2020 • 24 • 3

Print ISSN 2500-0462
Online ISSN 2500-3259

Научный рецензируемый журнал

ВАВИЛОВСКИЙ ЖУРНАЛ ГЕНЕТИКИ И СЕЛЕКЦИИ

Основан в 1997 г.
Периодичность 8 выпусков в год
DOI 10.18699/VJ20.615

Учредители

Федеральное государственное бюджетное научное учреждение «Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук»

Межрегиональная общественная организация Вавиловское общество генетиков и селекционеров

Сибирское отделение Российской академии наук

2020 • 24 • 3

Print ISSN 2500-0462
Online ISSN 2500-3259

Scientific Peer Reviewed Journal

VAVILOV JOURNAL OF GENETICS AND BREEDING

VAVILOVSKII ZHURNAL GENETIKI I SELEKTSII

Founded in 1997
Published 8 times annually
DOI 10.18699/VJ20.615

Founders
Federal State Budget Scientific Institution "The Federal Research Center Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences"
The Vavilov Society of Geneticists and Breeders
Siberian Branch of the Russian Academy of Sciences

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DOI 10.18699/VJ20.39-o

Constructing the constitutively active ribosomal protein S6 kinase 2 from *Arabidopsis thaliana* (*At*RPS6K2) and testing its activity *in vitro*

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Abstract. Ribosomal protein S6 (RPS6) is the only phosphorylatable protein of the eukaryotic 40S ribosomal subunit. Ribosomes with phosphorylated RPS6 can selectively translate 5′TOP-(5′-terminal oligopyrimidine)-containing mRNAs that encode most proteins of the translation apparatus. The study of translational control of 5′TOP-mRNAs, which are preferentially translated when RPS6 is phosphorylated and cease to be translated when RPS6 is de-phosphorylated, is particularly important. In *Arabidopsis thaliana*, *At*RPS6 is phosphorylated by kinase *At*RPS6K2, which should in turn be phosphorylated by upper level kinases (*At*PDK1 – at serine (S) 296, *At*TOR – at threonine (T) 455 and S437) for full activation. We have cloned *AtRPS6K2* cDNA gene and carried out *in vitro* mutagenesis replacing codons encoding S296, S437 and T455 by triplets of phosphomimetic glutamic acid (E). After the expression of both natural and mutated cDNAs in *Escherichia coli* cells, two recombinant proteins were isolated: native *At*RPS6K2 and presumably constitutively active *At*RPS6K2(S296E, S437E, T455E). The activity of these variants was tested *in vitro*. Both kinases could phosphorylate wheat (*Triticum aestivum* L.) *Ta*RPS6 as part of 40S ribosomal subunits isolated from wheat embryos, though the non-mutated variant had less activity than phosphomimetic one. The ability of recombinant non-mutated kinase to phosphorylate *Ta*RPS6 can be explained by its phosphorylation by bacterial kinases during the expression and isolation steps. The phosphomimetically mutated *At*RPS6K2(S296E, S437E, T455E) can serve as a tool to investigate preferential translation of 5′TOP-mRNAs in wheat germ cell-free system, in which most of 40S ribosomal subunits have phosphorylated TaRPS6. Besides, such an approach has a biotechnological application in producing genetically modified plants with increased biomass and productivity through stimulation of cell growth and division.
Key words: wheat (*Triticum aestivum*); S6 protein (*Ta*RPS6) of 40S ribosomal subunits; *Arabidopsis thaliana*; RPS6-kinase 2 (*At*RPS6K2); phosphomimetic mutation; *Ta*RPS6 phosphorylation.

For citation: Zhigailov A.V., Stanbekova G.E., Beisenov D.K., Nizkorodova A.S., Polimbetova N.S., Iskakov B.K. Constructing the constitutively active ribosomal protein S6 kinase 2 from *Arabidopsis thaliana* (*At*RPS6K2) and testing its activity *in vitro*. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):233-238. DOI 10.18699/VJ20.39-o

Конструирование постоянно активной киназы 2 рибосомного белка S6 из *Arabidopsis thaliana* (*At*RPS6K2) и тестирование ее активности *in vitro*

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Аннотация. Рибосомный белок S6 (RPS6) – единственный белок 40S субчастиц эукариотических рибосом, способный фосфорилироваться. Рибосомы с фосфорилированным RPS6 могут селективно транслировать 5′TOP (5′-terminal oligopyrimidine)-содержащие мРНК, которые кодируют большинство белков трансляционного аппарата клеток. Исследование трансляционного контроля 5′TOP-мРНК, которые преимущественно транслируются, когда RPS6 фосфорилирован, и перестают транслироваться, когда RPS6 дефосфорилируется, является особенно важным. В клетках *Arabidopsis thaliana* *At*RPS6 фосфорилируется киназой *At*RPS6K2, для активации которой, в свою очередь, требуется ее фосфорилирование киназами верхнего уровня (*At*PDK1 – по серину (S) 296, *At*TOR – по треонину (T) 455 и также по S437). Мы клонировали кДНК-ген *AtRPS6K2* и провели его мутагенез *in vitro*, заменив кодоны S296, S437 и T455 на триплеты, кодирующие фосфомиметическую глутаминовую кислоту (E). После экспрессии обеих кДНК в клетках *Escherichia coli* были выделены два рекомбинантных белка: немутированный вариант – *At*RPS6K2 и мутированный вариант – *At*RPS6K2(S296E, S437E, T455E), предположительно, находящийся в стабильно активном состоянии. Активность этих киназ была протестирована *in vitro*. Показано, что обе киназы способны фосфорилировать рибосомный белок TaRPS6 в составе 40S рибосомных субчастиц, выделенных из зародышей пшеницы (*Triticum aestivum* L.), но активность нативной киназы была ниже в сравнении с ее фосфомиметической формой. Способность рекомбинантной нативной киназы фосфорилировать *Ta*RPS6 может быть объяснена ее фосфорилированием бактериальными киназами на стадиях экспрессии и выделения. Фосфоми-

метически мутированная киназа *At*RPS6K2(S296E, S437E, T455E) может служить удобным средством для исследования избирательной трансляции 5′TOP-содержащих мРНК в бесклеточной системе из зародышей пшеницы, в которой большинство 40S рибосомных субчастиц имеет фосфорилированную форму *Ta*RPS6. Кроме того, такой подход может найти биотехнологическое применение для создания генетически модифицированных растений с увеличенной биомассой и продуктивностью за счет стимуляции роста и деления клеток.
Ключевые слова: пшеница (*Triticum aestivum*); белок S6 (*Ta*RPS6) 40S субчастицы рибосом; *Arabidopsis thaliana*; *At*RPS6-киназа2; фосфомиметическая мутация; фосфорилирование *Ta*RPS6.

Introduction

Growth and division of cells depending on the availability of nutrients, energy resources, as well as responding to internal and external stimuli are coordinated by signaling system based on a multilevel cascade of serine-threonine protein kinases. These kinases transmit signals from internal and external events to the protein synthesis apparatus, causing inhibition or enhancement of protein synthesis (Turck et al., 2004; Wolters, Jürgens, 2009; Henriques et al., 2014; Rexin et al., 2015; Roustan et al., 2016). The target of rapamycin (TOR) kinase – is the master signaling integrator, central hub synchronizing cell growth according to the nutrient and energy status as well as environmental influences (Caldana et al., 2019). In mammals, TOR forms two functionally distinct protein complexes: mTORC1 containing RAPTOR (regulatory-associated protein of mTOR), and mTORC2 containing RICTOR (rapamycin-insensitive companion of mTOR) (Roustan et al., 2016). In favorable conditions mTORC1 phosphorylates RPS6K (Wolters, Jürgens, 2009; Henriques et al., 2014; Rexin et al., 2015). Complete activation of mammalian RPS6K by phosphorylation is dependent on another upper level PDK1 kinase (Otterhag et al., 2006). The fully activated RPS6K in turn phosphorylates the S6 ribosomal protein (RPS6) (Williams et al., 2003).

At transcriptional level, phosphorylation of pRPS6 in nucleolus leads to activation of rRNA gene promoter and ribosomogenesis (Ren et al., 2011; Kim et al., 2014). In cytosol, RPS6 phosphorylation promotes the selective translation of special group of cellular mRNAs, containing 5′-terminal oligo-pyrimidine tract (5′TOP) in their 5′-untranslated regions (5′UTRs) (Meyuhas, Kahan, 2015). The number of these 5′TOP-containing mRNAs, according to various estimates, ranges from one hundred to two hundred and forty (Turck et al., 1998; Meyuhas, Kahan, 2015). They encode almost all the proteins of the translation apparatus (all ribosomal proteins, all elongation factors and many of the translation initiation factors, poly(A)-binding proteins, etc.) (Turck et al., 1998), as well as other protein families associated with lysosome functions, metabolism and proliferation (Meyuhas, Kahan, 2015).

As in yeast and animals, TOR kinase is involved in controlling plant growth and cell division (Ryabova et al., 2019). But in plants, only orthologs of genes encoding mTORC1 were found (Xiong, Sheen, 2015; Wu et al., 2019). No clear orthologs of the RICTOR have yet been found in plants (Xiong, Sheen, 2015; Ryabova et al., 2019). TOR proteins are highly conserved in eukaryotes. For example, in *A. thaliana* and *Homo sapiens* they share 73 % amino acid sequence identity in the kinase domains (Xiong, Sheen, 2015).

Although functioning of this main regulator of cell processes has been well studied in other eukaryotes, knowledge of the regulation of translation and gene expression in plants is very limited. Most studies of the regulation of cellular process by plant RPS6-kinase were performed on a model object *A. thaliana* containing two very similar forms – *At*RPS6K1 and *At*RPS6K2. It was shown that only *At*RPS6K2 is able to phosphorylate RPS6 (Turck et al., 1998; Werth et al., 2019) and stimulate an increase in cell size (Rexin et al., 2015). For the complete activation of *At*RPS6K2, it is necessary that it be phosphorylated by pPDK1 kinase (at Ser296), pTOR kinase (at Thr455), as well as by one more, unknown, kinase (at Ser437) (Turck et al., 1998; Otterhag et al., 2006).

Although pTOR→S6K signaling plays multiple roles in translational control (Rexin et al., 2015), mechanisms used by TOR kinase to impact global protein synthesis in plants are not well understood (Xiong, Sheen, 2015; Ryabova et al., 2019; Wu et al., 2019). New data are currently appearing on the involvement of pRPS6K1 in the promotion of translation reinitiation of upstream open reading frame (uORF)-containing viral and cellular mRNAs via phosphorylation of eIF3h (Schepetilnikov et al., 2013) and in regulation of translation initiation under energy-deficient conditions via formation of the functional eIF4F complex (Lee et al., 2017). Nevertheless, the role of plant pRPS6K2 and pRPS6 phosphorylation in translation regulation in the cytosol remains unclear (Xiong, Sheen, 2015; Ryabova et al., 2019; Wu et al., 2019).

It is practically impossible to control the multiple and simultaneous phosphorylation of *At*RPS6K2 kinase by the kinases of the upper regulatory level for experimental purposes. Therefore, we decided to use a different approach to achieve the phosphorylation of plant RPS6 using the mutated form of *At*RPS6K2, which should be stably active. We have cloned the *AtRPS6K2* cDNA gene and performed *in vitro* mutagenesis of this cDNA by replacing codons encoding serines at positions 296 and 437, as well as threonine at position 455 with triplets encoding the phosphomimetic amino acid – glutamic acid. After expression of non-mutated and mutated cDNA gene in *E. coli* cells the native *At*RPS6K2 and the phosphomimetic *At*RPS6K2(S296E, S437E, T455E) recombinant protein was obtained. The second one is expected to have stable kinase activity, regardless of the upper-level kinases, that could be used as a unique tool for the artificial phosphorylation of *Ta*RPS6 in a wheat germ cell-free translation system. Mutated version of cDNA gene encoding the constantly active form of *At*RPS6K2 may also be used to obtain genetically modified plants with increased productivity, earlier ripening and a higher rate of biomass accumulation.

Materials and methods

Cloning of *AtRPS6K2* cDNA gene. The total RNA was isolated from *A. thaliana* (Col-0 ecotype) leaves using Tri-reagent (Sigma). The reverse transcription reaction was performed using Maxima Reverse Transcriptase (Thermo) and '*At*S6K2-rev-3UTR' primer (5′-GAATTCGAGAAATAGGTTTCTTC AAACAACCGTTGATTTTG), which allowed to differentiate *At*RPS6K2 from *At*RPS6K1 mRNAs. RT-PCR was per-

formed in 25 µl reaction using Phusion High-fidelity DNA polymerase (Thermo), 0.2 pM primers 'Nde-*At*S6K2-for' (5′-GGGCGAATTGGGTCATATGGTTTCTTCTCAGTG) and '*At*S6K2-Xho-rev' (5′-AAACTCGAGCTACAAGTTG GATGTGGTCCGATGA) and 2.5 µl of RT-reaction mixture. Temperature regime: stage 1–5 min at 94 °C, 1 cycle; stage 2–10 s at 98 °C, 20 s at 49 °C, 45 s at 72 °C, 4 cycles; stage 3–10 s at 98 °C, 20 s at 52 °C, 45 s at 72 °C, 30 cycles; stage 4–5 min at 72 °C, 1 cycle. The PCR product (~1425 bp) was digested with *Nde*I/*Xho*I and cloned into pET19b vector digested with the same enzymes resulting 'Pet19b-His-*At*RPS6K2' plasmid.

Mutagenesis. *In vitro* mutagenesis was performed in three steps using QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies) according to the manufacturer's protocol. At the first step 'Pet19b-His-*At*RPS6K2' plasmid was amplified entirely using Pfu Ultra High-Fidelity DNA polymerase (Thermo) and complementary primers: 'S296-Glu-dir' (5′-AAACACAAGATCAAACGAAATGTGTGGGACTA CGGA) and 'S296-Glu-rev' (5′-TCCGTAGTCCCACACAT TTCGTTTGATCTTGTGTTT) containing corresponding nucleotide substitutions. Temperature regime: stage 1–30 s at 95 °C, 1 cycle; stage 2–30 s at 95 °C, 1 min at 55 °C, 7 min 30 s at 68 °C, 18 cycles. The reaction mixture was further treated with restriction enzyme *Dpn*I, which cleaves methylated DNA into fragments at 5′-Gm^{6}ATC-3′ sequences. Since the original plasmid was methylated (dam$^+$ *E. coli* strain DH5 was used for plasmid enrichment), the restriction enzyme *Dpn*I had cleaved the original non-mutated plasmid, whereas 'Pet19b-His-*At*RPS6K2(S296E)' plasmid synthesized during PCR-step remained intact. Subsequently, the competent *E. coli* cells (XL1-Blue strain) were transformed with the reaction mixture. Another two mutagenesis steps for the production of 'Pet19b-His-*At*RPS6K2(S296E, S437E)' and 'Pet19b-His-*At*RPS6K2(S296E, S437E, T455E)' plasmids were done in the same manner using 'S437-Glu-dir' (5′-ACATGTCTGTT TTGGATGAACCAGCAAGTAGTCCCA)/'S437-Glu-rev' (5′-TGGGACTACTTGCTGGTTCATCCAAAACAGAC ATGT) or 'T455-Glu-dir' (5′-ACCCTTTTACAAACTTCG AATACGTCAGGCCTCCTCA)/'T455-Glu-rev' (5′-TGAG GAGGCCTGACGTATTCGAAGTTTGTAAAAGGGT) primers respectively. Resulting DNA-constructs were used as templates for the next *in vitro* mutagenesis step. The inserts cloned into the recombinant plasmids were sequenced from both ends by the dideoxy chain termination method using Big Dye Terminator v.3.1 sequencing kit (Thermo) on the 310 genetic analyzer (Applied Biosystems) according to manufacturer's recommendations.

