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CLONNING OF *DREB1* GENE IN WHEAT WILD RELATIVES AND DEVELOPMENT OF A DNA MARKER FOR ITS MONITORING IN WHEAT BACKGROUND

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Abstract

The *DREB* gene encodes the transcription factor DREB involved in the response of the plant to drought, salinity and heat. The DREB transcription factors induce the expression of multiple genes linked by signal transmission, abscisic acid-dependent and independent, in response to abiotic stress. Many wild species have evolved under extreme environmental conditions (drought, salinity), so they can serve as sources of new genetic variants of *DREB* in breeding wheat for stress resistance. The study of *DREB* orthologous genes in wild relatives of wheat will permit to expand the set of genes in its breeding improvement using wide hybridization, and the design and application of molecular markers will facilitate transfer of these genes into a genome of bread wheat. Among the diversity of genes encoding DREB proteins, *DREB1* is of the greatest interest due to its involvement in control of plant resistance to various abiotic factors, such as drought, salinity, low temperatures. We were the first to study the *DREB1* orthologs in members of the genera *Thinopyrum*, *Dasyphyrum* and *Pseudoroegneria*. Using primers designed on the basis of *DREB1* conserved regions we amplified fragments of the *DREB1* orthologs in *Thinopyrum intermedium*, *Th. ponticum*, *Th. bessarabicum*, *Dasyphyrum villosum*, *Pseudoroegneria spicata*, and *P. stipifolia*. The obtained PCR products were cloned and sequenced. As a result, 30 unique sequences were shown to be highly homologous (92-98 %) to the *TaDREB* genes of bread wheat. Between the sequences, we identified multiple single-nucleotide polymorphisms (SNPs) and several large insertions/deletions. The resulting DNA sequences were translated in silico into hypothetical amino acid sequences. All nucleotide sequences found by us are capable of encoding a complete protein that has a DNA-binding domain specific for DREB AP2. Comparison of the amino acid sequences of the AP2 DNA-binding domain in the studied samples showed the presence of polymorphisms for individual amino acids. In all hypothetical amino acid sequences, except for one the sequence described, amino acids conserved for the DREB AP2-domain of cereals were found. We developed the CAPS marker P18_FokI, which in most cases can differentiate the *DREB1* orthologs between wheat and wild relatives due to the presence of polymorphisms in the restriction sites, the fragment amplified from the genome of bread wheat has a size of about 570 bp. A *DREB1* ortholog was localized in the homeological group 3 of *Th. elongatum* (3Je) using P18_FokI and a series of addition bread wheat lines with *Th. elongatum* chromosomes. Analysis of 10 wheat-wheatgrass hybrids revealed the presence of both *TaDREB* bread wheat and the *DREB1* ortholog in all analyzed accessions. In this case, the wheat-type fragment was absent in bread wheat with a substituted chromosome 6J (6D), which also serves as a proof of localization of the *DREB1*

ortholog on the chromosome of homeological group 3. Thus, the CAPS marker P18_FokI developed by us helps to effectively transfer the *DREB1* gene from the wild cereals to the genome of bread wheat, so that we can study the effect of the alien *DREB1* gene on the resistance of bread wheat to drought, salinity, low temperatures, and, farther, to create valuable breeding forms using MAS.

Keywords: bread wheat, *Triticum*, *Thinopyrum*, *Pseudoroegneria*, *Dasyphyrum*, resistance genes, orthologous genes, drought, salinity, DREB1, DREB, TADREB1, polymerase chain reaction, molecular markers, sequencing

