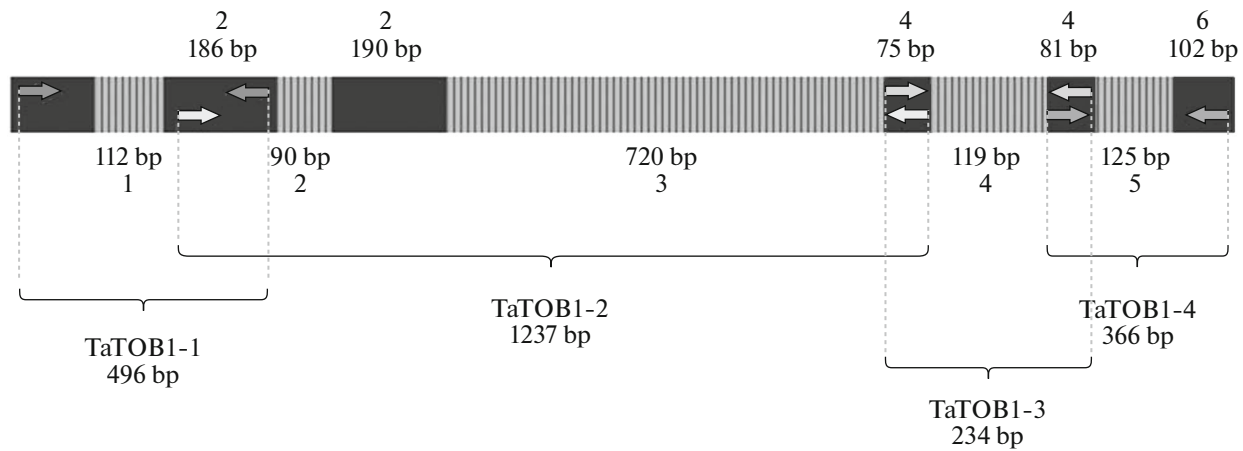


Fig. 1. Structure of the *TaTOB1-B* homoeoallele and the localization of the primers used in the work. Dark shades indicate exons (element number and length are above the figure), light shades indicate introns (element number and length under the figure).

introns. The difference in the length of the exons did not exceed 5 bp, while the differences were greater for the introns: the first and third introns of the *TaTOB1-B* gene were 20 bp longer than the two homoeologs, and the last, the fifth intron of *TaTOB1-D*, was shorter by 25 bp than in homoeologs.

The *TaDL* gene of bread wheat was localized in three subgenomes on the fourth group of chromosomes and its structure contains six exons and five introns (Table 1). The sequence of the gene located in subgenome B is inverted relative to the genes located in other subgenomes. Single-nucleotide polymorphisms of the lengths between homoeoalleles were found in exon 6 as well as in introns 1 and 4. Intron 2 in the *TaDL-B* gene was longer by 38 bp than in other homoeoalleles.

Differences in the number of introns and exons were revealed for homoeoalleles of the *TaETT2* gene localized on the third group of chromosomes (Table 1). In particular, the *TaETT2-A* homoeoallele had two exons and two introns at the beginning of the sequence that were not found in the homoeoalleles of the subgenomes B and D. Accordingly, *TaETT2-A* consisted of 11 exons and ten introns, and two other homoeoalleles had nine exons and eight introns. Such a polymorphism may be due to the fact that only the *TaETT2-A* homoeoallele is functional, and the other two are pseudogenes with deletions. This assumption is supported by the inversion of the *TaETT2-B* and *TaETT2-D* sequences with respect to the *TaETT2-A* direction and the 3.3 kb insertion in the eighth intron of *TaTT2-D* (Table 1). It is known that insertions of such size are usually due to insertions of retroelements, and they often lead to altered functional activity of the gene [27].