Expression and purification of recombinant proteins. *E. coli* strain BL21(DE3) cells transformed with recombinant 'Pet19b-His-*At*RPS6K2' or 'Pet19b-His-*At*RPS6K2(S296E, S437E, T455E)' plasmid were grown in 100 ml of LB medium containing ampicillin (100 µg/ml) at 30 °C to A_{600} of 0.5 unit. The expression of recombinant proteins was induced by 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C for 4 h. Cells were collected by centrifugation, resuspended in His-buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing 10 mM imidazole, and then lysed by addition of lysozyme (1 mg/ml) and sonication. The cell debris was removed by centrifugation at 10000 g for 20 min at 4 °C. Supernatant was combined with PerfectPro Ni–NTA resin suspension (5-Prime), shaken at 4 °C for 1 h followed by flow throw in column. The resin was washed twice by His-buffer containing 20 mM imidazole at 4 °C. His-tagged proteins bound to the resin were eluted with His-buffer containing 250 mM imidazole and dialyzed against dialysis buffer (20 mM TrisAc, 90 mM KAc, 2.5 mM $Mg(OAc)_2$, pH 7.6) at 4 °C for 12 h. Dialyzed proteins were concentrated by centrifugation in 10,000 MWCO HY columns (Sartorius) according to the manufacturer's instructions. Protein concentration was estimated by the Bradford protein assay (Bradford, 1976).

Gel-electrophoresis. Proteins were separated by standard SDS-PAGE in Tris-Glycine gel system (12.5 % T, 0.5 % C separating gel; 5.2 % T, 2.5 % C stacking gel) according to U.K. Laemmli (1970). After electrophoresis, the gels were fixed and stained in PageBlue Protein Staining Solution (Thermo) or subjected to semi-dry blotting in transfer buffer (102 mM glycine, 25 mM Tris base, 20 % (v/v) ethanol) for 1 h at 0.8 mA/cm^2 and 20 V using 0.22 µm pore NitroBind nitrocellulose membranes (GVS).

Western blotting. For immunodetection of His-*At*RPS6K2 and His-*At*RPS6K2(S296E, S437E, T455E) proteins, the blots were first 'blocked' by submerging them in blocking solution (TBST buffer (20 mM Tris-HCl; 150 mM NaCl, 0.05 % (v/v) Tween 20, pH 7.5) containing 5 % skim milk) for 1 h at 25 °C with gentle shaking. The blots were then incubated with Penta-His mouse antibodies (5 Prime) diluted (1:2,000) in the blocking solution for 1 h at 25 °C, thoroughly washed three times with TBST buffer, and incubated for 1 h at 25 °C with horseradish peroxidase-conjugated goat anti-mouse antibodies (Santa Cruz) diluted (1:2,000) in blocking solution. After double washes in TBST and double washes in TBS, the blots were chemiluminescence developed using Chemiluminescent Peroxidase Substrate-3 detection reagents (Sigma). An image of the membrane was then produced on X-ray film. Monoclonal Anti-Phosphoserine Mouse Antibodies (Sigma) and Monoclonal Anti-Phosphothreonine Mouse Antibodies (Sigma) were used as 1st antibodies (at 1:300 dilution in TBST containing 5 % BSA) for the detection of phosphorylation status of proteins.

40S ribosomal subunits isolation. 40S ribosomal subunits were isolated from wheat (*T. aestivum* L., Kazahkstanskaya-10 cultivar) embryos, purified from endosperm, as described previously for ribosomal subunits isolation from human placenta (Matasova et al., 1991) with the ratio of buffer to embryos of 6:1. It was considered that 1 A_{260} unit corresponds to 50 pmol of 40S subunits.

Kinase assay. The reaction mixture in 20 µl contained 20 mM TrisAc (pH 7.6), 90 mM KAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT, 10 pmol of 40S ribosomal subunits, 0.1 mM ATP. Purified His-*At*RPS6K2 or His-*At*RPS6K2(S296E, S437E, T455E) were added in amount of 2.5 ng/µl. The mixtures were incubated for 20 min at 26 °C.

Results

Cloning and mutagenesis of *AtRPS6K2* cDNA gene. A total RNA preparation was isolated from *A. thaliana*, and reverse transcription was performed using '*At*S6K2-rev-3UTR' primer, complementary to 3′UTR of *AtRPS6K2* mRNA, but not *AtRPS6K1* mRNA, allowing to discriminate between

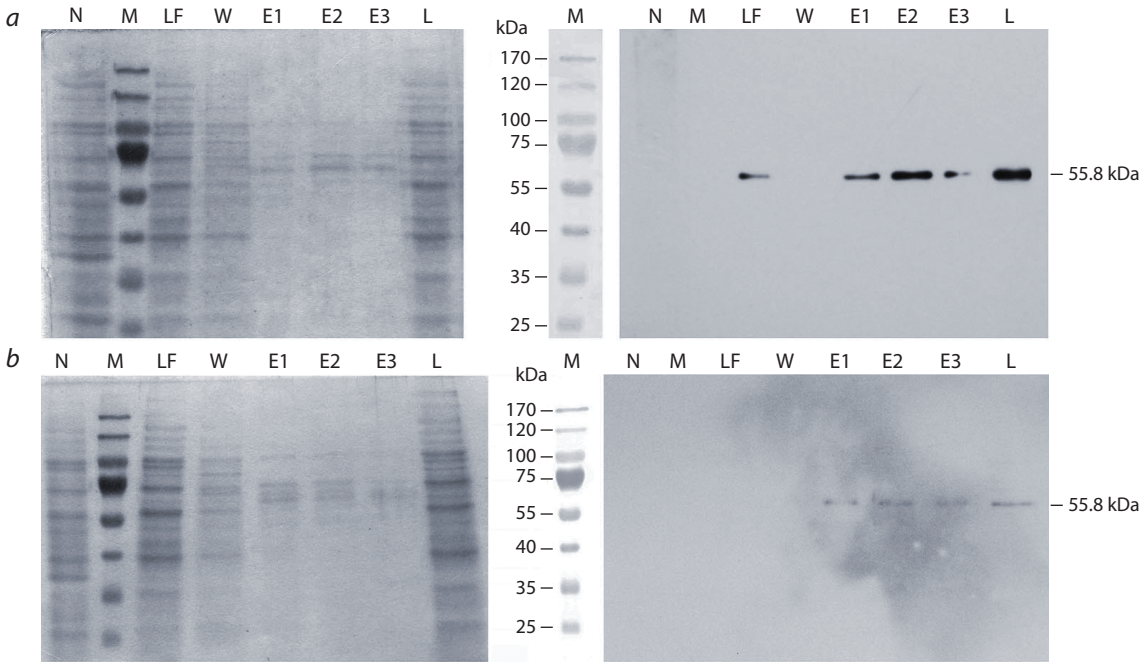

Fig. 1. Electrophoregrams (at the left) and western blot analysis (at the right) of recombinant proteins from different fractions of IMAC chromatography.

a – His-*At*RPS6K2; *b* – His-*At*RPS6K2(S296E, S437E, T455E). Left panels represent 12.5 % PAA-gels stained with Coomassie G250; right panels represent X-ray films developed after exposure with immunoblotting membranes (Penta-His Ab were used as the first antibodies). Tracks: M – protein markers; N – negative control (lysate of bacteria containing empty pET19b vector); L – lysate of bacteria synthesizing recombinant proteins; LF – proteins of wash-through fractions after loading of bacterial lysates onto Ni-NTA agarose; W – proteins eluted from Ni-NTA agarose with His-buffer containing 20 мМ of imidazole; E1, E2, E3 – proteins eluted from Ni-NTA agarose with His-buffer containing 250 мМ of imidazole.

them. Then, *AtRPS6K2* cDNA was amplified by RT-PCR and cloned into pET19b vector. According to sequencing analysis, *AtRPS6K2* cDNA corresponded to #AT3G08720 (GeneBank) sequence.

Thus obtained 'Pet19b-His-*At*RPS6K2' plasmid was mutated *in vitro* in three steps to introduce three phosphomimetic mutations into *AtRPS6K2* cDNA. At the first step, the TCC triplet encoding serine at position 296 was replaced by the GAA triplet, which encodes glutamic acid that imitates phosphorylated serine. In a second step, the TCT triplet of obtained mutated *AtRPS6K2*(*S296E*) cDNA encoding serine at position 437 was mutated to GAA triplet to form *AtRPS6K2*(*S296E*, *S437E*) cDNA. In the third step, the ACA triplet of *AtRPS6K2*(*S296E*, *S437E*) cDNA encoding threonine at position 455 was replaced by the GAA triplet to form the mutated *AtRPS6K2*(*S296E*, *S437E*, *T455E*) cDNA.

Expression and purification of recombinant kinases. *AtRPS6K2* and *AtRPS6K2*(*S296E*, *S437E*, *T455E*) cDNA genes were expressed in *E. coli* cells, then recombinant His-tagged proteins (His-*At*RPS6K2 and His-*At*RPS6K2(S296E, S437E, T455E) respectively) were isolated using immobilized metal ion affinity chromatography (IMAC) followed by immunoblotting analysis (Fig. 1).

Isolated proteins were purified by dialysis and concentrated. Preparations isolated under native conditions contained a certain amount of impurity polypeptides. Content of recombinant proteins in preparations was corrected according to densitometric analysis data (by ImageJ 1.42). The yield of purified and concentrated full-length recombinant proteins His-*At*RPS6K2 and His-*At*RPS6K2(S296E, S437E, T455E) was 5.22 mg and 4.52 mg per L of media respectively.

Testing the activity of recombinant kinases. Both forms of kinase (the intact one and that carrying three phosphomimetic substitutions) were tested for their ability to phosphorylate *Ta*RPS6 in the composition of 40S ribosomal subunits isolated from wheat embryos. The phosphorylation state of proteins was tested using monoclonal antibodies against phosphoserine (Fig. 2).

As can be seen from the data presented in Fig. 2, both kinases are able to phosphorylate the plant ribosomal protein S6 (*Ta*RPS6) in composition of 40S ribosomal subunits, although activity of His-*At*RPS6K2(S296E, S437E, T455E) is obviously higher than that of non-mutated His-*At*RPS6K2 (compare tracks *4* and *5* with tracks *2* and *3*, respectively in Fig. 2). In wheat germ, there are at least two forms of the S6 ribosomal protein (A and B); therefore, two bands are observed (see e. g. track *5* in Fig. 2).

Initially, we expected that non-mutated kinase should have no activity since for its activation in plant cells phosphorylation at three sites is required by upper-level kinases. The phosphorylation state of purified recombinant kinases was checked using monoclonal antibodies against phosphoserine and phosphothreonine (Fig. 3).

As can be seen from the data presented in Fig. 3, the non-mutated recombinant His-*At*RPS6K2 kinase produced in *E. coli* cells was phosphorylated both at serine residues (track *1* in Fig. 3, *a*) and threonine residues (track *1* in Fig. 3, *b*). Thus, some bacterial kinases were able to phos-

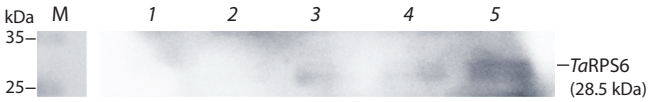

Fig. 2. Investigation of the ability of His-*At*RPS6K2 and His-*At*RPS6K2(S296E, S437E, T455E) to phosphorylate *Ta*RPS6.

Monoclonal Anti-phosphoserine Mouse Antibodies diluted (1:300) in 1× TBST containing 5 % BSA were used as the 1st antibodies. All reactions contained 5 pmol of 40S ribosomal subunits isolated from wheat embryos. Tracks: *1* – negative control (without kinases); *2* – 20 ng of His-*At*RPS6K2; *3* – 200 ng of His-*At*RPS6K2; *4* – 20 ng of His-*At*RPS6K2(S296E, S437E, T455E); *5* – 200 ng of His-*At*RPS6K2(S296E, S437E, T455E); M – protein markers.

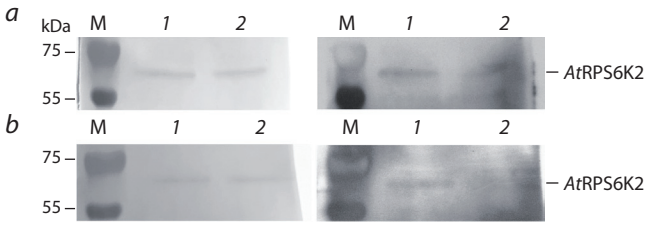

Fig. 3. Analysis of the phosphorylation state of His-*At*RPS6K2 and His-*At*RPS6K2(S296E, S437E, T455E) recombinant proteins.

a – analysis using monoclonal antibodies to phosphoserine; *b* – analysis using monoclonal antibodies to phosphothreonine. Left panels represent membranes stained with Ponso-S; right panels are immunoblots of the same membranes. Tracks: *1* – 5 μg of His-*At*RPS6K2; *2* – 5 μg of His-*At*RPS6K2(S296E, S437E, T455E). M – PageRuler Plus marker proteins.

phorylate His-*At*RPS6K2 protein resulting in its activation. It should be noted that certain non-mutated serine residues of mutated His-*At*RPS6K2(S296E, S437E, T455E) recombinant kinase were also phosphorylated (track *2* in Fig. 3, *a*), although this kinase was not phosphorylated at threonine residues (track *2* in Fig. 3, *b*).

Discussion

The interest in studying the mechanisms of TOR-mediated regulation of mRNA translation in plants is high because other mechanisms of regulation of protein biosynthesis, which are well described for mammals and yeast, either do not work or function within very narrow limits in plants. Indeed, in plant cells eIF4E binding proteins (eIF4E-BPs) were not found, and there are no genes for these proteins in plant genome (Immanuel et al., 2012). The mechanism of translation suppression by phosphorylation of peEF2 is not realized in plants. Then, out of four protein-kinases (PKR, HCR, PERK, GCN2) that phosphorylate α-subunit of meIF2 in mammalian cells, only pGCN2-kinase was detected in plants, that can be activated under several but not all stresses. Moreover, it was shown, that factor eIF2B is not necessary for cyclic functioning of plant peIF2 (Shaikhin et al., 1992), and neither its biochemical activity nor peIF2B-like factor orthologs were detected in plants till now (Immanuel et al., 2012). These circumstances make the TOR system one of the few currently known effective regulators of protein biosynthesis in plants.

Having obtained the constitutively active protein kinase *At*RPS6K2(S296E, S437E, T455E) with phosphomimetic substitutions of key amino acids, we acquire a convenient tool that allows to considerably increase phosphorylation of *Ta*RPS6 in the composition of 40S ribosomal subunits in wheat germ cell-free system. This allows studying important mechanisms of preferential translation of a specific group of cellular 5′TOP-containing mRNAs, which is preferably translated when pRPS6 is phosphorylated and ceases to be translated when RPS6 is de-phosphorylated (Williams et al., 2003). In addition to fundamental interest the use of cDNA encoding constitutively active RPS6-protein kinase would open novel routes for increasing crop yield through stimulation of ribosomogenesis and subsequent growth and division of plant cells. It is known that augmented expression of the *At TOR* gene results in a dose-dependent decrease or increase, in organ and cell size, seed production (Deprost et al., 2007; Enganti et al., 2017; Bakshi et al., 2019). In addition to regulating the protein synthesis process, TOR acts as a master regulator of the cell cycle, coordinator of rRNA transcription, activation of ribosomal protein genes, ribosome assembly (Shi et al., 2018) and may also regulate long non-coding RNAs (lncRNAs) expression (Song et al., 2019). Therefore, artificial increasing of *TOR* gene expression in plant cells can lead to serious undesirable consequences while using of *At*RPS6K2(S296E, S437E, T455E) cDNA may help to avoid these complications.