Significant losses in the agrarian sector are due to such abiotic factors as soil salinity and moisture deficit. The tolerance of plants to these stresses is complex, that is, it is the result of the interaction of many genes and biochemical factors. A key role in plant resistance is played by various transcription factors [1-2]. Among them, the DREB group (dehydration-responsive element binding) can be distinguished from the AP2/ERF family proteins. The transcription factors of DREB, in response to the abiotic stress, induce the expression of multiple genes associated with the abscisic acid-dependent (ABA-dependent) and ABA-independent signal transmission [3-5]. The transcription factor of DREB specifically binds to the regulatory sequence Dre (5'-TACCGACAT-3'), which was first detected in the promoter of the *rd29A* gene [6]. The DREB protein contains an alkaline N-terminal amino acid region acting as a nuclear localization signal. To the right of the N-terminal region, there is the AP2 DNA-binding domain, which consists of three-chain β -folds and one α -helix almost parallel to β -folds [7]. The seven amino acid residues in the AP2 domain [8] are key for binding to the DNA GCC box. The conserved region with a high content of serine and threonine (ST-rich region), which can serve as a phosphorylation site [9], adjoins the AP2/ERF DNA-binding domain. DREB has an acidic C-terminal region, which is believed to possess transcriptional activation activity [10, 11].

Among the diversity of genes, encoding DREB proteins, *DREB1* is of the greatest interest due to involvement in plant resistance to various abiotic factors, such as drought, salinity and low temperatures [12-14]. *DREB1* genes are sequenced in many plant species, including bread wheat [15-17]. B. Wei et al. [17] localized homologous genes *TaDREB1* of bread wheat on the chromosomes of homeologous group 3 and revealed polymorphism by DNA sequences. Various single nucleotide polymorphisms (SNPs) in *DREB1* of wheat are associated with resistance to drought and salinity [18, 19]. In addition to bread wheat, *DREB* genes have been characterized in the related wild-growing species *Aegilops tauschii*, *Leymus chinensis* and *Elymus spicatus* [20-22].

The use of genetic engineering approaches permitted to demonstrate the role of *DREB* genes in enhancing resistance to abiotic stress. In particular, the gene of wild barley *Hordeum spontaneum* was inserted into the genome of *Paspalum notatum* [23]. In this and other similar experiments, the obtained transgenic plants differed from the control ones by increased resistance to salt and drought [24-27].

The use of the genetic potential of wild-growing species, closely related to wheat, can also be useful for its genetic improvement, including increasing resistance to biotic and abiotic factors. Evolutionally, many species were formed in extreme ecological and geographic conditions (drought and salinity) [28, 29]. Therefore, the genetically closest wild relatives can serve as a source of new genetic variants of *DREB* for stress-resistance selection.

Allopolyploid species, e.g. intermediate wheatgrass *Thinopyrum intermedium* (genomic constitution J^JJ^{Vs}St, $2n = 6 \times = 42$) and rush wheatgrass *Th. ponticum* (genomic constitution JJJJ^SJ^S, $2n = 10 \times = 70$) are widely used in distant hybridization of wheat [29, 30]. They carry valuable genes of resistance to

salinity, drought and low temperatures [31, 32], as well as to pre-harvest sprouting [33]. Forms of wheat with the genetic material of wheatgrass have been developed, which have resistance to leaf rust [34-36], stem rust [37], fusariosis [38], wheat streak mosaic virus [39] and other diseases. Chromosomes of rye and intermediate wheatgrass are relatively easy to conjugate and exchange patches [40]. According to current representations, the donors of the subgenomes of these species are *Dasypyrum villosum* (V, $2n = 2x = 14$), *Th. bessarabicum* (J^b, $2n = 2x = 14$) and *Pseudoroegneria spicata* (St, $2n = 2x = 14$); the first two species are also widely used in distant wheat hybridization [41-43].

This article presents the DNA sequences of the *DREB1* orthologues, which were obtained for the first time in representatives of the genera *Thinopyrum*, *Dasypyrum*, *Pseudoroegneria*, and also the CAPS marker P18_FokI was developed, which permits to distinguish the *TaDREB1* wheat gene and the gene of the listed species.

The purpose of this study was to sequence and analyze *DREB1* orthologues of wild species related to wheat and to create a PCR marker, which would allow distinguishing the *DREB1* gene of wild species and bread wheat.