Identification of the *TaTOB1* gene in the wheat genome by PCR. Four pairs of primers were designed

to amplify *TaTOB1* gene fragments from genomic DNA of wheat. In each of the pairs, the left and right primers were localized in the neighboring exons, thus, the entire intron and part of the encoding sequence were amplified in PCR (Fig. 1). For the pair of TaTOB1-1 primers, the left and right primers were in exons 1 and 2, with intron 1 in-between. For the second pair, the left and right primers were in exons 2 and 4 and were delimited by two introns and a third exon. For the pair of primers TaTOB1-3, the left and right primers were in exons 4 and 5, with intron 4 in-between. For the pairs of primers TaTOB1-4, the left and right primers were in exons 5 and 6, with intron 5 in-between.

Based on analysis of the bread wheat genomic sequence, the expected lengths of the amplicons were 467, 496, and 467 bp for the first pair, 1221, 1237, and 1224 bp for the second pair, 226, 234, and 247 bp for the third pair, and 369, 366, and 337 bp for the fourth pair for homoeoalleles on subgenomes A (*TaTOB1-A*), B (*TaTOB1-B*), and D (*TaTOB1-D*), respectively. Separation of DNA amplification products of the studied genotypes in agarose gel gave us the following information. The spectra resulted from separation of amplification products obtained with each of the studied primers did not differ between *T. aestivum* and *T. durum* (Fig. 2). When separating the genomic DNA amplification products obtained with the pair TaTOB1-1, three components were observed with estimated sizes of 335, 314, and 307 bp. Electrophoretic spectra of bread and durum wheat obtained by separating the products with the second pair of primers TaTOB1-2 contained one component with estimated size of 1120 bp, and the spectra obtained using the third pair of primers TaTOB1-3 contained one component with estimated size of 375 bp. Separation of amplification products with the fourth pair of primers to the *TaTOB1* gene produced two components in