Conclusion

We have cloned the *AtRPS6K2* cDNA gene encoding kinase 2 of ribosomal protein S6 from *A. thaliana* and performed its mutagenesis to obtain the *At*RPS6K2(S296E, S437E, T455E) kinase containing phosphomimetic substitutions. Such mutated enzyme with constant RPS6-kinase activity may be used to study specific molecular mechanisms mediating efficient translation of 5′TOP-mRNAs depending on phosphorylation of RPS6 in plant cells. At the same time, the cDNA gene *AtRPS6K2*(*S296E*, *S437E*, *T455E*) may be used to obtain genetically modified plants with increased productivity and earlier ripening.

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Acknowledgements. Current work was carried out in the framework of the following scientific projects: AP05132532 "New molecular genetic approaches to improve crop productivity" and AP05130800 "Identification and study of universal translation enhancers suitable for plant and bacterial expression".

Conflict of interest. The authors declare no conflict of interest.

Received October 28, 2019. Revised December 23, 2019. Accepted December 24, 2019. Published online February 5, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):239-243
DOI 10.18699/VJ20.616

Рекомбинантный укороченный белок TNF-BD вируса натуральной оспы проявляет специфическую фармакологическую активность в экспериментальной модели септического шока

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Аннотация. Фактор некроза опухолей (TNF) – один из основных цитокинов, медиаторов иммунной системы, обеспечивающих защиту организма человека от вирусных инфекций. В процессе эволюции антропогенный вирус натуральной оспы (*Variola virus*, VARV) освоил эффективные механизмы преодоления иммунологических барьеров человека, кодируя в своем геноме белки, способные взаимодействовать с рецепторами цитокинов организма-хозяина, блокируя таким образом их активность. В частности, ген *G2R* этого вируса кодирует белок CrmB, который эффективно связывает TNF человека и мышей. При этом данный белок является двухдоменным и наряду с TNF-связывающим N-концевым доменом содержит С-концевой хемокин-связывающий домен. При использовании методологии молекулярного клонирования нами ранее получен рекомбинантный бакуловирус, продуцирующий в клетках насекомых рекомбинантный белок CrmB вируса натуральной оспы (VARV-CrmB), и показан его TNF-нейтрализующий потенциал в различных тестах *in vitro* и *in vivo*. С целью снижения иммуногенности этого вирусного белка при его многократном введении для терапии хронических воспалительных заболеваний получена рекомбинантная плазмида, направляющая в клетках *Escherichia coli* биосинтез укороченного однодоменного TNF-связывающего (TNF-BD) белка VARV. Методом металл-хелатной аффинной хроматографии из клеток выделен рекомбинантный белок TNF-BD. Терапевтический потенциал белка TNF-BD изучен в экспериментальной модели септического шока, индуцированного в организме мышей введением бактериального липополисахарида (ЛПС). После индукции септического шока животным вводили разные дозы рекомбинантного белка TNF-BD и регистрировали их гибель в течение 7 сут. Все мыши, не получавшие препарат белка TNF-BD после инъекции ЛПС, погибли через 3 сут, а в группах животных, которым вводили TNF-BD, в зависимости от дозы этого белка выжили 30, 40 или 60 % мышей. Результаты исследования демонстрируют наличие специфической фармакологической активности у рекомбинантного белка TNF-BD, синтезированного в бактериальных клетках, в мышиной модели ЛПС-индуцированного септического шока.
Ключевые слова: белок VARV-CrmB; вирус натуральной оспы; ЛПС; септический шок; TNF-связывающий домен.

Для цитирования: Гилева И.П., Якубицкий С.Н., Колосова И.В., Щелкунов С.Н. Рекомбинантный укороченный белок TNF-BD вируса натуральной оспы проявляет специфическую фармакологическую активность в экспериментальной модели септического шока. Вавиловский журнал генетики и селекции. 2020;24(3):239-243. DOI 10.18699/VJ20.616

Recombinant short TNF-BD protein from smallpox virus is pharmacologically active in an experimental septic shock model

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Abstract. Tumor necrosis factor (TNF) is one among the key cytokines that mediate the immune system to protect humans against viral infections. Throughout evolution, anthropogenic *Variola virus* (VARV) has developed effective mechanisms to overcome human defense reactions. The viral genome encodes soluble proteins imitating the structure of cellular cytokine receptors. These proteins compete with cellular receptors for cytokine binding, thus blocking the antiviral immune response. In particular, the *G2R* gene of VARV encodes the TNF decoy receptor, VARV-CrmB protein. This protein consists of N-ended TNF-biding (TNF-BD) and C-ended chemokine binding (Ch-BD) domains. Recombinant VARV-CrmB protein has been produced in insect cells using molecular cloning methods and its TNF neutralizing activity has been shown *in vitro* and *in vivo*. To decrease the immunogenicity of this protein, a recombinant plasmid coding for shortened TNF-BD protein of VARV in *Escherichia coli* cells has been constructed. Using the method of immobilized metal affinity chromatography, recombinant TNF-BD protein corresponding to the TNF-biding domain

of VARV-CrmB protein was purified from *E. coli* cells. The therapeutic potential of TNF-BD was studied using an experimental model of LPS-induced septic shock. After septic shock induction, several doses of recombinant TNF-BD were injected and the mortality of experimental animals was observed during 7 days. All mice not injected with TNF-BD had been dead by day 3 of the experiment, but 30, 40 and 60 % of the experimental animals, who received different TNF-BD doses, survived in a dose-dependent manner. Data obtained demonstrate that recombinant TNF-BD protein is pharmacologically active in the experimental model of LPS-induced septic shock.
Key words: VARV-CrmB protein; variola virus; LPS; septic shock; TNF-biding domain.

For citation: Gileva I.P., Yakubitskiy S.N., Kolosova I.V., Shchelkunov S.N. Recombinant short TNF-BD protein from smallpox virus is pharmacologically active in an experimental septic shock model. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):239-243. DOI 10.18699/VJ20.616 (in Russian)

Введение

В процессе коэволюции у вируса натуральной оспы (ВНО) появились многочисленные эффективные механизмы преодоления иммунологических барьеров человека. В частности, вирусный геном детерминирует синтез секретируемых белков, имеющих структурное сходство с клеточными рецепторами цитокинов. Вирусные белки функционируют как связывающие цитокины рецепторы, блокируя таким образом их активность (Shchelkunova, Shchelkunov, 2016). Способность вирусных белков эффективно взаимодействовать с иммунной системой человека открывает перспективу их использования для разработки иммуномодулирующих терапевтических средств нового поколения.

Некоторые TNF-блокаторы – Remicade® (Centocor, Inc., Malvern, PA, США), Enbrel® (Immunex Corp, Seattle, WA, США), Humira™ (Abbott Laboratories, Abbott Park, IL, США), действующим началом которых являются моноклональные антитела или химерные белки, состоящие из TNF-рецепторных и иммуноглобулиновых доменов, прошли клинические испытания и разрешены для применения в медицинской практике, но не один из перечисленных препаратов не оказался эффективным против септического шока (Monaco et al., 2015). Продемонстрированная *in vivo* и *in vitro* высокая TNF-нейтрализующая активность белка VARV-CrmB вируса натуральной оспы (Gileva et al., 2006; Shchelkunova, Shchelkunov, 2016) позволяет надеяться, что основой терапевтических препаратов нового поколения может стать этот белок.

Эндотоксический шок, индуцированный липополисахаридом (ЛПС) грамотрицательных бактерий (реакция Шварцмана), – широко используемая экспериментальная модель септического шока (Leturcq et al., 1995; Lamping et al., 1998; Fei et al., 2011). Введение рекомбинантного белка VARV-CrmB мышам с ЛПС-индуцированным септическим шоком приводило к достоверному увеличению выживаемости мышей и отчетливому снижению гистопатологических изменений внутренних органов по сравнению с контрольными животными (Gileva et al., 2006).

Полноразмерный рекомбинантный белок VARV-CrmB имеет молекулярную массу 47 кДа, и в его структуре выделяют N-концевой TNF- (TNF-BD) и С-концевой хемокин-связывающий (Ch-BD) домены (Alejo et al., 2006). С целью уменьшения иммуногенности рекомбинантного белка VARV-CrmB ранее нами получен его укороченный однодоменный вариант TNF-BD. Показаны биологическая активность белка TNF-BD *in vitro* (ингибирование как цитотоксичности TNF для культуры клеток фибробластов мыши L929, так и TNF-индуцированной окислительно-метаболической активности лейкоцитов крови мышей) (Цырендоржиев и др., 2014; Трегубчак и др., 2015), а также пониженная иммуногенность относительно полноразмерного белка (Непомнящих и др., 2017).

Цель настоящего исследования – изучение специфической фармакологической активности укороченного однодоменного рекомбинантного белка TNF-BD вируса натуральной оспы *in vivo*, а именно – в экспериментальной модели ЛПС-индуцированного септического шока.

Материалы и методы

Реагенты. Для приготовления жидких и твердых питательных средств использовали бактопептон, дрожжевой экстракт и бактоагар (Difco, США), антибиотики ампициллин, тетрациклин и канамицин (Sigma, США). Рекомбинантную плазмидную ДНК выделяли с помощью набора Qiagen Plasmid Midi Kit (QIAGEN, Германия). В работе были применены также реактивы фирмы Sigma (США): имидазол, ЛПС *Escherichia coli* 055:B5, мочевина, фенилметилсульфонилфторид (PMSF), IPTG.

Рекомбинантные плазмиды и бактериальные штаммы. Рекомбинантная плазмида pQE60-TNF-BD любезно предоставлена Т.В. Трегубчак (Трегубчак и др., 2015). Для амплификации этой плазмиды использовали штамм *E. coli* XL10-Gold (QIAGEN, Германия), а для наработки рекомбинантного белка TNF-BD – штамм *E. coli* SG13009[pRep4] (QIAGEN, Германия). Компетентные клетки *E. coli* XL10-Gold и *E. coli* SG13009 получали по методу (Mandel, Higa, 1970).

Бактериальные среды. Для культивирования штамма *E. coli* SG13009[pRep4], содержащего pQE60-TNF-BD, использовали стандартную LB-среду (Sambrook et al., 1985).

Электрофорез, хроматография. Фракционирование белков проводили в SDS-ПААГ по стандартной методике (Laemmli, 1970), с помощью маркеров молекулярных масс белков PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific, США) (11, 17, 28, 36, 55, 72, 95, 130, 250 кДа). Металл-хелатную афинную хроматографию проводили, используя Ni-NTA агарозу (QIAGEN, Германия), на колонке объемом 1 мл. Концентрацию белка определяли по методу (Bradford, 1976).

Животные. В эксперименты брали самок мышей линии BALB/c с массой тела 19–21 г (возраст 8–9 нед), полученных из вивария Государственного научного центра вирусологии и биотехнологии «Вектор» Роспотребнадзора (Кольцово, Новосибирская область, Россия). Мышей содержали при естественном световом режиме и

постоянном доступе к воде и пище. Животных выводили из эксперимента в соответствии с правилами, принятыми Европейской конвенцией по защите животных, используемых для экспериментальных целей.

Результаты

Наработка рекомбинантного белка TNF-BD. Для получения инокулятов были взяты индивидуальные колонии Ap^r-, Km^r-, Tc^r-трансформантов *E. coli* SG13009[pRep4]. Инокулят разводили свежим L-бульоном (1:50), содержащим 100 мкг/мл ампициллина и по 30 мкг/мл канамицина и тетрациклина. Бактериальную культуру инкубировали при 37 °C и интенсивной аэрации до достижения оптической плотности культуры A_{550} = 0.3–0.5, затем добавляли индуктор IPTG до концентрации 1 мМ (продолжительность индукции 4 ч). Клетки собирали низкоскоростным центрифугированием и ресуспендировали в 5 мл буфера следующего состава: 10 % сахароза, 100 мМ Трис-HCl, pH 7.3, 5 мМ ЕДТА, 20 мкг/мл PMSF. Затем бактериальные клетки разрушали ультразвуком на установке Soniprep (MSE, США; 10 циклов по 1 мин с интервалами между циклами в 30 с). Как было показано ранее (Трегубчак и др., 2015), рекомбинантный белок TNF-BD синтезируется в клетках *E. coli* в виде «телец включения». Для выделения целевого продукта тельца включения солюбилизовали в буфере А (100 мМ NaH_2PO_4, 10 мМ Трис-HCl, pH 8.0, 300 мМ NaCl, 10 мМ имидазол, 6 М мочевина) и супернатант наносили на колонку с Ni-NTA агарозой (V = 1 мл), уравновешенную 5 мл этого же буфера. Колонку промывали (2 мл × 3) буфером В (100 мМ NaH_2PO_4, 10 мМ Трис-HCl, pH 6.3, 300 мМ NaCl, 20 мМ имидазол, 6 М мочевина) и элюировали целевой продукт буфером С (100 мМ NaH_2PO_4, 10 мМ Трис-HCl, pH 8.0, 300 мМ NaCl, 250 мМ имидазол) (0.3 мл × 5). Фракции выделения анализировали в 10 % ПААГ. Результаты выделения представлены на рис. 1. Фракции, содержащие целевой продукт, объединяли. Таким образом, был выделен препарат рекомбинантного белка TNF-BD, чистота которого, по данным электрофореза, составляла около 90 %.

Исследование токсичности рекомбинантного белка TNF-BD. Для того чтобы определить способность препарата укороченного однодоменного белка TNF-BD предотвращать взаимодействие мышиного TNF со специфическими рецепторами эукариотических клеток, изучали его активность в тесте нейтрализации цитотоксического действия mTNF на культуре клеток мышиных фибробластов L929, как описано в работе (Трегубчак и др., 2015). Для определения токсичности рекомбинантного белка взяли две группы мышей линии BALB/c (каждая группа содержала пять животных). Одной группе вводили внутрибрюшинно физиологический раствор, другой – рекомбинантный белок в дозе 50 мкг/мышь. Наблюдение вели в течение 7 сут, за этот период не было обнаружено проявлений токсичности рекомбинантного белка.

Исследование специфической фармакологической активности белка TNF-BD в экспериментальной модели септического шока. Предварительно для мышей линии BALB/c была определена доза ЛПС *E. coli* 055:B5, вызывающая гибель 100 % животных ($ЛД_{100}$), равная 20 мг/кг веса. Животные были объединены в пять групп

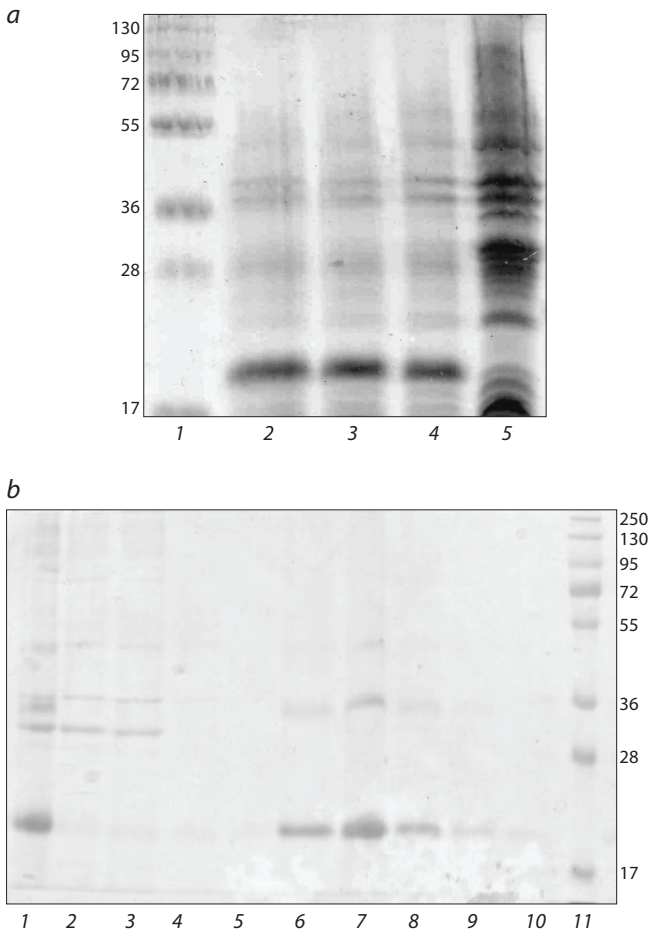

Fig. 1. Electrophoretic resolution of proteins.