Techniques. The authors used samples of *Thinopyrum bessarabicum* (Savul. & Rayss) Á. Löve (PI 531711), *Th. intermedium* (Host) Barkworth & D.R. Dewey (PI 401200), *Th. ponticum* (Podp.) Z.-W. Liu & R.-C. Wang (PI 508561), *Pseudoroegneria spicata* (Pursh) Á. Löve (PI 537371), *P. stipifolia* (Czern. ex Nevski) Á. Löve (PI 325181), *Dasypyrum villosum* (L.) Borbás (W6 21717), seeds of which were obtained from Germplasm Research International Network (GRIN, USA), and *Th. ponticum* (1158A/19), obtained from N.V. Tsitsin Main Botanical Garden (MBG) of the Russian Academy of Sciences (Moscow, Russia). *DREB1* gene mapping was performed on a set of bread wheat lines Chinese Spring supplemented with *Th. elongatum* chromosomes. Moreover, 10 samples of wheat-wheatgrass hybrids (WWH) containing wheatgrass and wheat chromatin were used from the collection of N.V. Tsitsin Main Botanical Garden of the RAS, the 5542, 2087, 548, 1674, 4082, ZP26, M3202, 4044, 4015 and M12. The bread wheat (*Triticum aestivum* L.) varieties Nemchinovskaya 24, Aivina, as well as bread wheat varieties Tulaikovskaya zolotistaya, Tulaikovskaya 10, Tulaikovskaya 100, Tulaikovskaya 110 with a substituted chromosome from intermediate wheatgrass 6J (6D) [35] served as the control ones.

The DNA was extracted from young leaves by the method of R. Bernatzky et al. [44]. In PCR, the primers P18F were used (5'-CCC AAC CCA AGT GAT AAT AAT CT-3'), P18R (5'-TTG TGC TCC TCA TGG GTA CTT-3'), P20F (5'-TCG TCC CTC TTC TCG CTC CAT-3'), P20R (5'-GCG GTT GCC CCA TTA GA CAT AG-3'), P21F (5'-CGG AAC CAC TCC CTC CAT CTC-3'), P21R (5'-CGG TTG CCC CAT TAG ACG TAA-3'), P22F (5'-CTG GCA CCT CCA TTG CCG CT-3'), P25F (5'-CTG GCA CCT CCA TTG CTG CC-3'), PRa (5'-AGT ACA TGA ACT CAA CGC ACA GGA CAA C-3'), developed by B. Wei et al. [17]. PCR was performed on a DNA Engine Tet-rad 2 (Bio-Rad, USA) by the following protocol: 5 min at 95 °C (1 cycle); 30-45 s at 95 °C, 30-60 s at 60 °C, 40-120 s at 72 °C (34 cycles); and 10 min at 72 °C (1 cycle). Samples were stored at 10 °C. The PCR products were separated in 1.5% agarose gel with TBE buffer at a field strength of 6 V/cm.

To clone the amplification products, they were purified using the GeneJET™ PCR Purification Kit (Fermentas, Lithuania) according to the manufacturer's instructions. Purified DNA was ligated into the pGEM®-T Easy vector (Promega, USA). The vector was transformed into *Escherichia coli* strain DH10B (Life Technologies, USA). Transformation was executed on the electroporator GenePulser (Bio-Rad, USA). Recombinant clones were detected by the

blue-white selection method.

Clones for sequencing were selected by PCR with primers M13 flanking the insertion site. Sequencing was performed on an ABI-3130XL device (Applied Biosystems, USA). The alignment of the nucleotide and amino acid sequences was performed with ClustalW2 program [45]. To obtain hypothetical amino acid sequences, the resource ExPASy [46] was used. The nuclear localization signal was found using the cNLS Mapper algorithm [47]. To determine the GCC-binding boxes and the AP2-binding domain, the Conserved Domain Database [48] was used.

The restriction endonuclease (Fok1) was selected based on the nucleotide alignment analysis of the region amplified with the primer P18. PCR products (10 μ l) were restricted for 12 hours at 37 °C, and the restriction fragments were separated by electrophoresis in a 2% agarose gel.