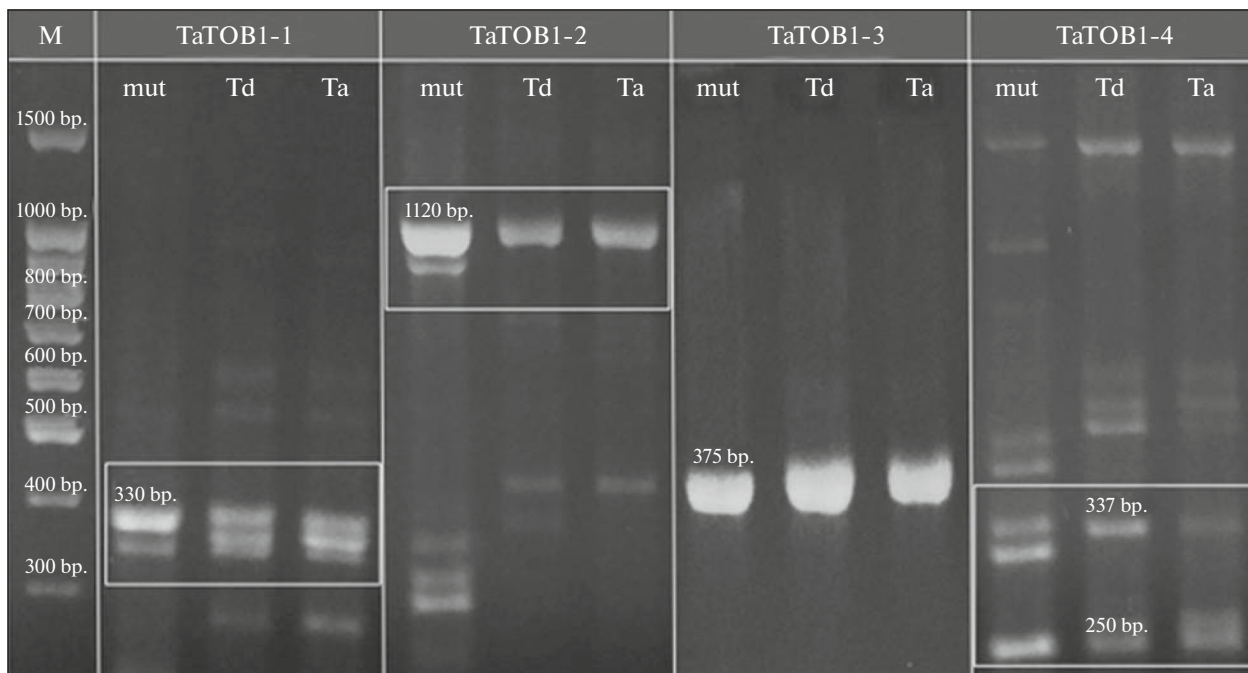


Fig. 2. Results of separating the *TaTOB1* amplification products with four pairs of primers in agarose gel. Ta—*T. aestivum* cultivar Aurora; Td—*T. durum* cultivar Chernomor; mut, *Ae. mutica*; M, DNA ladder M-27 SibEnzyme 100 bp + 1.5 kb + 3 kb DNA Ladder.

the wheat spectra, 337 and 251 bp. In the diploid species *Ae. mutica*, the spectra obtained after separating the amplification products with the first and fourth pairs of primers to the *TaTOB1* gene differed from the spectra of durum and bread wheat (Fig. 2). When amplifying with the first pair of primers, instead of the three components, two components of 335 and 307 bp were obtained. In amplification with the fourth pair, the spectrum of *Aegilops*, in addition to the two amplicones inherent in the wheat DNA spectra, had another component, which may be the result of a non-specific amplification. Agarose gel does not allow separating the fragments that were expected to be obtained by amplifying the homoeoalleles of the *TaTOB1* gene of bread wheat due to a rather small difference in their size. Therefore, for the separation of these fragments and their subgenomic identification, the PCR products were further separated in 6% polyacrylamide gel (PAGE).

Subgenomic identification of amplification products separated by PAGE. For the purpose of subgenomic identification of the amplicons, PAGE separation was carried out for the amplification products obtained with the *TaTOB1* primer and DNA of diploid wheat of the *Aegilops* genus, genotypes of tetraploid wheat *T. durum* (A^uA^uBB), bread hexaploid wheat cultivars (A^uA^uBBDD), and hexaploid synthetic species *Triticum miguschovae* (A^bA^bGGDD). The spectra of diploid, tetraploid, and hexaploid species were compared, and conclusions about the subgenomic origin of the components were made. *Ae. sharonensis* (S^{sh}) belongs

to the same Sitopsis section that includes *Ae. speltooides* (S), the hypothetical ancestor of wheat subgenomes B and G [28]. Therefore, when DNA spectra of *Ae. sharonensis*, *T. aestivum*, *T. durum*, and *T. miguschovae* shared the amplicon, it was considered to be a product of subgenome B of the species *T. aestivum* and *T. durum* and a product of subgenome G of the species *T. miguschovae*. The components common to the DNA spectra of durum and bread wheat were attributed to the products of subgenome A^u , and those common to the DNA of *T. miguschovae* and bread wheat were attributed to the products of subgenome D.

When separating the PCR products obtained with the pair of primers *TaTOB1-1* in 6% PAGE, the spectra of diploid *Aegilops* had one component each, which is double for the DNA of *Ae. sharonensis*, the spectra of durum wheat cultivars had three components, the spectra of bread wheat cultivars had four components, and the spectrum of *T. miguschovae* had five components (Fig. 3). According to the above-described approach, the lightest double component found in the spectra of durum and bread wheat and *T. miguschovae*, whose mass matched with the double component of the spectrum of *Ae. sharonensis*, were attributed to the amplification products of homoeoalleles of subgenomes B and G. This part of the spectrum also contains amplification products of DNA from the three other *Aegilops* species studied. The larger component, common to bread wheat and *T. miguschovae*, was attributed to the amplification product of the homoeoallele of subgenome D. The heaviest compo-