(*a*) PAGE resolution of *E. coli* cell lysates with 10 % SDS. Lanes: *1*, protein ladder; *2–4*, SG13009[pRep4, pQE60-TNF-BD]; *5*, SG13009[pRep4]. (*b*) PAGE resolution of recombinant TNF-BD protein fractions with 12 % SDS. Lanes: *1*, fraction of insoluble cell proteins of *E. coli* SG13009[pRep4, pQE60-TNF-BD] solubilized in buffer A; *2, 3*, loading onto a column with Ni-NTA agarose equilibrated with buffer A; *4, 5*, washing the column with buffer B; *6–10*, elution of the target product with buffer C; *11*, protein ladder.

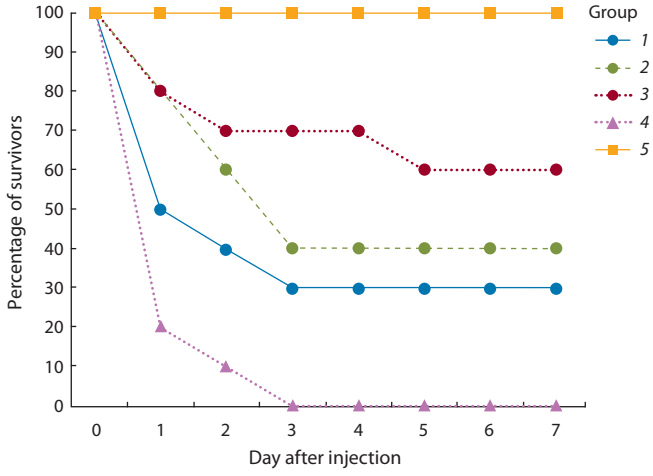

Fig. 2. Survival of experimental animals with LPS-induced endotoxic shock after administration of the recombinant TNF-BD protein.

(по 10 особей в группе). Мышам в группах 1–3 внутрибрюшинно вводили рекомбинантный белок TNF-BD в дозах 0.2, 2 или 20 мкг/мышь. Через 30 мин этим животным внутрибрюшинно инъецировали раствор ЛПС *E. coli* (O55:B5) в дозе 1 $ЛД_{100}$. Группе сравнения (группа 4) вводили только ЛПС, а группе отрицательного контроля (группа 5) – натрий-фосфатный буфер. После введения препаратов мышам регистрировали их гибель в течение 7 сут. Результаты эксперимента показаны на рис. 2: на конец эксперимента выживаемость животных в группе 5 составила 100 %, в группе 4 – 0 %, а в группах 1–3 – 30, 40 и 60 % соответственно.

Обсуждение

Сигнальная система, активируемая TNF, – один из основных факторов, обусловливающих развитие воспалительного процесса. Гиперпродукция TNF может приводить к гибели организма в результате развития системной воспалительной реакции или септического шока. Сепсис и септический шок до настоящего времени представляют одну из основных проблем здравоохранения. Ежегодно они являются причиной смерти более миллиона человек (Seymour, Rosengart, 2015). TNF-антагонисты, действующим началом которых были моноклональные антитела (Infliximab, Adalimumab) или аналог клеточных рецепторов (Etanercept), прошли клинические испытания и разрешены для применения в медицинской практике, но не один из этих препаратов не проявил терапевтической активности против септического шока (Monaco et al., 2015). Поэтому поиск новых классов соединений, обладающих TNF-нейтрализующей активностью, остается актуальной проблемой.

Существование вирусных белков, способных связывать цитокины и, в частности TNF, блокируя таким образом его активность (Shchelkunova, Shchelkunov, 2016), позволяет предположить возможность разработки нового поколения TNF-антагонистов на основе TNF-связывающего белка вируса натуральной оспы (VARV-CrmB). Ранее нами получен рекомбинантный бакуловирус BVi67, несущий ген белка VARV-CrmB, а из инфицированных им клеток насекомых линии Sf21 выделен рекомбинантный вирусный белок. Рекомбинантный белок VARV-CrmB эффективно нейтрализует эффекты TNF в экспериментальных системах *in vitro* и *in vivo* (Gileva et al., 2006). Особенно следует подчеркнуть его выраженный терапевтический эффект на проявление ЛПС-индуцированного септического шока у мышей, что отчетливо снижает гистопатологические изменения внутренних органов и достоверно увеличивает выживаемость животных (Gileva et al., 2006). Новый TNF-антагонист, кроме TNF-нейтрализующего потенциала, должен обладать низкой иммуногенностью, так как эффективность применения препарата может быть снижена из-за развития иммунного ответа на терапевтический белок (Chen et al., 2015; Eng et al., 2015).

Полноразмерный рекомбинантный белок VARV-CrmB (47 кДа) обладает способностью к олигомеризации, это может снизить его терапевтический потенциал при повторном или многократном применении. Белок VARV-CrmB состоит из TNF- и хемокин-связывающего доменов (Alejo et al., 2006). Нами сделано предположение, что однодоменный укороченный TNF-связывающий белок сохранит свою терапевтическую активность и будет обладать существенно меньшей иммуногенностью. Был получен бактериальный продуцент укороченного белка TNF-BD, соответствующего TNF-связывающему домену белка VARV-CrmB, и экспериментально доказана его способность ингибировать цитотоксичность TNF на клетках мышиных фибробластов L929 и TNF-индуцированную окислительно-метаболическую активность лейкоцитов крови мышей (Цырендоржиев и др., 2014; Трегубчак и др., 2015). При этом рекомбинантный TNF-BD проявлял сниженную иммуногенность относительно белка VARV-CrmB при многократном введении экспериментальным животным (Непомнящих и др., 2017).

В настоящем исследовании изучен терапевтический потенциал короткого рекомбинантного однодоменного белка TNF-BD в экспериментальной модели септического шока. С этой целью белок TNF-BD выделили из клеток *E. coli* методом металл-хелатной аффинной хроматографии, как это показано на рис. 1, *а* и *б*. При его внутрибрюшинном введении мышам линии BALB/c установлено отсутствие токсичности. Для мышей этой линии экспериментально определена доза ЛПС *E. coli* (O55:B5), вызывающая 100 % гибель животных ($ЛД_{100}$). При внутрибрюшинном введении ЛПС и белка TNF-BD в нашей работе впервые продемонстрирована дозозависимая специфическая фармакологическая активность низкоиммуногенного укороченного рекомбинантного белка TNF-BD в модели ЛПС-индуцированного септического шока (см. рис. 2).

Следует отметить, что вопрос правомерности использования мышиной модели эндотоксимии (реакции Щварцмана) для выявления новых препаратов для терапии септического шока у людей остается дискуссионным. Основанием для скептического отношения к мышиной модели служат отличия результатов транскриптомных анализов, механизмов развития врожденного и приобретенного иммунных ответов, гетерогенность человеческой популяции versus гомогенности инбредных линий мышей (Efron et al., 2015; Stortz et al., 2017). Не исключено, что препараты, проявившие высокую эффективность в мышиной модели сепсиса, не будут эффективными для человека, как это случилось с препаратом Centocor (Thayer, 1993). Однако простота проведения эксперимента и воспроизводимость результатов делают эту модель незаменимой для первичного скрининга TNF-связывающих терапевтических препаратов.

Заключение

На основании проведенного исследования можно сделать вывод, что рекомбинантный белок TNF-BD вируса натуральной оспы является перспективным для разработки нового поколения TNF-антагонистов. Этот белок может служить модельным объектом для изучения взаимодействия вирусных белков с компонентами иммунной системы организма человека. Методами делеционного анализа и мутагенеза, рентгеноструктурного анализа, компьютерного моделирования возможно идентифицировать в структуре белка TNF-BD аминокислотные остатки, осуществляющие взаимодействие с TNF и составляющие конкуренцию клеточным рецепторам.

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Acknowledgements. This work was supported by State Budgeted Project 0324-2019-0041 for ICG and the Russian Foundation for Basic Research, project 18-04-00022A. The authors are grateful to T.B. Tregubchak for providing plasmid pQE60-TNF-BD.

Conflict of interest. The authors declare no conflict of interest.

Received December 6, 2019. Revised January 27, 2020. Accepted January 30, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):244-251
DOI 10.18699/VJ20.617

Determination of the breeding value of collection chickpea (*Cicer arietinum* L.) accessions by cluster analysis

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Abstract. Assessment of the genetic resources of chickpea (*Cicer arietinum* L.) in a zone that is atypical for its cultivation (eastern forest-steppe of Ukraine) gives an opportunity to identify valuable starting material for priority breeding areas. The article presents the results of a cluster analysis on chickpea accessions from the National Center for Plant Genetic Resources of Ukraine (NCPGRU) for a set of agronomic characteristics. In 2005–2017, 653 chickpea accessions from the NCPGRU's core collection were studied: 369 *kabuli* accessions and 284 *desi* accessions. One hundred and fifty two sources of valuable traits were identified for 11 parameters: drought tolerance, resistance to Ascochyta leaf and pod spot, early ripening (vegetation period length), yield, performance, number of productive pods and seed number per plant, response to nitrogenization, protein content, seed size, and cooking quality. These accessions (77 *kabuli* accessions are light-colored and 75 *desi* ones are dark-colored) were grouped by a set of valuable economic characteristics using cluster analysis with the Euclidean distance as a measure. The study showed that this sample consisted of 4 clusters. Cluster 1 contained mainly *kabuli* accessions with optimal combinations of valuable traits: drought tolerance, resistance to Ascochyta leaf and pod spot, large seeds, high yield capacity and performance, pod and seed numbers as well as protein content in seeds. This cluster includes standards and most of reference varieties, which are well-adapted to the conditions of the eastern forest-steppe of Ukraine. The accessions of cluster 2 are characterized by high resistance to Ascochyta leaf and pod spot, late ripening, small seeds, low protein content, moderate response to nitrogenization, high performance attributed to a large number of productive pods and seeds per plant. Most of the accessions of this cluster are small-seeded late-ripening *kabuli* accessions. Cluster 3 consists of 3 accessions, which have large seeds and high protein content in them, give moderate yields, are highly responsive to nitrogenization and poorly resistant to Ascochyta leaf and pod spot. Cluster 4 comprises mainly *desi* accessions (63 %), which are mid-ripening, with small seeds, low performance, moderate yield capacity, medium protein content, poor cooking quality, moderate resistance to Ascochyta leaf and pod spot, and low drought tolerance. Representatives of this cluster are predominantly sources of one trait and may have restricted application in specialized breeding programs. Based on the data obtained, we concluded that the accessions of cluster 1 were preferable in breeding programs to develop chickpea varieties for the forest-steppe zone.
Key words: chickpea; cluster analysis; genetic resources; sources of valuable traits; breeding.

For citation: Vus N.A., Kobyzeva L.N., Bezuglaya O.N. Determination of the breeding value of collection chickpea (*Cicer arietinum* L.) accessions by cluster analysis. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):244-251. DOI 10.18699/VJ20.617

Определение селекционной ценности коллекционных образцов нута (*Cicer arietinum* L.) методом кластерного анализа

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Аннотация. Оценка генетических ресурсов нута (*Cicer arietinum* L.) в нетипичной для выращивания зоне (восточная часть лесостепи Украины) дает возможность выделить ценный исходный материал для приоритетных направлений селекции. В статье представлены результаты кластерного анализа образцов нута Национального центра генетических ресурсов растений Украины (НЦГРРУ) по комплексу агрономических характеристик. В период 2005–2017 гг. были изучены 653 образца базовой коллекции нута НЦГРРУ: 369 образцов морфотипа *kabuli* и 284 – *desi*. Выделены 152 источника ценных признаков по 11 показателям: засухоустойчивости, устойчивости к аскохитозу, скороспелости (длительности вегетационного периода), урожайности, продуктивности, количеству продуктивных бобов, количеству семян с одного растения, реакции на нитрагинизацию, содержанию белка, крупности семян, разваримости. Эти образцы (77 типа *kabuli* – светлоокрашенные и 75 *desi* – темноокрашенные) были сгруппированы по комплексу ценных хозяйственных

признаков с помощью кластерного анализа методом евклидовых расстояний. Результаты исследования показали, что представленная выборка состоит из четырех кластеров. В кластере 1 преобладают образцы типа *kabuli* с оптимальным сочетанием ценных признаков: засухоустойчивость, устойчивость к аскохитозу, крупносемянность, высокая урожайность и продуктивность, количество бобов и семян, содержание белка в семенах. В этот кластер вошли стандарты и большинство эталонов, которые характеризуются высокой адаптивностью к условиям восточной части лесостепи Украины. Образцы кластера 2 отличаются высокой устойчивостью к аскохитозу, позднеспелостью, мелкосемянностью, низким содержанием белка, средней реакцией на нитрагинизацию и высокой продуктивностью за счет большого количества продуктивных бобов и семян с одного растения. Большая часть образцов этого кластера – мелкосемянные позднеспелые образцы типа *kabuli*. Кластер 3 состоит из трех образцов, отличающихся высокой крупностью семян, средним уровнем урожайности, высокой реакцией на нитрагинизацию и повышенным содержанием белка при низкой устойчивости к аскохитозу. Кластер 4 объединяет преимущественно образцы морфотипа *desi* (63 %), среднеспелые со средними показателями урожайности, содержания белка и устойчивости к аскохитозу, с невысокой крупностью семян и продуктивностью, низкой разваримостью и засухоустойчивостью. Представители этого кластера – преимущественно источники одного признака – и могут иметь узкое применение в специализированных селекционных программах. На основе полученных данных предлагается приоритетное использование образцов первого кластера в селекционных программах по созданию сортов нута для лесостепной зоны.

Ключевые слова: нут; кластерный анализ; генетические ресурсы; источники ценных признаков; селекция.

Introduction

The world chickpea production is growing with every passing year, especially in arid regions, where it is the main source of protein for the population. Lack of varieties suitable for cultivation in different geographical zones of the country, which would combine high seed quality and resistance to bio- and abiotic factors, is the main limitation that hinders the spread of chickpea in Ukraine. The leading countries for chickpea cultivation have already gone this way, have develop and continue to develop plastic varieties, which allows expanding their cultivation regions.

Exploration of genetic diversity is very useful, when one works with genetic resources and for breeding programs, and includes labeling, identification and/or removal of duplicates in the gene pool and creation of core collections (Aliu et al., 2016).

Chickpea is a cheap source of high-quality protein in the diet of millions of people, which is a good alternative to animal protein for a balanced nutrition. The quality of chickpea protein is second only to milk protein. It is the second most important grain legume in the world, and in some parts, such as the Indian subcontinent, the first (Singh et al., 2015). Two main chickpea types are distinguished in the crop: *desi* and *kabuli*. *Desi* chickpea seeds are small, angular with dark seed coats; *kabuli* seeds are relatively large, smooth, yellow, yellow-pink or cream in color.