Results. The involvement of the genetic resources of the tertiary pool of the tribe *Triticeae* plays an important role in the selective improvement of wheat. In this respect, wild-growing representatives of the genera *Thinopyrum*, *Dasypyrum*, *Pseudoroegneria* are considered promising. They are characterized by resistance to salt and drought, resistance to phytopathogens of viral and fungal origins, pests and by other economically significant characteristics [28, 31, 49]. *DREB1* genes of wild species closely related to wheat are important in wheat breeding for increased tolerance to salinity and drought.

As a result of cloning PCR products amplified with various primer combinations and subsequent sequencing, we obtained 30 unique nucleotide sequences for six examined species of wild cereals: *Th. bessarabicum* (Thbe1, Thbe2, Thbe3), *Th. intermedium* (Thin1, Thin2, Thin4, Thin5, Thin6, Thin7), *Th. ponticum* (Thpo1, Thpo2, Thpo3, Thpo4, Thpo5, Thpo6, Thpo7, Thpo8 – from the sample PI 508561; Thpo9, Thpo10, Thpo11, Thpo12, Thpo13, Thpo14, Thpo15, Thpo16 – from the sample 1158A/19), *P. spicata* (Pssp1, Pssp2), *P. stipifolia* (Psst) and *D. villosum* (Davi). All sequences were obtained with primers P18F/R, except Thbe1 (P20F + PRa is a full-length gene sequence), Thbe2, Thbe3, Thpo1, Thpo2, Thin1, Pssp1 and Davi (P21F + PRa).

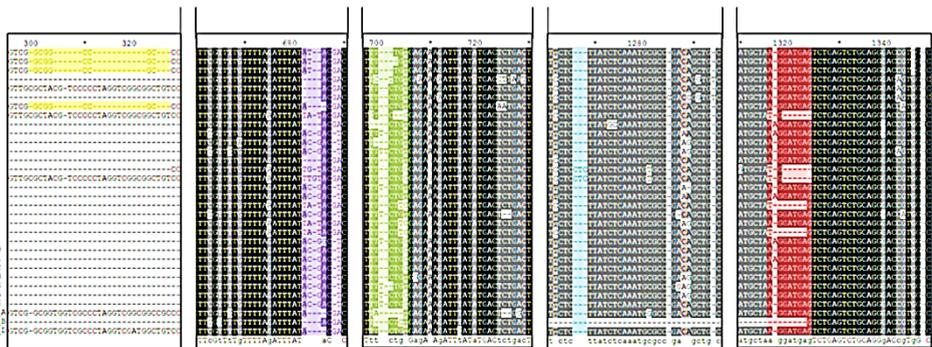


Fig. 1. Fragments of the aligned sequences of *DREB1* orthologous gene in *Thinopyrum bessarabicum* (lines 1-3), *Pseudoroegneria stipifolia* (line 4), *P. spicata* (lines 5-6), *Dasypyrum villosum* (line 7), *Th. intermedium* (lines 8-13), *Th. ponticum* (lines 14-29) and *TaDREB* of three subgenomes of bread wheat (three lower lines). The largest indels are highlighted in color: four indels in positions 299-327 (highlighted in yellow); region 682-689 (highlighted in purple); region 698-705 (highlighted in green); region 1266-1268 (highlighted in blue); region 1316-1324 (highlighted in red).

The alignment of DNA sequences with *DREB1* genes of bread wheat revealed 92-98% homology between them. The main differences between the sequences of the species studied and the sequences of bread wheat were numerous SNPs and insertions/deletions (indels) (Fig. 1). The largest ones were four indels in the positions 299-327 in *Th. bessarabicum* (1 bp, 6 bp, 11 bp and 3 bp) and

the indel in *D. villosum* (see Fig. 1, highlighted in yellow), indels in all studied species in the positions 682-689 (see Fig. 1, highlighted in purple), 698-705 (highlighted in green), 1266-1268 (see Fig. 1, highlighted in blue) and 1316-1324 (see Fig. 1, highlighted in red). Despite a significant number of DNA sequences we obtained the polymorphism of the *DREB1* orthologous gene is not limited to the detected SNPs and indels, since even within a single specimen of a wild species there can be intrapopulation diversity.