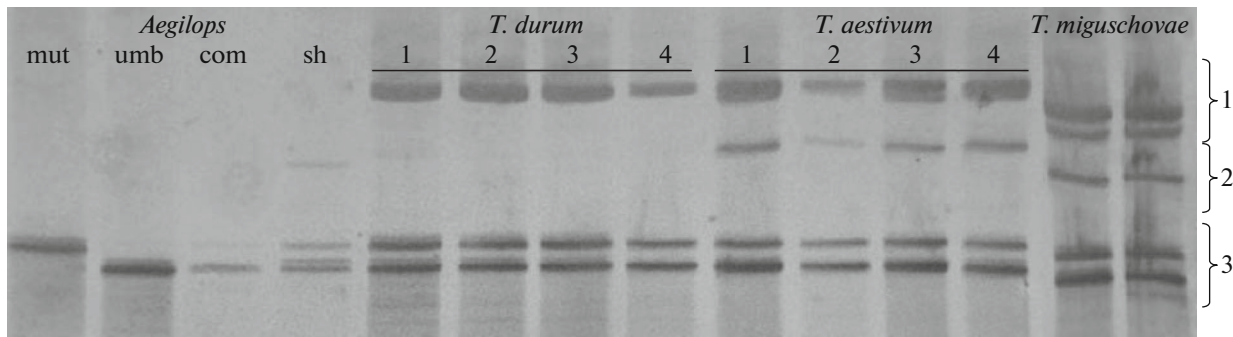


Fig. 3. Results of separating the TaTOB1-1 amplification products in PAGE. Subgenomic attribution of the amplicons: 1— A^u and A^b ; 2—B, G, T, U, M, and S^{sh} ; 3—D. *T. aestivum* cultivars: 1—Aurora; 2—Lybid; 3—Tira; 4—Yuvileyna. *T. durum* genotypes: 1—Rubrum; 2—Candicans; 3—Leucurum; 4—Chernomor. *Aegilops* species: mut—*mutica*; umb—*umbellulata*; com—*comosa*; sh—*sharonensis*.