Kabuli chickpea seeds are considered more valuable due to high contents of protein, dietary fiber, complex carbohydrates and minerals. Both decorticated (removal of the seed coat) and non-decorticated chickpea seeds are used. Use without decortication involves boiling, frying or grinding to a paste (for example, in hummus manufacture). *Desi* chickpea is used both as split beans (dhal) and as flour (besan). Chickpea flour mixed with wheat or rice flour is used for making bread (chapati) and in confectionery (Tripathi et al., 2012). High drought tolerance plays an important role in the significant spread of chickpea. This feature of chickpea allows its growing in risky farming areas with limited precipitation and obtaining high yields of valuable dietary protein. With the expansion of arid zones and extension of rainless periods, this feature of the crop widens the prospects for its cultivation. However, to increase the sown acreage, it is necessary to develop new chickpea varieties that are adapted to specific conditions.

Food and Agriculture Organization (FAO) informs that the implementation of innovative breeding programs has increased the total yield of chickpea from 0.71 t/ha in 1996 to 0.96 t/ha in 2014 (FAOSTAT, 2016).

In the last two years, the chickpea production in Ukraine has grown from 6.5 thousand tons to 19.2 thousand tons due to introduction of domestic varieties, such as Pamiat, Triumf, Budzhak, and Odissei, with a potential yield of 1.8–2.3 t/ha (Kernasyuk, 2018). Ukrainian breeders were able to achieve such results owing to active involvement of the chickpea gene pool, which is studied in the National Center for Plant Genetic Resources of Ukraine (NCPGRU, Kharkiv). The NCPGRU's collection of chickpea comprises 1,897 accessions, with foreign varieties accounting for 91 % of the collection. One hundred and thirty four accessions are of Ukrainian origin; 38 of them are breeding varieties. FAO regards the NCPGRU's chickpea collection as one of the most important in the world by volume and diversity. All accessions are studied for three years and evaluated for phenological and morphological characteristics, disease resistance, quality and chemical composition of seeds, and, as a result of this work, sources of valuable traits are identified for further breeding. Based on results, working, trait, genetic and other collections are formed (The Second Report..., 2010).

To develop new varieties, it is important to use well-studied and selected starting material. In addition, numerous domestic and foreign studies show that breeding varieties have a narrow genetic base, despite a wide assortment of chickpea accessions stored in genebanks of different countries (Akinina, Popov, 2012; Khamassi et al., 2012; Benzohra et al., 2013;Vus et al., 2017a). Expansion of the genetic base by involving new parents in crossing is an important mechanism in breeding, but this material should be well-studied and adapted to a specific climatic zone.

Work with a collection of plant genetic resources implies evaluating large numbers of accessions for a wide range of different qualitative traits, which was applied to explore collections of corn (Kroonenberg et al., 1995), rice (Nandini et

al., 2017), lentil (Shikhalieva et al., 2018), chickpea (Malik et al., 2014) and other crops. For the convenience of breeding, different genotypes are grouped into clusters according to the on genetic diversity, and the degree of genetic divergence between them is assessed. For this purpose, cluster analysis is one of the most acceptable tools to assess the relative contribution of different traits – constituents to the total diversity, to quantify the degree of divergence and to choose genetically diverse parents to generate desirable recombinants. This was successfully done by N. Gupta et al., who studied 20 grape accessions for 9 yield traits (Gupta et al., 2017) and by E.J. Oliveira et al., who analyzed the cassava genebank in Brazil (Oliveira et al., 2016). The use of this method for evaluating breeding material at the early stages of breeding can accelerate the development of new varieties (Vilchinskaya et al., 2017). It is used to analyze agronomic, phenological, and morphological features in different crops, both to evaluate hybridization results and to assess the gene pool diversity (Motavassel, 2013). To solve this problem, researchers use different variants of cluster analysis. For example, evaluating 11 cotton accessions, L.F. Araújo et al. concurrently applied several variants (single-link method, complete link method, median, average linkage within the cluster and average linkage between the clusters) and found that the average linkage between the clusters was the most effective for their purpose (Araújo et al., 2014).

Cluster analysis is widely used in the breeding practice for assessment of different crops and comparison of their parameters. G. Evgenidis et al. applied this method, working with tomato starting material (Evgenidis et al., 2011). M. Khodadadi used the Euclidean distance method and Ward's method (Khodadadi et al., 2011) for assessing the genetic diversity and selection of parental pairs from 36 winter wheat accessions. The Euclidean distance method was also used by A. Subramanian and N. Subbaraman to group 38 corn accessions by 25 traits, which allowed them to identify the most distant pairs for crossing and showed that the geographical origin was not associated with expression of the studied traits (Subramanian, Subbaraman, 2010). P.M. Kroonenberg et al. clearly demonstrated the effectiveness of cluster analysis approaches for investigating collections of corn resources (Kroonenberg et al., 1995).

To assess the genetic diversity of wheat, B. Mecha et al. used cluster analysis and principal component method (Mecha et al., 2017). V. Nandini used the K-average method to assess 14 agronomic traits in rice accessions (Nandini et al., 2017). To select starting material for breeding for the quality of green beans, the Mahalanobis method was used (Haralayya et al., 2017). A. Kahraman et al. used the Euclidean distance method, which enables one to assess the similarity between accessions and identify groups of similar accessions, to group 35 bean accessions (Kahraman et al., 2014). The same method was used to analyze the results of studying major economically valuable traits in lentil accessions (Shikhalieva et al., 2018). S.R. Malik used cluster analysis to evaluate 113 *desi* chickpea accessions for 11 agronomic traits, which enabled him to identify a group of accessions with a set of traits combining the most valuable genotypes for further breeding (Malik et al., 2014). S. Aliu et al. investigated the qualitative composition of chickpea seeds and its association with yield indicators (Aliu et al., 2016).

Cluster analysis methods allow comparing various numbers of accessions: from 6 (Kayan, Adak, 2012) to over 300 (Naghavi et al., 2012) by different quantity and quality of characteristics under investigation: both qualitative and quantitative ones. Scientists from countries where chickpea is an important crop have used cluster analysis to evaluate genetic or selection material. For cluster analysis, both phonological/morphological and genetic features are used (Hajibarat et al., 2014; Aggarwal et al., 2015). For comparison of diverse agronomically valuable traits, such as yield capacity, resistance to diseases, growing period length and seed quality, the Euclidean distance method, which enables one to identify the hierarchical structure among the studied accessions, group them by a set of traits and select the most promising pairs for crossing, is most often used to study chickpea accessions. This method allows evaluating both similarity and distinction between accessions and characterizes the degree of expression of a trait under investigation (Syed et al., 2012; Malik et al., 2014). This makes it possible to distinguish the most distant accessions for crossing, which can positively affect the heterosis effect.

Cluster analysis allows one to compare accessions, using genetic markers, and characterize expression levels of the studied traits, for example, in inbred corn lines or in offspring from one cross (Subramanian, Subbaraman, 2010; Shrestha, 2016). K. Khamassi et al.'s studies proved that clustering by morphological characteristics was close to genotypic clustering by SSR markers ($r = 0.554$; $p = 0.001$). This makes it possible to conduct an effective selection of parental pairs for crossing based on assessments of morphological characteristics only (Khamassi et al., 2012).

Our purpose was to group chickpea accessions using cluster analysis and to select sources from clusters that have several valuable economic characteristics for further breeding.

Materials and methods

Six hundred and fifty three chickpea accessions from the core collection of the NCPGRU were taken as the test material for studies conducted in 2005–2017. The studied accessions were represented by two non-taxonomic groups: 369 *kabuli* accessions (light-seeded – white, cream, beige) and *desi* 284 accessions (dark-seeded – red, brown, green, black and other colors). Geographically the studied accessions originate from 20 countries. The majority of the *kabuli* accessions are from Ukraine (21 %); 20 % – from India; 13 % – from Syria; 11 % – from Afghanistan; and 10 % – from Iran. The vast majority of *desi* accessions originate from India (46 %); 12 % – from Canada; 7 % – from Syria; and 7 % – from Ukraine. Biologically, the accessions are represented by old and modern commercial domestic and foreign varieties and lines. The vast majority of the studied assortment were breeding lines: *kabuli* – 63 % and *desi* – 77 %.

Eleven parameters were assessed: drought tolerance, resistance to Ascochyta leaf and pod spot, early ripening (vegetation period length), yield capacity, performance, number of productive pods and number of seeds per plant, response to nitrogenization, protein content, seed size, and cooking quality.

The field experiments were conducted in scientific crop rotation 1 in the collection nursery of the Laboratory of Genetic Resources of Grain Legumes and Groat Crops of the Plant

Production Institute named after V.Ya. Yuriev of National Academy of Agrarian Sciences of Ukraine, which is located in the Kharkiv district of Kharkiv region in the North-East of the Left-Bank forest-steppe of Ukraine. Farmingtechniques were conventional for grain legume cultivation in this zone. Winter wheat was the forecrop. Chickpea was sown with manual planters; the record area was 1 m^2; the sowing scheme: 10 × 30 cm. Check varieties were sown every other 20 collection accessions. Variety Rozanna (Ukraine) was taken as the check variety for *kabuli*-type; Krasnokutskiy 123 (Russia) – for *desi*-type.

The collection chickpea accessions were studied in accordance with "Methodical Recommendations for Studies into Grain Legume Genetic Resources of the All-Union Research Institute of Plant Breeding" (1975) and "Methodological Recommendations for Studies into Grain Legume Genetic Resources" (Kobyzeva et al., 2016). The accessions were categorized by economic and biological characteristics using the classifier of the genus *Cicer* L. (Bezuglaya et al., 2012). The collection accessions were assessed for resistance to Ascochyta leaf and pod spot in accordance with "Methodical Guidelines for Studies of Resistance of Grain Legumes to Diseases" (1976). Provocative background was used to simulate epiphytoties of Ascochyta leaf and pod spotin 2005 and 2016 (Kosenko, Bezuglaya, 2006; Bezuglaya et al., 2007; Vus et al., 2017a; Vus, Kobyzeva, 2018). The average scores for the epiphytotic years were used for the calculation matrix.

Drought tolerance was assessed by tolerance indices developed by foreign researchers (Fisher, Maurer, 1978; Rosielle, Hamblin, 1981; Bouslama, Schapaugh, 1984; Gavuzzi et al., 1997; Ribaut, Poland, 1999; Yücel, Mart, 2014). Previously we had evaluated drought tolerance of chickpea accessions using drought tolerance indices (Vus et al., 2017b) and developed a rating scale for assessing drought tolerance. Exceedance of median for each individual index is defined as one point. The total points give a seven-point score: from 0 to 7 points, where 0 – no exceedance of median for any index, 7 – exceedance of median for seven indices. Thus, in this work, we used the following drought tolerance scale: 0 points – the accession is very susceptible to drought, 7 – the accession is drought tolerant.

Response of the accessions to pre-sowing nitrogenization of seeds was studied in plots of 2 m^2 without repetitions; the sowing scheme was 10 × 30 cm. The accessions were sown within the optimum timeframe, as described in (Didovich et al., 2010). Seeds were treated with rhizobophyte based on *Mesorhizobium cicero* (strain 065) immediately before sowing. The control was sown without seed treatment with rhizobophyte. The accessions' responses to nitrogenization were expressed as a percentage increase in the yield related to the control.

The protein content in seeds was determined by Kjeldahl digestionin the Laboratory of Genetics, Biotechnology and Guality of the Plant Production Institute named after V.Ya. Yuriev of NAAS (Yermakov, 1987). The cooking quality was determined in accordance with "Methodological Recommendations. Technological Evaluation of Pea, Lentil, and Bean Grain" (Komarov, Proreshneva, 1992).

As a result of evaluating 653 accessions of the NCPGRU's chickpea core collection by 11 parameters, 152 sources of valuable traits were identified: 77 *kabuli* and 75 *desi* accessions. These accessions were further used for grouping by the Euclidean distance method.

The experimental data were statistically processed by calculus of variations, analysis of variance and cluster analysis in Microsoft Excel (license No. 44208338, release date 06.27.2008) and Statistics 6.0 (license No. BXXR502C631 824NET3).

Results

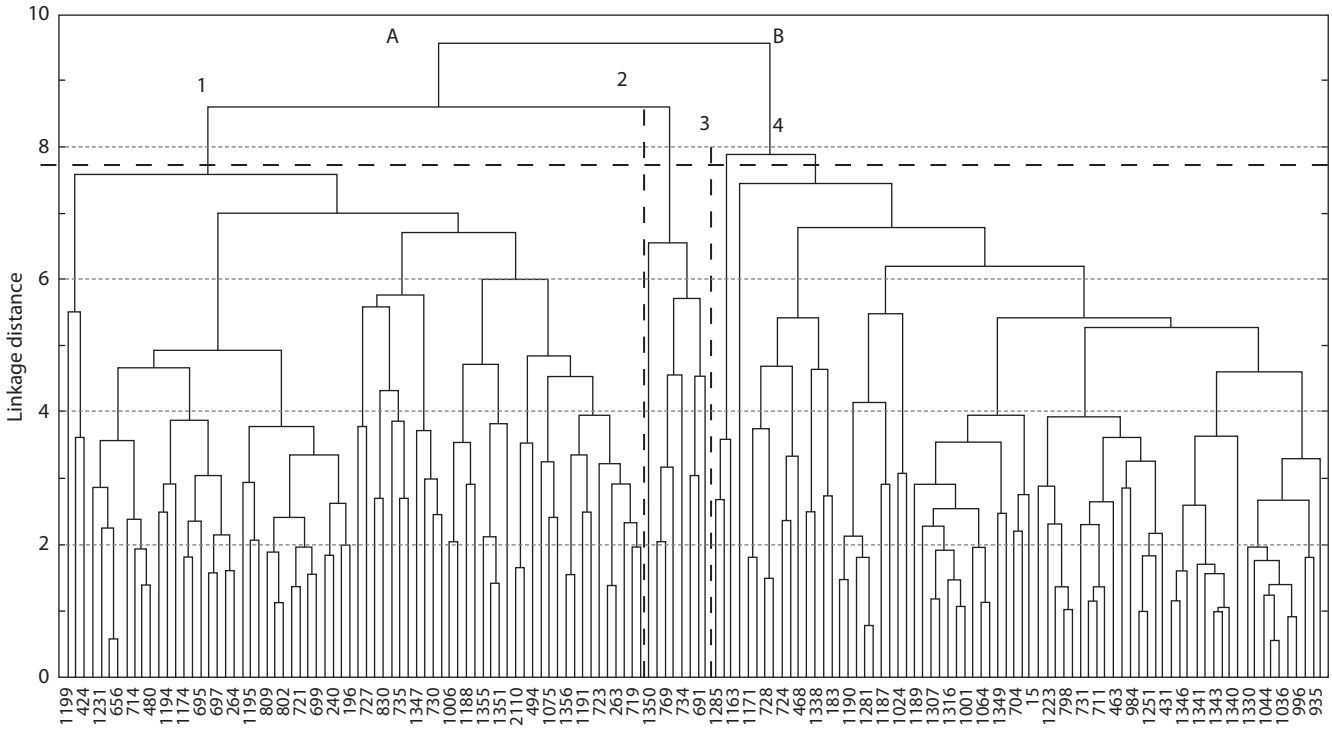
The primary differentiation of 152 accessions identified two clusters: A and B with similar numbers of accessions (78 and 74, respectively) (see the Figure). Most of the accessions in cluster A belong to *kabuli*-type (63 %), unlike cluster B, where 62 % of the accessions are *desi*. In this study, the types were only defined by the seed coat color: *kabuli* – light-colored and *desi* – dark-colored, which allowed us to establish relationships between valuable economic traits and seed color.

Further, each of the primary clusters was divided into two unequal secondary clusters: the first cluster (A) – into cluster 1 (70 accessions) and cluster 2 (8 accessions) and the second cluster (B) – into cluster 3 (3 accessions) and cluster 4 (71 accessions) (Table).