The obtained DNA sequences were translated in silico into hypothetical sequences of amino acids (AA). None of them contained stop codons. All the predicted AA sequences have the same structural organization, which is characteristic of DREB protein. In the N-terminal domain, there is a nuclear localization signal (NLS). Near the AP2 DNA-binding domain, a conserved ST-rich region is located. The C-terminal domain is enriched with glutamic and aspartic acids, which indicates the presence of a transcriptional activation domain.



Fig. 2. Alignment of the hypothetical amino-acid sequence of the AP2 DNA-binding domain of DREB1 protein in *Thinopyrum bessarabicum* (lines 1-3), *Pseudoroegneria stipifolia* (line 4), *P. spicata* (lines 5-6), *Dasypyrum villosum* (line 7), *Th. intermedium* (lines 8-13), *Th. ponticum* (lines 14-29) and sub-genomes A, B and D of bread wheat (three lower lines). The arrows indicate polymorphic amino acids in the AP2 domain; the amino acid residues that interact with the GCC box are highlighted in green, the replacement by one such amino acid residue is highlighted in red; asterisks indicate the amino acids that are important for DRE-specific binding.

An important role in the functioning of the DREB protein belongs to the AP2 DNA-binding domain; polymorphisms within this region can have a critical effect on the activity of the DREB protein. In all hypothetical AA sequences, there was an AP2 DNA-binding domain consisting of 59 amino acid residues (Fig. 2). The comparison of the AA sequences of the AP2 DNA-binding domain in the studied samples showed the presence of single amino acid polymorphisms (SAP). Since wild species are able to occupy different ecological and geographical niches and exhibit significant population diversity, it should be assumed that the SAP over the DREB1 protein may be wider than we have identified. In the sequences obtained by us all conserved amino acids of the AP2 domain were present which are characteristic of DREB proteins of cereals [50], with the probable exception of the Thpo11 sequence: in the position 25-27, the

specific WLK motif [25-27] was substituted by RLG (replacement of tryptophan with arginine) (see Fig. 2). The W(R) substitution that we revealed occurred in one of the seven conserved amino acids of the GCC-binding box in the AP2 domain. The presence of such a substitution in the AP2 domain may disorder protein configuration which, in turn, may affects the DNA-binding ability [51].

Comparative analysis of the DNA sequences of the *DREB1* gene of six wild species of cereals (*Th. intermedium*, *Th. ponticum*, *D. villosum*, *P. spicata*, *P. stipifolia*, *Th. bessarabicum*) with DNA sequences of the *DREB1* gene of bread wheat revealed SNPs which are characteristic for each species. This allowed the development of a PCR marker that can be used to identify most of the *DREB1* genes of the species which were studied in the genetic background of bread wheat.

We used the primers P18F/R, proposed by B. Wei et al. [17], to amplify the conserved region of the *TaDREB1* gene of bread wheat encoding the DNA-binding domain of AP2. By comparison of the nucleotide sequences of the *DREB1* orthologous genes obtained with a pair of primers P18F/R in the five examined species, the *Th. intermedium*, *Th. ponticum*, *D. villosum*, *P. spicata*, *Th. bessarabicum*, and the sequences of *DREB1* genes of bread wheat, three sites for FokI endonuclease restriction were identified (Fig. 3, highlighted in turquoise). The absence of one of the restriction sites and the presence of the other two were characteristic of most *DREB1* gene sequences in wild species, which distinguished them from the *DREB1* gene sequences of the subgenomes A and D of bread wheat. The *DREB1* gene of the subgenome B contained only one FokI restriction site; in addition, there was a large deletion (over 35 bp) in the same region. This difference allowed us to create CAPS (cleaved amplified polymorphic sequences) marker P18_FokI which in most cases can differentiate the *DREB1* orthologous genes of wheat and wild cereals.



Fig. 3. Fragments of the aligned sequences of the *DREB1* orthologous gene in *Thinopyrum bessarabicum* (lines 1-3), *Pseudoroegneria stipifolia* (line 4), *P. spicata* (lines 5-6), *Dasyphyrum villosum* (line 7), *Th. intermedium* (lines 8-13), *Th. ponticum* (lines 14-29) and *TaDREB1* of three subgenomes of bread wheat (three lower lines). The turquoise color shows restriction sites for FokI endonuclease.