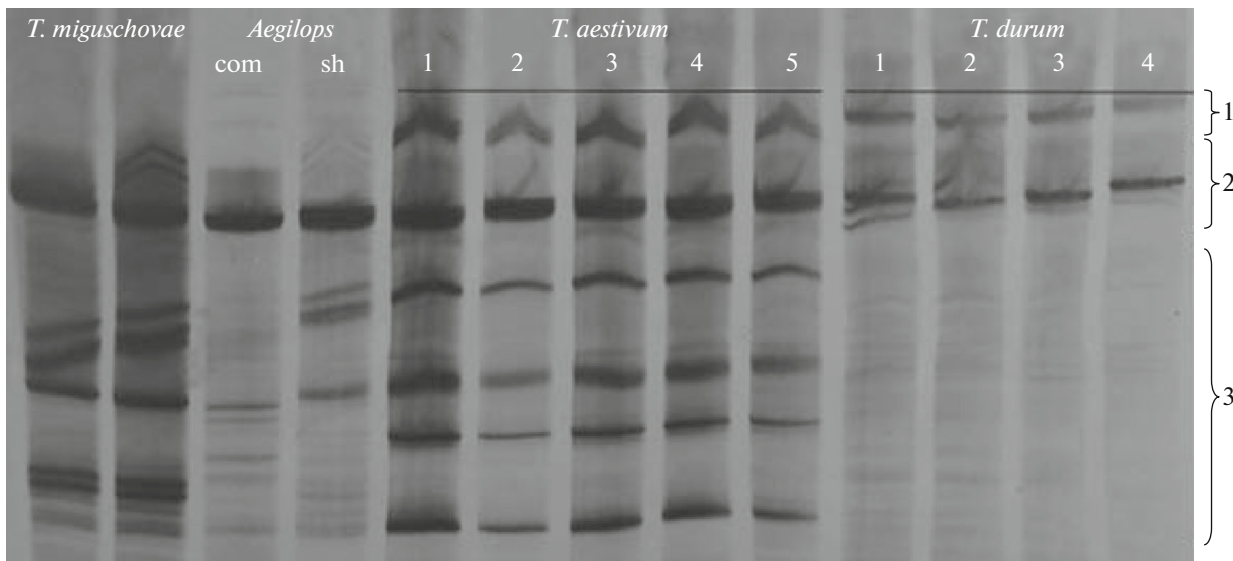


Fig. 4. Results of separating the TaTOB1-3 amplification products in PAGE. Subgenomic attribution of the amplicons: 1— A^u ; 2—B, G, M, and S^{sh} ; 3—D (for *T. miguschovae*, A^b and D). *T. aestivum* cultivars: 1—Aurora; 2—Lybid; 3—Tira; 4—Yuvileyna; 5—Nikoniya. *T. durum* genotypes: 1—Rubrum; 2—Candicans; 3—Leucurum; 4—Chernomor. *Aegilops* species: com—*comosa*; sh—*sharonensis*.

nent inherent to durum and bread wheat, as well as *T. miguschovae*, was attributed to amplification products of the homoeoalleles of subgenomes A^u and A^b . Thus, amplification products of the *TaTOB1* gene homoeoalleles are polymorphic when using primers to the 5'-part of the gene, and this pair of primers can be used in the study of the genomic structure of introgressive wheat lines with respect to the *TaTOB1* gene.

For the second pair of primers (Fig. 1), the size of amplification products turned out to be too large, and, therefore, it was not possible to adjust the PAGE separation conditions. When separating the PCR products with the TaTOB1-3 primers in 6% PAGE, one component was detected in each of *Aegilops* spectra,

two in each of durum wheat cultivars, six in each bread wheat cultivars, and five in the spectrum of *T. miguschovae* (Fig. 4). A component common for all spectra was identified as an amplification product of the homoeoalleles of subgenomes B and G. The lighter component found in the bread and durum wheat spectra is likely to be formed when amplifying the homoeoallele from subgenome A^u common for these two species. The four heavy components in spectra of the bread wheat cultivars and the *T. miguschovae* genotype are the result of amplification of homoeoalleles of subgenome D, although the components for the two wheat species differ by size. In the case of the *TaTOB1-A* homoeoallele for *T. miguschovae*, which