Cluster 1 consists of 70 accessions, of which 43 belong to *kabuli*-type. These accessions optimally combine 8 of the 11 traits under investigation: drought tolerance (4.30 points), resistance to Ascochyta leaf and pod spot (6.14 points), seed size (271.54 g/1000 seeds), high yield (328.17 g/m^2), performance (14.47 g/plant), the numbers of pods (37.50) and seeds (53.13), protein content in seeds (18.00 %). This cluster includes the check varieties for the *kabuli*- and *desi*-types: Rozanna (Ukraine) and Krasnokutskiy 123 (Russia). It also includes the NCPGRU's reference accessions for resistance to Ascochyta leaf and pod spot (UD0500196 (Azerbaijan), UD0500264 (Ukraine) and UD0500240 (Syria)); for high palatability (UD0500417 (Ukraine)); and for suitability for mechanized harvesting (UD0500444 (Ukraine)). The accessions of this cluster are highly adapted to the conditions of the eastern forest-steppe of Ukraine and the most promising material for breeding programs to develop varieties with several useful features.

Cluster 2 is represented by eight accessions; six of them are of *kabuli*-type, and two – of *desi*-type. The accessions of this cluster are characterized by high resistance to Ascochyta leaf and pod spot (7.00 points), late ripening (vegetation period = 93.13 days), small seeds (1000-seed weight = 209.69 g), low protein content (17.57 %), moderate response to nitrogenization (108.38 % related to the control), and high performance (14.70 g/plant) attributed to the large numbers of productive pods (59.47) and of seeds (86.92) per plant. All the six *kabuli* accessions of this cluster are small-seeded, late-ripening and the most resistant to Ascochyta leaf and pod spot (Reddy, Singh, 1984). These are local accessions from Moldova (UD0500691, UD0500692, UD0500734, and UD0500762), Russia (UD0500769) and India (UD0501256). In addition, this cluster contains reference accessions of *desi*-type for the traits of "high number of seeds" and "high number of productive pods per plant": UD0500022 (Georgia) and UD0501350 (India).

Cluster 3 has only three accessions: two of them belong to *kabuli*-type (UD0501163 (Ukraine) and UD0501268 (India))



Clustering of chickpea (*Cicer arietinum* L.) accessions by a set of valuable economic characteristics using the Euclidean distance method.

Distribution of the chickpea accessions between clusters

Parameter	Cluster 1 (70 accessions)		Cluster 2 (8 accessions)		Cluster 3 (3 accessions)		Cluster 4 (71 accessions)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DT, score	4.30	1.87	1.00	1.41	2.96	1.78	1.85	1.65
RA, score	6.14	1.89	7.00	2.39	3.67	2.31	4.49	1.55
VP, days	91.54	4.41	93.13	2.90	88.00	1.73	88.54	3.70
P, g	14.47	3.53	14.70	4.48	11.75	1.20	11.82	2.91
W1000, g	271.54	71.31	209.69	75.78	291.35	91.14	225.38	92.01
PP/P	37.50	7.22	59.47	5.77	27.43	6.81	34.21	7.82
S/P	53.13	17.15	86.92	24.72	34.46	15.43	52.12	18.33
Y, g/m^2	328.17	78.59	289.48	57.37	294.50	38.27	272.54	62.89
CQ, min	131.37	17.16	114.83	16.27	134.00	2.83	132.08	20.37
PC, %	18.00	1.61	17.57	1.03	19.22	1.75	18.49	1.59
N, % to control	101.10	5.60	108.38	9.13	141.67	3.79	102.93	6.80
T, % kabuli	61		75		67		37	

Note. DT – drought tolerance; RA – resistance to Ascochyta leaf and pod spot; VP – vegetation period; P – performance; W1000 – 1000-seed weight; PP/P – number of productive pods per plant; S/P – number of seeds per plant; Y – yield; CQ – cooking quality; PC – protein content; N – nitrogenization; T – type; SD – standard deviation.

and one accession is a *desi* one (UD0501285 (Syria)). The accessions of this cluster are characterized by large seeds (1000-seed weight = 291.35 g), moderate yield (294.50 g/m^2), strong response to nitrogenization (141.67 % related to the control), increased protein content (19.22 %), and low resistance to Ascochyta leaf and pod spot (3.67 points).

Cluster 4 mainly comprises *desi* accessions (63 %), which are characterized by mid-ripening (vegetation period = 88.54 days), small seeds (1000-seed weight = 225.38 g), low performance (11.82 g), moderate yield (272.54 g/m^2), medium protein content (18.49 %), poor cooking quality (132.08 min), moderate resistance to Ascochyta leaf and pod spot (4.49 points), and low drought tolerance (1.85 points). The representatives of this cluster are mainly sources of one trait and can have only narrow application in specialized breeding.

Discussion

The number of seeds and pod weight per plant are determining factors of the chickpea plant performance (Zali et al., 2011; Kazydub et al., 2015). Therefore, when selecting for performance, one should pay attention to these traits. In our study, few accessions (8) of cluster 2 produce the maximum numbers of productive pods and seeds per plant, and they have low indices of drought tolerance. At the same time accessions of cluster 1 with the maximum yield and above-average performance constituent in combination with resistance to Ascochyta leaf and pod spot and drought are of high value for breeding. Parameters such as performance, plant height, protein content and the pod number per plant are closely interrelated and positively correlate with yield (Kayan, Adak, 2012). In our studies, such characteristics were intrinsic to accessions of clusters 1 and 2.

Many researchers found that the growing period length, plant height, the numbers of branches and seeds per plant are mainly controlled by additive genes, directly and strongly correlating with the phenotypic trait of seed yield (Syed et al., 2012). Therefore, such traits-oriented breeding can be effective for increasing yield capacity. Stepwise regression analysis showed that the number of seeds per plant and 1000-seed weight accounted for 96 % of the total change in the yield. Therefore, the chickpea yield can be improved by choosing an idiotype having larger numbers of secondary and primary branches, larger numbers of pods and seeds per plant and a higher1000-seed weight of (Zali et al., 2011). We observed that the largest seeds were produced by accessions of cluster 3, however, they had the fewest productive pods and seeds per plant, while accessions of cluster 1, though having slightly smaller seeds than the average size, had larger numbers of seeds and productive pods. Therefore, priority should be given to accessions of cluster 1 in breeding for yield.

Often, researchers in chickpea study accessions of a single type A. Taleei studied *desi* accessions in Iran (Taleei, Shaabani, 2016), S.R. Malik – in Pakistan (Malik et al., 2014). M. Aarif (Aarif et al., 2017) and S. Aliu (Aliu et al., 2016) studied *kabuli* accessions in India and in Kosovo, respectively. We evaluated accessions of two types under the same conditions, and they were clearly differentiated in two primary clusters, A and B, and then in clusters 1 and 4, where the overwhelming majority of accessions were concentrated (70 and 71, respectively), *kabuli* accessions prevailed in cluster 1, and *desi* ones – in cluster 4. The involvement of accessions of different types from distant clusters in breeding will allow using seed color as a marker and expanding the genetic base of breeding varieties.

Large-seeded varieties are usually more susceptible to environmental conditions, therefore, selection of accessions and development of starting material with a high 1000-seed-weight that are tolerant to biotic factors is important for breeding (Gridnev et al., 2012). To obtain breeding material with increased resistance to Ascochyta leaf and pod spot and several valuable traits, it is advisable to cross large-seeded accessions cluster 3 with accessions of clusters 1 and 2, which are highly-resistant to Ascochyta leaf and pod spot.

The systematic selection of parental pairs used in modern breeding upon crossing, the principles of which had been shaped by N.I. Vavilov (1967) and further developed by scientists from different countries (Serebrovskiy, 1969; Vural, Karasu, 2007; Aarif et al., 2017; Haralayya et al., 2017), shows that genotypes from clusters with a maximum distance between them can be used as parents in breeding to generate varieties with combining high yields and good seed quality, especially when accessions of different eco-geographical origin are crossed.

Conclusions

Cluster analysis is an effective method for evaluating a large number of collection chickpea accessions for several parameters, which enables one to select parental pairs for different breeding programs. Starting material with a set of features was selected and grouped into 4 clusters. Accessions of cluster 1 are preferable for breeding chickpea varieties adapted to the eastern forest-steppe of Ukraine. Accessions of cluster 2 are important in breeding for resistance to Ascochyta leaf and pod spot and high performance. Accessions of cluster 3 (UD0501163 (Ukraine), a reference accession of positive response to nitrogenization, with large seeds and high protein content in seeds; UD0501268 (India) with strong response to nitrogenization and moderate resistance to Ascochyta leaf and pod spot; and UD0501285 (Syria) with large seeds, moderate resistance to Ascochyta leaf and pod spot) are highly valuable for developing commercial chickpea varieties, however, they require augmenting their adaptive features. Accessions of cluster 4 are sources of one trait may have narrow application in specialized breeding programs.

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Conflict of interest. The authors declare no conflict of interest.

Received August 15, 2019. Revised February 11, 2020. Accepted February 11, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):252-258
DOI 10.18699/VJ20.618

Metabolomic approach to search for fungal resistant forms of *Aegilops tauschii* Coss. from the VIR collection

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Abstract. Broadening of the genetic diversity of donors of resistance to biotic environmental factors is a challenging problem concerning *Triticum* L., which can be solved by using wild relatives of wheat, in particular, *Aegilops tauschii* Coss., in breeding programs. This species, believed to be the donor of D genome of common wheat (*T. aestivum* L.), is a source of some traits important for breeding. This greatly facilitates the possibility of crossing *Ae. tauschii* with common wheat. *Aegilops* L. species are donors of effective genes for resistance to fungal diseases in wheat. For instance, genes that determine resistance to rust agents in common wheat were successfully introgressed from *Ae. tauschii* into the genome of *T. aestivum* L. The aim of our study was to identify differences in metabolomic profiles of *Ae. tauschii* forms (genotypes), resistant or susceptible to such fungal pathogens as *Puccinia triticina* f. sp. *tritici* and *Erysiphe graminis* f. sp. *tritici*. These indicators may be used as biochemical markers of resistance. A comparative analysis of groups of *Ae. tauschii* accessions showed that metabolomic profiles of the forms with or without resistance to fungal pathogens differed significantly in the contents of nonproteinogenic amino acids, polyols, phytosterols, acylglycerols, mono- and oligosaccharides, glycosides, phenolic compounds (hydroquinone, kempferol), etc. This fact was consistent with the previously obtained data on the relationship between *Fusarium* resistance in oats (*Avena sativa* L.) and certain components of the metabolomic profile, such as acylglycerols, nonproteinogenic amino acids, galactinol, etc. Thus, our studies once again confirmed the possibility and effectiveness of the use of metabolomic analysis for screening the genetic diversity of accessions in the VIR collection, of *Ae. tauschii* in particular, in order to identify forms with a set of compounds in their metabolomic profile, which characterize them as resistant. *Ae. tauschii* accessions with a high content of pipecolic acids, acylglycerols, galactinol, stigmasterol, glycerol, azelaic and pyrogallic acids, campesterol, hydroquinone, etc., can be used for creating wheat and triticale cultivars with high resistance to fungal pathogens causing powdery mildew, brown rust, and yellow rust.
Key words: *Aegilops tauschii* Coss.; metabolomic approach; disease resistance; fungal pathogens.

For citation: Shelenga T.V., Malyshev L.L., Kerv Yu.A., Diubenko T.V., Konarev A.V., Horeva V.I., Belousova M.K., Kolesova M.A., Chikida N.N. Metabolomic approach to search for fungal resistant forms of *Aegilops tauschii* Coss. from the VIR collection. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):252-258. DOI 10.18699/VJ20.618

Использование метаболомного подхода для поиска форм *Aegilops tauschii* Coss. из коллекции ВИР им. Н.И. Вавилова, устойчивых к грибным патогенам

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Аннотация. Расширение генетического разнообразия доноров устойчивости *Triticum* L. к биотическим факторам среды – актуальная задача, которую возможно решить благодаря использованию в селекционных программах дикорастущих родичей пшеницы, в частности *Aegilops tauschii* Coss. По существующим представлениям, последний – донор генома D мягкой пшеницы *T. aestivum* L. и носитель ряда ценных селекционных признаков. Это значительно облегчает скрещивание *Ae. tauschii* с мягкой пшеницей. Виды рода *Aegilops* L. являются донорами эффективных генов устойчивости пшеницы к грибным болезням. Так, от видов *Ae. tauschii* в геном *T. aestivum* L. успешно интрогрессированы гены, детерминирующие устойчивость мягкой пшеницы к возбудителям ржавчины. Целью нашего исследования было выявление различий по метаболомным профилям форм (генотипов) *Ae. tauschii*, устойчивых и неустойчивых к грибным патогенам

(*Puccinia triticina* f. sp. *tritici* и *Erysiphe graminis* f. sp. *tritici*), что позволит использовать такие показатели в качестве биохимических маркеров устойчивости. Сравнительный анализ групп образцов *Ae. tauschii* показал, что метаболомные профили устойчивых и неустойчивых к грибным патогенам форм достоверно различаются между собой по содержанию небелковых аминокислот, многоатомных спиртов, фитостеролов, ацилглицеролов, моно- и олигосахаридов, гликозидов, фенольных соединений (гидрохинона, кемпферола) и др. Последнее подтверждается ранее полученными нами данными о связи устойчивости овса (*Avena sativa* L.) к возбудителям фузариоза с определенными компонентами их метаболомного профиля: ацилглицеролами, небелковыми аминокислотами, галактинолом и др. Таким образом, наши исследования еще раз подтвердили возможность и эффективность применения метаболомного анализа для скрининга генетического разнообразия образцов коллекции ВИР, в частности *Ae. tauschii*, с целью выделения форм, имеющих в метаболомном профиле набор соединений, характеризующих их как устойчивые. К таковым относятся образцы *Ae. tauschii* с высоким содержанием пипеколиновых кислот, ацилглицеролов, галактинола, стигмастерола, глицерола, азелаиновой и пирогалловой кислот, кампестерола, гидрохинона и др., которые могут быть использованы для создания сортов пшеницы и тритикале, высокоустойчивых к грибным патогенам – возбудителям мучнистой росы, бурой и желтой ржавчины.
Ключевые слова: *Aegilops tauschii* Coss; метаболомный подход; устойчивость к болезням; грибные патогены.

Introduction

Wheat (*Triticum* L.) is one of the most significant crops in the world, including the Russian Federation. For the majority of the world's population, it is one of the staple foods. The yield and quality of wheat largely depend on the resistance of cultivars to environmental stress factors, including fungal diseases. Most of the cultivated foreign and domestic cultivars are susceptible to diseases caused by agents of stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Erikss. et Henn), brown rust (*Puccinia recondita* Rob. ex Desm f. sp. *triticina* Eriks.), mildew (*Blumeria graminis* (DC.) Speer f. sp. *tritici* Marchal.) and septoria leaf blotch (*Mycosphaerella graminicola* (Fuckel) J. Schroet. (=*Septoria tritici*); *Phaeosphaeria nodorum* (E. Muell.) Hedjar. (=*Leptosphaeria nodorum* E. Muell., =*Septoria nodorum* (Berk.)). Crop losses can reach up to 40 % (Afanasenko, 2010; Kolomiets et al., 2017). The creation of wheat cultivars resistant to the most harmful fungal pathogens is one of the effective ways to combat them.

Wild relatives of cultivated forms of wheat, rye, barley, oats, etc. serve as an inexhaustible source of resistance genes for creating cultivars combining high yields and resistance to environmental factors. Evolutionary, *Aegilops* L. species are close to those of the genus *Triticum* L. (Dorofeev, 1971; Migushova, 1975; Konarev, 1980; Liu et al., 2015; Arora et al., 2017). However, the donors used in breeding programs, in most cases are characterized by the same resistance genes. With time, it leads to the appearance of "adapted" forms of pathogens that infect cultivars previously considered to be resistant. Many known resistance genes from *Ae. tauschii* Coss. are not used in practice to improve wheat cultivars, as their protective effect is considered low (Pretorius, 1997; Kolmer, Anderson, 2011). Expansion of the genetic diversity of donors of resistance to wheat fungal diseases will help breeders solve this problem, and the global collection of wheat wild relatives at the N.I. Vavilov All-Russian Research Institute of Plant Industry (VIR) plays a crucial role in this task (Vavilov, 1919).