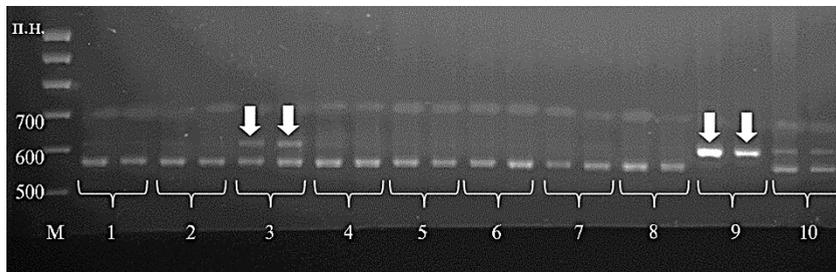


Fig. 4. An electropherogram showing the results of the analysis of Chinese Spring (CS) wheat lines with *Thinopyrum elongatum* chromosomes using the CAPS marker P18_FokI associated with the *DREB1* gene: 1 – CS + 1J^e, 2 – CS + 2J^e, 3 – CS + 3J^e, 4 – CS + 4J^e, 5 – CS + 5J^e, 6 – CS + 6J^e, 7 – CS + 7J^e, 8 – *Triticum aestivum*, Aivina variety, 9 – *Th. bessarabicum*; M is a marker of DNA fragment length (GeneRuler 100 bp DNA Ladder, Thermo Fisher Scientific, USA). The arrow indicates an additional fragment of ~600 bp amplified from orthologous wheatgrass *DREB1* gene.

We established chromosomal localization of the *DREB1* gene in the genome J with the developed P18_FokI marker as a tool. For this, we used a series of wheat lines with alien chromosomes of tall wheatgrass *Th. elongatum* ($2n = 2 \times = 14$, genome J^eJ^e). The control was wheatgrass *Th. Bessarabicum* carrying the genome J^bJ^b ($2n = 2 \times = 14$), highly homologous to the genome of *Th. elongatum*. In collections of genetic resources noted as *Th. Elongatum* we revealed only representatives of the polyploid species *Th. ponticum* ($2n = 10 \times = 70$, genomic composition JJJJJJ^sJ^sJ^sJ^s). These accessions were formerly called the Eastern European species of *Th. elongatum* [52, 53], which is a common thing for genetic collections of species with complicated botanical identification [54].