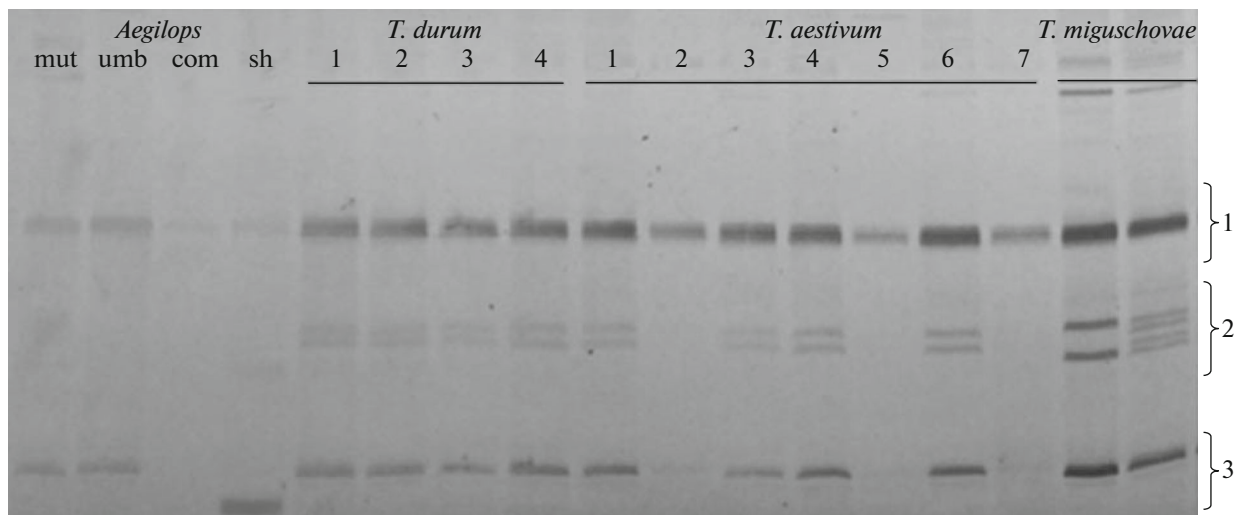


Fig. 5. Results of separating the TaTOB1-4 amplification products in PAGE. Subgenomic attribution of the amplicons: 1—A^u and A^b; 2—B, G, M, and S^{sh}; 3—D. *T. aestivum* cultivars: 1—Tira; 2—Nikoniya; 3—Odeska 267; 4—Yuvileyna; 5, 7—Lybid; 6—Aurora. *T. durum* genotypes: 1—Chernomor; 2—Leucurum; 3—Candicans; 4—Rubrum. *Aegilops* species: mut—*mutica*; umb—*umbellulata*; com—*comosa*; sh—*sharonensis*.

has subgenome A^b in contrast to subgenome A^u of bread and durum wheat, it may not form an amplification product with the pair of TaTOB1-3 primers due to changes in nucleotide sequences of the fourth and/or fifth exons of the *TaTOB1-A^b* homoeoallele compared to the *TaTOB1-B* homoeoallele, based on which this pair of primers was designed. Moreover, according to the data of genomic sequencing of bread wheat for exon 5, length polymorphisms are found even between homoeoalleles of this species. However, this pair of primers can also be used in the study of the structure of introgressive wheat lines with respect to the *TaTOB1* gene.

The spectra obtained by separating the PCR products from DNA of the studied genotypes with the TaTOB1-4 primers revealed the following components: one in *Ae. comosa*, two in *Ae. mutica* and *Ae. umbellulata*, two or three in *Ae. sharonensis*, four in the bread and durum wheat cultivars, and four in *T. miguschovae* (Fig. 5). The resulting picture is difficult to interpret. Two relatively common (in the spectrum of *Ae. sharonensis* amplicons, the mobility of the lightest component is higher than that in other spectra) components for all spectra were attributed to amplification products of the homoeoalleles of subgenomes B and G. Two components that lie between the *TaTOB1-B* and *TaTOB1-G* amplification products in the spectra of bread and durum wheat and *T. miguschovae* were attributed to amplicons of the homoeoalleles of subgenomes A^u, A^b, and D. However, this is only a preliminary assumption, which has no evidence for this material. One of the primers of the TaTOB1-4 pair is located in the fifth exon, whose variation was already suggested when considering the amplification

products obtained using the pair of TaTOB1-3 primers. Therefore, the marker potential of amplification products obtained using the pair of TaTOB1-4 primers for studying the introgressive lines with respect to the *TaTOB1* gene alleles is limited.

Comparison of the amplification spectra of DNA of awned and awnless cultivars of bread and durum wheat obtained with the use of three pairs of primers (Figs. 3–5), did not reveal any differences in the spectra of cultivars that differ for the awnedness trait. At first glance, it seems that there is no effect of the studied gene on the development of awns. Of course, one can assume that there are changes in the sequence of nucleotides that transform the functional awn development gene-promoter into a nonfunctional allele localized in that part of the *TaTOB1* gene that was amplified by the pair of TaTOB1-2 primers and whose amplification products we could not analyze by separation in PAGE. However, we think that it is more correct to accept the following explanation for the absence of polymorphic products in the DNA amplification spectra of the studied genotypes polymorphic for the awnedness trait with the use of intragenic primers to the *TaTOB1* gene. First, the key polymorphism could consist not in a change in the length of amplification products but in a change in the sequence, which does not lead to variation in the lengths of amplification products, such as single nucleotide substitution polymorphisms, which is why we could not identify it. Second, the polymorphism could affect the regulatory regions located upstream or downstream the coding parts of the gene, which were not investigated. Such a polymorphism may lead to a change in gene expression without altering its coding region. Therefore, as the next step, one should consider the study of this

gene's functioning in wheat through determining the level of its expression in genotypes that differ for the presence of awns.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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