The VIR collection of the genus *Aegilops* L. contains over 5,000 accessions of various ecological and geographical origins, and it includes thirteen diploid, ten tetraploid, and five hexaploid species. Since 1956, species possessing complex immunity to fungal diseases have been identified in the collection: diploid *Ae. mutica* Boiss., *Ae. speltoides* Tausch., *Ae. aucheri* Boiss., *Ae. bicornis* (Forsk.) Jaub. et Spach., *Ae. comosa* Sibth. & Sm., *Ae. uniaristata* Vis., *Ae. heldreichii* Hozm.; tetraploid *Ae. ovata* L., *Ae. triaristata* Wild., *Ae. ventricosa* Tausch., *Ae. variabilis* Eig. Such a diversity of genetic material makes it possible to select appropriate genotypes for the subsequent production of wheat cultivars with improved biological characteristics. *Ae. tauschii* is a carrier of D genome, which is close to the polyploid wheat genome, and this fact greatly facilitates the crossing of *Ae. tauschii* with common wheat when transmitting effective disease resistance genes (Dobrotvorskaia et al., 2017). Besides, flour of such cultivars has high baking quality (Semenova et al., 1973). By now, most of the effective genes that determine resistance to rust and wheat spot blotch agents have been successfully introgressed into the genome of *T. aestivum* L. (McIntosh et al., 1995; Mujeeb-Kazi et al., 2001; Yang et al., 2003; Adonina et al., 2012).

Recently, nonspecific metabolomic analysis has found wide application for plant species phenotyping and resistance studies, which provides a unique opportunity to scan a wide range of compounds that make up the metabolomic profile in the source material and give an objective assessment (using metabolomic markers) of the plant's response to environmental factors (Konarev et al., 2015). This approach is increasingly used to identify individual metabolites or their groups that can characterize the protective status of the studied object, which makes it possible to identify accessions resistant to environmental stressors (Taji et al., 2006; Chakraborty, Newton, 2011; Valitova et al., 2016; Loskutov et al., 2017). Currently, metabolomic profiles of various crops from the VIR collection are investigated. Wild and cultivated forms of oats resistant and susceptible to *Fusarium* were studied, and significant differences among them were shown for a number of compounds (acylglycerols, nonproteinogenic amino acids, galactinol, etc.) (Loskutov et al., 2017, 2019). Total screening of wild forms of various crops will make it possible to shape a model "metabolomic profile of a resistant cultivar".

The aim of this study was the identification of metabolomic markers to be used in screening the genetic diversity of wild relatives for the forms with effective resistance genes, which can be efficiently introduced into the common wheat genome

for the use in breeding programs. The research objectives included the study of metabolomic profiles of *Ae. tauschii* accessions with and without resistance to leaf rust and powdery mildew pathogens, in order to detect metabolites marking the susceptibility of *Ae. tauschii* to fungal pathogens. The results of the study can shorten and optimize the selection of source material when creating wheat cultivars highly resistant to fungal pathogens.

Materials and methods

Fifty *Ae. tauschii* accessions from the VIR collection (see the Table), grown at the Dagestan Experimental Station of VIR (DES VIR) in 2017 and harvested at full ripeness, were used as the material for the study. The sample was composed from the main of *Ae. tauschii* botanical varieties in the VIR collection, taking into account the most complete ecogeographical representation.

Field studies of *Ae. tauschii* accessions were conducted at DES VIR on irrigated 1-m^2 plots according to the method accepted in VIR (Merezhko et al., 1999). During the growing season, the air temperature averaged +20.4 °C, the amount of precipitation was 15.4–16.3 mm, and the total of active temperatures amounted to 3400–4500 °C. Field assessment of the infection of *Ae. tauschii* accessions by fungal pathogens was carried out in fields of DES VIR, and the laboratory evaluation of the degree of infection was carried out at the VIR Department of Genetics in compliance with methods employed at VIR (Tyryshkin et al., 2004). Resistance was determined on a 9-point scale, where 9 means the absence of disease symptoms or presence of small necrotic spots; 7, microscopic pustules surrounded by necrotic zone; 5, small pustules surrounded by a wide necrotic zone; 3, medium-size pustules surrounded by chlorotic tissue; and 1, large pustules forming continuous lesion zones. Plants scoring 9–7 were classified as resistant, and those with 5–1 points as susceptible. Plants were examined at 5-day intervals throughout the growing season. For each accession, an integral score was derived from the highest infection point (see the Table).

Metabolomic profiles of grain from *Ae. tauschii* accessions were studied at the Department of Biochemistry and Molecular Biology of VIR (in 5 biological and 3 analytical replications) (Loskutov et al., 2017). The grains were cleaned of glumes and ground; 50 mg of the flour of an accession were homogenized with 500 μL of methanol, and the sample was kept at 5–6 °C for 30 days. A 100-μL portion of the extract was evaporated to dryness using a Labconco CentriVap Concentrator (USA). The dry residue was silylated using bis(trimethylsilyl)trifluoroacetamide at 100 °C for 40 minutes. The separation of trimethylsilyl ethers of metabolites was carried out using an HP-5MS 5 % phenyl–95 % methylpolysiloxane capillary column (30.0 m, 250.00 μm, 0.25 μm) on an Agilent 6850 gas chromatograph with an Agilent 5975B VL quadrupole mass selective detector MSD (Agilent Technologies, USA). The analysis was performed at 1.5 mL/min inert gas flow rate through a column. The column was heated from +70 up to +320 °C at 4 °C/min heating rate. The temperature of the mass spectrometer's detector was +250 °C, and that of the injector was +300 °C. The injected sample volume was

Leaf disease resistance in *Ae. tauschii* Coss. accessions

VIR Catalog No.	Subspecies (ssp.), variety (var.)	Field disease resistance, points		
		Mildew	Rust	
			brown	yellow
	Azerbaijan			
k-101	var. *typica*	9	3	3
k-108	ssp. *strangulata*	9	7	7
k-112		9	9	9
k-113		9	9	9
k-291		9	3	7
k-315		9	9	3
k-336		9	9	9
k-338		9	3	3
k-340		9	3	3
k-520		9	9	9
k-617		9	5	3
k-1098	var. *typica*	9	5	9
k-1102	var. *meyeri*	9	9	9
k-1111	ssp. *strangulata*	9	9	3
k-1112		9	9	9
k-1155		9	9	1
k-1723		9	9	9
k-1783	var. *meyeri*	9	3	3
	Armenia			
k-527	ssp. *strangulata*	9	9	5
k-1520	var. *typica*	9	7	9
k-2271		9	9	7
k-3187		9	9	9
	Afghanistan			
k-994	var. *typica*	9	3	3
k-1619		9	3	5
k-1659		9	9	9
k-1740		9	9	9
	Georgia			
k-608	var. *meyeri*	9	3	3
k-1216		9	9	9
	Dagestan			
k-1022	var. *typica*	9	5	3
k-1770	ssp. *strangulata*	9	9	9
	Iran			
k-1657	ssp. *strangulata*	9	3	3
k-1662		9	9	9
k-1958	var. *typica*	9	9	9
k-1966	ssp. *strangulata*	9	9	7
k-4043		9	9	7
k-4049		9	9	7
k-4056		9	9	7
	Kirghizia			
k-674	var. *meyeri*	9	5	1
k-677		9	5	1
	Nakhchivan			
k-553	var. *meyeri*	9	3	1
k-560		9	3	1
k-1172		9	9	1
k-1498		9	1	3
	Nagorno-Karabakh Autonomous Region			
k-494	ssp. *strangulata*	9	9	7
k-497		9	9	9
	Syria			
k-4564	ssp. *strangulata*	9	3	3
	Turkmenistan			
k-423	ssp. *strangulata*	9	7	3
	Uzbekistan			
k-394	var. *meyeri*	9	5	3
k-396		9	3	3
k-663		9	1	3

1.2 μL. Pyridine solution of tricosan (1 μg/μL) served as the internal standard.

The results were processed using UniChrom and AMDIS software. The peaks were identified using the NIST 2010 mass spectra library and as well as libraries of the St. Petersburg University Research Park and of the V.L. Komarov Botanical Institute of the Russian Academy of Sciences (Puzanskiy et al., 2015). The biochemical parameter values are given in ppm (μg/g).

Statistical data were evaluated with the Statistica 7.0 software package. The initial set of characters was screened by the method of one-way analysis of variance to identify the characters (metabolites) whose contents reliably discriminated *Ae. tauschii* accessions resistant and susceptible to the studied pathogens. The discriminant analysis was used to assess informativeness of resistance characters (metabolites) of *Ae. tauschii* accessions.

Results

Metabolomic profiles of caryopses of *Ae. tauschii* accessions with and without resistance to fungal pathogens differed in several indicators. Higher contents of organic acids were observed in the resistant forms of *Ae. tauschii* (1190 ppm) compared with the susceptible ones (1090 ppm). The dominating organic acids in the metabolomic profile of *Ae. tauschii* caryopses were malic and methylmalonic acids (203 vs. 164 and 168 vs. 153 ppm, respectively). The caryopses of resistant and susceptible forms were found to contain, respectively, the following contents of organic acids (ppm): 102 and 79 galacturonic acid, 78 and 76 lactic, 43 and 48 gulonic, 25 and 44 gluconic, 30 and 47 azelaic, 32 and 38 succinic, 28 and 27 oxalic, 42.8 and 41.0 fumaric, 19.9 and 13.0 ribonic, and 11.5 and 10.0 glyceric. The sum of minor acids with concentrations no higher than 10 ppm each was 24.0 ppm in both resistant and susceptible forms of *Ae. tauschii*. All metabolic reactions in plants occur with the participation of phosphoric acid and its derivatives, which explains its rather high content in the studied accessions (320.2 in resistant and 277.0 ppm in susceptible forms), the amounts of methylphosphate being 64.0 and 56.0 ppm, respectively.

Three nonproteinogenic amino acids constituted 60 % of free amino acids in resistant and susceptible *Ae. tauschii* accessions: 3-hydroxypipecolic (137.2 and 116.6), pipecolic (0.5 and 0.3), and 5-hydroxypipecolic (0.7 and 0.7 ppm, respectively). The remaining amino acids were represented by essential (valine, isoleucine, threonine, phenylalanine, and tryptophan) and non-essential ones (α-alanine, glycine, serine, proline, hydroxyproline, asparagine, glutamine, tyrosine, aspartic acid, and glutamic acid). The dominating ones were asparagine (18.7 and 13.4 ppm), valine (15.6 and 16.0), α-alanine (10.6 and 13.1), glutamine (12.3 and 9.0) and glutamic acid (5.7 and 5.5 ppm), respectively. The amounts of the remaining amino acids did not exceed 4.0 ppm. The sums of free amino acids (except nonproteinogenic) did not differ significantly between the studied forms of *Aegilops* resistant and susceptible to fungal pathogens: 88.4 and 85.3 ppm, respectively.

Higher concentrations of polyols and phytosterols were recorded in the susceptible forms of *Ae. tauschii* (341.8 and 336.5 ppm, respectively). Glycerol, xylitol, dulcitol, myo-inositol and inositol derivatives (59.5, 84.4, 70.4, 23.4, 9.6 ppm, respectively) prevailed in caryopses of the susceptible forms, while galactinol (92.5 ppm) dominated in the resistant ones. Phytosterols were mainly represented by sitosterol, stigmasterol, and campesterol; their contents were 219.8, 85.8, and 30.9 ppm in the susceptible forms, and 160.3, 44.5, and 22.7 ppm in the resistant ones, respectively.

The dominating fatty acids in caryopses of both resistant and susceptible forms of *Ae. tauschii* were palmitic (584.7 and 602.0), stearic (173.5 and 187.0), oleic (493.9 and 520.0), and linoleic (1515.0 and 1545.0 ppm), respectively. No significant difference in the contents of individual fatty acids or their total values (2943.3 and 3064.0 ppm) was noted.

The caryopses of resistant forms of *Ae. tauschii* were found to have higher contents of acylglycerols (954.0 vs. 745.6 ppm), mainly due to diacylglycerol (DAG) amounting to 631.0 and 464.0 ppm, respectively. The amounts of monoacylglycerols, i. e., MAG-2 C18:3 and MAG-1 C18:1, were also higher in resistant forms: 188.0 vs. 152.7, 54.0 vs. 34.2 ppm. On the contrary, the content of MAG-1 C16:0 was higher in the susceptible forms: 94.7 vs. 81.0 ppm in resistant.

Monosugars in *Ae. tauschii* caryopses were represented mainly by hexoses (over 80 %). These values were higher (1164.8 ppm) for resistant forms of *Aegilops* than for susceptible ones (1026.7 ppm). Of the hexoses, glucose (819.8) was predominant in resistant forms, while in the susceptible ones these were glucose (455.4) and fructose (318.1 ppm). The total pentose contents (ribose and xylose) did not exceed 40 ppm. The contents of glycerol-3 phosphate did not differ significantly between resistant and susceptible forms of *Aegilops*. They amounted to 42.8 and 37.4 ppm, respectively.

Oligosaccharides in caryopses of resistant and susceptible forms of *Ae. tauschii* amounted to 13480.6 and 14920.0 ppm, respectively. They were mainly represented by sucrose and raffinose. The content of sucrose was higher in susceptible forms (11604.9), while that of raffinose was higher (4882.0 ppm) in resistant ones.

Derivative sugars, identified as methyl-D-galactopyranoside, were found in both resistant and susceptible forms of *Ae. tauschii* (169.2 and 84.4 ppm, respectively).

In the group of phenolic compounds, the contents of hydroquinone (93.6 in resistant and 74.0 ppm in susceptible accessions) were found to be the highest. The values for kempferol; pyrogallic, 2,3-dihydroxybenzoic, salicylic, and caffeic acids; and α-tocopherol were 29.1, 1.5, 0.2, 0.3, 0.4, 0.4 ppm in resistant and 14.0, 1.3, 0.2, 0.2, 0.4 and 0.3 ppm in susceptible forms.

The above data reflect the activity of metabolic processes in *Ae. tauschii* caryopses. This activity characterizes primary and secondary metabolism, i. e., exchange of nitrogen-containing compounds, including amino acids; the Krebs cycle; carbohydrate metabolism; glycolysis; pentose phosphate cycle; exchange of signal (inositol) compounds; shikimate and glyoxylate pathways; etc.