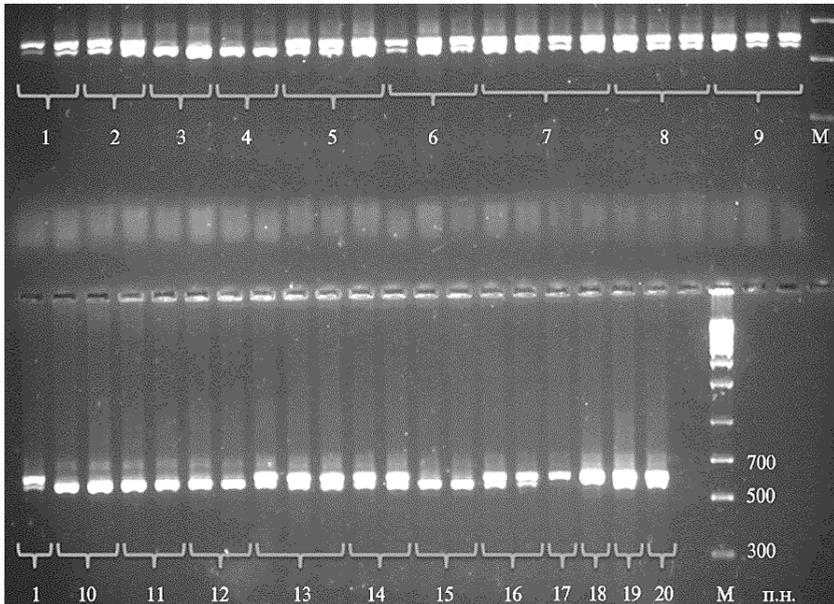


Fig. 5. Electrophoregram showing the results of analysis of wheat and wheatgrass hybrids and varieties using the CAPS marker P18_FokI for *DREB1* gene: 1 – 5542, 2 – 2087, 3 – Tulaikovskaya 110, 4 – Nemchinovskaya 24, 5 – 548, 6 – 1674, 7 – 4082, 8 – ZP26, 9 – M3202, 10 – Aivina, 11 – Tulaikovskaya 10, 12 – Tulaikovskaya 100, 13 – 4044, 14 – 4015, 15 – Tulaikovskaya zolotistaya, 16 – M12, 17 – *Thinopyrum intermedium*, 18 – *Th. ponticum*, 19 – *Pseudoroegneria spicata*, 20 – *Th. intermedium*; M – marker of DNA fragment length (Fisher BioReagents™ exACTGene™ DNA Ladders, Thermo Fisher Scientific, USA).

With the marker P18_FokI, a fragment of about 570 bp in size was found in wheat and about 600 bp in wheatgrass *Thinopyrum bessarabicum* (Fig. 4, indicated by an arrow). In all bread wheat lines with chromosomes of tall wheatgrass *Th. elongatum*, a fragment of ~ 570 bp specific for wheat was found and only CS + 3E line with 3J^e chromosome had a fragment of ~600 bp specific for wheatgrass. That is, the *DREB1* orthologous gene in *Th. elongatum* is located in the homeologous group 3, as in bread wheat, which confirms the correct work of the marker we elaborated.

To approbate the obtained marker P18_FokI, sequential PCR and restriction in 10 samples of wheatgrass hybrid was conducted. Using the marker P18_FokI, two fragments were identified in all the test samples of wheat and wheatgrass hybrids, pf ~ 570 bp and ~ 600 bp, that is, the samples carry the *DREB1* gene of both wheat and wheatgrass type (Fig. 5). All control samples of bread wheat, including varieties of bread wheat with a substituted chromosome from intermediate wheatgrass 6J(6D), i.e. Tulaikovskaya zolotistaya, Tulaikovskaya 10, Tulaikovskaya 100, Tulaikovskaya 110, a fragment of ~ 600 bp was absent. Hence, the fragment of ~ 600 bp of the wheatgrass type is specific for the

DREB1 gene localized in the homeologous group 3.

The marker-assisted selection increases the effectiveness of breeding. The use of molecular markers makes it possible to determine the valuable alleles in the splitting population with the least expenditures, to quickly and accurately identify any gene in case of introgression from the donor line to the recipient genome; it also provides a convenient tool for back-crossing [55]. With the help of the authors' CAPS marker P18_FokI associated with the *DREB1* orthologous gene it is possible to increase the efficiency of transferring this gene from wild cereals to bread wheat, to study the effect of the foreign *DREB1* gene on bread wheat resistance to drought, salinity and low temperatures, as well as in the long term to select valuable selection forms.

Thus, in this work we obtained DNA sequences of genes, orthologous to the *DREB1* wheat gene in the species *Thinopyrum intermedium*, *Th. ponticum*, *Th. bessarabicum*, *Dasypyrum villosum*, *Pseudoroegneria spicata* and *P. stipifolia*. The research on these nucleotide sequences revealed a polymorphism between the samples of the examined species, i.e. the presence of single nucleotide polymorphisms (SNPs) and indels. The analysis of the hypothetical amino acid sequences encoded by the detected *DREB1* orthologous genes showed conservativeness: only in one sequence there is a substitution of W(R) which is important for the functioning of the AP2 domain. A CAPS marker P18_FokI has been created, by means of which it is possible to distinguish the *DREB1* orthologous gene of the studied wild species from *DREB1* of bread wheat. Using the marker P18_FokI, the *DREB1* orthologous gene is mapped on the chromosome 3J^e of tall wheatgrass *Thinopyrum elongatum*. In 10 wheat and wheatgrass hybrids, there was the *DREB1* orthologous gene of a wheatgrass origin.

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