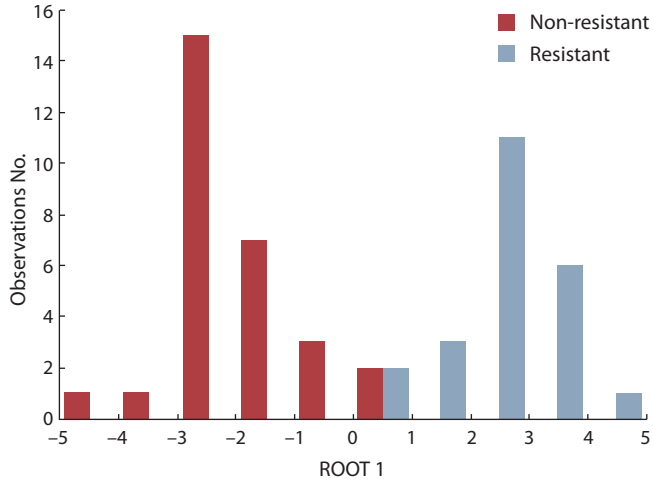
Statistical processing of the data showed that the metabolome of susceptible forms of *Ae. tauschii* differs with varying degrees of significance from that of resistant ones in a number of indicators. Resistant forms of *Ae. tauschii* were divided into

three groups: the first comprised accessions resistant to all the pathogens tested, the second included those resistant to only brown and yellow leaf rust, while the third included mildew resistant accessions. The metabolomes of all resistant forms differed with a high degree of confidence ($p = 0.05$) from those of susceptible ones in the contents of nonproteinogenic amino acids (pipecolic and 3-hydroxypipecolic), glycerol, ononitol, galactinol, sitosterol, stigmasterol, behenic acid, DAG, MAG-1 C16:1, ribose, sorbose, sucrose, maltose, and hydroquinone; and with confidence levels $0.1 > p > 0.05$ for fumaric, galacturonic, gluconic acids, threonine, dulcitol, mannitol, chiro- and myo-inositol, pelargonic and arachinic fatty acids, MAG-1 C18:1, and fructose. At $p = 0.05$, metabolomes of accessions from the second group differed in the content of the phosphoric, oxalic, pyrogallic, azelaic, 5-hydroxypipecolic acids; dulcitol; campesterol; undecyl and behenic fatty acids; and MAG-1 C18:1; while lower confidence levels ($0.1 > p > 0.05$) characterized the differences in the contents of 3-hydroxypipecolic acid, MAG-2 C18:3, and methyl-D-galactopyranoside. The third group was noted for the amounts of fumaric, pelargonic, pipecolic acids, ononitol, galactinol, and hydroquinone at $p = 0.05$, and for threonine, dulcitol, myoinositol, hydroxyoctacosenoic acid, and sucrose at $0.1 > p > 0.05$.

A significant correlation was found between indicators of resistance to fungal pathogens and the group of nonproteinogenic amino acids, polyols, phytosterols, acylglycerols, mono- and oligosaccharides, glycosides, and phenolic compounds (hydroquinone, kempferol). No relationship was found between the sum of free amino acids and organic acids, on the one hand, and sensitivity to fungal pathogens, on the other hand.

The discriminant analysis of metabolomic data on the studied caryopses of *Ae. tauschii* accessions demonstrated separation of the latter into two main groups – resistant and susceptible to fungal pathogens – according to the canonical variable coefficient. The most "informationally important" characters that confirmed the difference in metabolite profiles and determined the separation of *Ae. tauschii* accessions between the group of resistant or susceptible ones with the 98 % accuracy were the levels of stigmasterol, MAG, DAG; of pelargonic, galacturonic, and 3-hydroxypipecolic acids; and of galactinol, glycerol, sorbose, maltose, tyrosine and glycosides. Among the above characters, stigmasterol and maltose values were the most statistically significant for the susceptible forms, while for the resistant ones it was DAG.

The "informationally important" characters and the classification function were used to analyze how the assumptions as for the resistance or susceptibility of *Ae. tauschii* accessions matched the reality. As a result, the status was confirmed for all the studied accessions, except for k-527, an *Ae. tauschii* accession from Armenia. The canonical discriminant analysis of metabolomic characters made it possible to clarify the differentiation parameters of resistant and susceptible forms of *Ae. tauschii*. The histogram of the canonical variable eigenvalues distribution according to their magnitude shows that the values for resistant accessions fall within the 0 to +5 range, while for the susceptible ones these values are found in the 0 to –5 range (see the Figure).



Histogram of the distribution of resistant and susceptible *Ae. tauschii* forms by the magnitude of the canonical variable eigenvalues.

Discussion

The results of our work show that the metabolomic profiles of resistant and susceptible forms of *Ae. tauschii* differ significantly. When using the metabolomic analysis data, there is a high probability (up to 98 %) of identifying forms resistant to fungal pathogens among the accessions taken into the study without additional tests. We recommend that this approach be used to optimize the breeding process.

Analysis of metabolomic data for resistant and susceptible forms of *Ae. taushii* allows for a substantiated conclusion about the degree and nature of fungal pathogen influence on the main stages of primary and secondary metabolism in caryopses of accessions differing in the degree of resistance. To a greater or lesser degree, almost all pathogens affected metabolic processes, i. e., the Krebs cycle; glycolysis; and metabolism of fatty acids, acylglycerols, polyols, phytosterols, mono- and oligosaccharides. With all this, fungal pathogens had practically no effect on the contents of free amino acids (except for threonine and tyrosine). Since tyrosine is a precursor in the synthesis of many bioactive compounds that perform various functions – structural (lignin), protective (phenolic compounds, alkaloids, etc.), transport (electron transfer), and others – the effect of pathogens on the content of this amino acid in a caryopsis may be due to activation of defense mechanisms in response to the penetration of the pathogen into plant tissues, as confirmed by studies conducted outside Russia (Schenck, Maeda, 2018). Changes in the content of another amino acid, threonine, are associated by a number of authors with the influence of adverse biotic environmental factors, for example, insect pests. A plant reduces the concentration of substances necessary for the nutrition of the parasite, thereby affecting its population. The same mechanism may also act in cases of leaf rust and powdery mildew pathogens (Gonzales-Vigil et al., 2011). Azelaic acid, a product of oleic acid oxidation, and pipecolic acid (lysine catabolites) are intensely produced in response to the invasion of pathogens, in particular, fungi, into plant tissues; therefore, changes in the concentration of these compounds are well justified and

confirmed by other researchers (Navarova et al., 2012; Zoeller et al., 2012).

Among free phenolic compounds, a significant effect on the resistance to fungal pathogens was detected for hydroquinone (the dominant compound of this group) and pyrogallic acid. Phenolic compounds are known to be actively involved in the formation of plant immunity. The presence of free forms of phenolic compounds most often indicates intensity of glycoside synthesis, where they function as aglycones. According to our data, mainly hydroquinone and pyrogallic acid accumulate in the caryopsis of resistant forms of *Ae. tauschii*. Their presence in the free state is most likely associated with the destruction of active forms, glycosides, exemplified by arbutin. As for pyrogallic acid, its accumulation may also be due to the plant/fungal pathogen interaction (the role of signaling substances) (Seigler, 1998; cit.: Gilbert, 2001).

The data from the present study confirm those we obtained earlier investigating the relationship between the resistance of oat (*Avena sativa* L.) forms to the *Fusarium* agent and such components of the metabolomic profile as acylglycerols, nonproteinogenic amino acids, and galactinol (Loskutov et al., 2019).

Conclusion

The results of our study confirm the pertinence and effectiveness of the use of nonspecific metabolomic analysis for the search for and identification of plant forms with a set of compounds proposed as markers of resistance to certain pathogens. *Ae. tauschii* accessions with high contents of pipecolic acids, acylglycerols, galactinol, stigmasterol, maltose, tyrosine, sorbose, glycerol, azelaic and pyrogallic acids, methyl-D-galactopyranoside, etc. can be included in breeding programs for cultivars of main cereal crops with high resistance to mildew, brown rust, and yellow rust.

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Acknowledgements. The work was supported by State Budgeted Project 0662-2019-0006 for VIR.

Conflict of interest. The authors declare no conflict of interest.

Received September 20, 2019. Revised November 21, 2019. Accepted December 04, 2019.

Вавиловский журнал генетики и селекции. 2020;24(3):259-266
DOI 10.18699/VJ19.587

Использование гиперспектральной камеры Specim IQ для анализа растений

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Аннотация. Важной технологией для неразрушающего мониторинга пигментного состава растений, который тесно связан с их физиологическим состоянием или заражением патогенами, является дистанционное зондирование при помощи гиперспектральных камер. В работе представлен опыт применения мобильной гиперспектральной камеры Specim IQ для исследований заболевания проростков четырех сортов пшеницы обыкновенной корневой гнилью (возбудитель – гриб *Bipolaris sorokiniana* Shoem.), а также для анализа мякоти клубней картофеля 82 линий и сортов. Для проростков были получены спектральные характеристики и по данным определены наиболее информативные спектральные признаки (индексы) для обнаружения корневой гнили. У проростков контрольных вариантов в видимой части спектра наблюдается возрастание отражательной способности с небольшим пиком в зеленой области (около 550 нм), затем идет понижение из-за поглощения света пигментами растений с экстремумом при длине волны около 680 нм. Анализ гистограмм значений вегетационных индексов показал, что индексы TVI и MCARI наиболее информативны для обнаружения патогена на проростках пшеницы по данным гиперспектральной съемки. Для образцов картофеля были выявлены участки спектра, соответствующие локальным максимумам и минимумам отражения. Показано, что спектры сортов картофеля имеют наибольшие различия в области длин волн 900–1000, 400–450 нм, что в первом случае может быть связано с уровнем содержания воды, а во втором – с формированием в клубнях меланина. По характеристикам спектра исследованные образцы разделились на три группы, каждая из которых содержит повышенные либо пониженные уровни интенсивности для указанных участков спектра. Кроме того, для ряда сортов были установлены минимумы в спектрах отражения, соответствующих хлорофиллу *a*. Результаты демонстрируют возможности камеры Specim IQ для проведения исследований гиперспектрального анализа растительных объектов.
Ключевые слова: гиперспектральные данные; спектральные характеристики растений; заболевания пшеницы; корневая гниль; мякоть картофеля; хлорофилл; вегетационные индексы.

Для цитирования: Альт В.В., Гурова Т.А., Елкин О.В., Клименко Д.Н., Максимов Л.В., Пестунов И.А., Дубровская О.А., Генаев М.А., Эрст Т.В., Генаев К.А., Комышев Е.Г., Хлесткин В.К., Афонников Д.А. Использование гиперспектральной камеры Specim IQ для анализа растений. Вавиловский журнал генетики и селекции. 2020;24(3):259-266. DOI 10.18699/VJ19.587

The use of Specim IQ, a hyperspectral camera, for plant analysis

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Abstract. Remote sensing using hyperspectral cameras is an important technology for non-destructive monitoring of plant pigment composition, which is closely related to their physiological state or infection with pathogens. The paper presents the experience of using Specim IQ, a mobile hyperspectral camera, to study common root rot

(the pathogen is the fungus *Bipolaris sorokiniana* Shoem.) affecting the seedlings of four wheat varieties and to analyze the pulp of potato tubers of 82 lines and varieties. Spectral characteristics were obtained for seedlings and the most informative spectral features (indices) for root rot detection were determined based on the data obtained. Seedlings of control variants in the visible part of the spectrum show an increase in reflectance with a small peak in the green area (about 550 nm), then a decrease due to light absorption by plant pigments with an extremum at a wavelength of about 680 nm. Analysis of histograms of vegetation index values demonstrated that the TVI and MCARI indices are the most informative for detecting the pathogen on wheat seedlings according to hyperspectral survey data. For potato samples, regions of the spectrum were found that correspond to local maxima and minima of reflection. It was shown that the spectra of potato varieties have the greatest differences within wavelength ranges of 900–1000 nm and 400–450 nm, which in the former case may be associated with the level of water content, and in the latter, with the formation of melanin in the tubers. It was shown that according to the characteristics of the spectrum, the samples studied are divided into three groups, each characterized by increased or reduced intensity levels for the specified parts of the spectrum. In addition, minima in the reflection spectra corresponding to chlorophyll a were found for a number of varieties. The results demonstrate the capabilities of the Specim IQ camera for conducting hyperspectral analyses of plant objects.
Key words: hyperspectral data; spectral characteristics of plants; wheat diseases; root rot; potato pulp; chlorophyll; vegetation indices.

For citation: Alt V.V., Gurova T.A., Elkin O.V., Klimenko D.N., Maximov L.V., Pestunov I.A., Dubrovskaya O.A., Genaev M.A., Erst T.V., Genaev K.A., Komyshev E.G., Khlestkin V.K., Afonnikov D.A. The use of Specim IQ, a hyperspectral camera, for plant analysis. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):259-266. DOI 10.18699/VJ19.587 (in Russian)

Введение

Пигменты играют важную роль в жизни растений. Наиболее важные из них – хлорофиллы *a* и *b*, обеспечивающие процесс фотосинтеза, каротиноиды, ответственные за эффективность использования солнечной энергии при фотосинтезе, антоцианы, выполняющие защитные функции. Концентрации пигментов в растительных тканях могут быстро меняться в ответ на изменение физиологического состояния растений, и, таким образом, служить его маркерами. Состав и концентрацию пигментов можно исследовать химическими методами, однако более удобным способом, который активно развивается в последнее время, является метод дистанционной оценки на основе анализа спектров отраженного излучения (Мерзляк и др., 1997; Blackburn, 2007). Они основаны на том, что различные пигменты поглощают излучение разных длин волн неодинаково. В соответствии с этим у каждого пигмента есть свой характерный спектр отраженного излучения, который можно идентифицировать с использованием спектрометров высокого разрешения и интервала длин волн.

Развитие дистанционного мониторинга растений на основе мульти- и гиперспектральных сенсоров стало важным методом для оценки их физиологического состояния (Мерзляк и др., 2003), поражения заболеваниями (Mahlein et al., 2013), состояния плодов (Lorente et al., 2012) и корнеплодов (Rady et al., 2015; Pan et al., 2016). Такие сенсоры можно применять для оценки состояния растений как в лабораторных, так и в полевых условиях, а также устанавливать на беспилотные летательные аппараты (БПЛА) для широкого охвата посевов (Adão et al., 2017).

В настоящее время для получения мульти- и гиперспектральных изображений используют различные технические решения, которые отличаются технологией реализации сенсора, шириной спектрального интервала, спектральным и пространственным разрешением (Sellar, Boreman, 2005). Некоторые из них разработаны для установки на БПЛА, другие могут быть применены в лабораторных условиях. Однако все устройства требуют специальной технической настройки, установки и дополнительного оборудования для того, чтобы результаты можно было визуализировать. Гиперспектральная камера Specim IQ, применяемая в наших исследованиях, является компактным мобильным сенсором для наземной и лабораторной съемки (Bohnenkamp et al., 2018).

В нашей работе приведены результаты использования камеры Specim IQ для решения двух задач. Во-первых, проведено исследование проростков пшеницы четырех сортов в условиях заражения корневой гнилью и без заражения. Корневые гнили – наиболее вредоносные заболевания на пшенице в Западной Сибири. Из них существенное значение имеет гельминтоспориоз (возбудитель – гриб *Bipolaris sorokiniana* Shoem. = *Drechslera sorokiniana* Subram. et Jain, *Helminthosporium sativum* Pam.), поражающий практически все органы растения (первичные, вторичные корни, колеоптиле, стебель, листья, зерно). Болезнь приводит к гибели всходов, отставанию в росте, отмиранию продуктивных стеблей, пустоколосице, щуплости зерна. Потери урожая в среднем составляют 15 % в результате снижения продуктивной кустистости, озерненности колоса и массы зерна (Долженко и др., 2014). Мы провели оценку информативности оптических параметров различных сортов пшеницы при действии возбудителя обыкновенной корневой гнили злаков *B. sorokiniana* Shoem.

Второй задачей было исследование спектральных характеристик мякоти клубней картофеля (*Solanum tuberosum* L.), представленных в коллекции Института цитологии и генетики СО РАН (ИЦиГ) «ГенАгро» (Новосибирск).

Материалы и методы

Образцы пшеницы для исследования поражения корневой гнилью. Исследования проводили в лабораторных условиях (вегетационный опыт – водные культуры) на проростках районированных сортов мягкой яровой пше-

ницы селекции Сибирского научно-исследовательского института растениеводства – филиала ИЦиГ СО РАН: Новосибирская 18, Новосибирская 44, Сибирская 21 и Омского АНЦ – Омская 18.

Схема опыта включала варианты: контроль – семена без искусственной инфекции; опыт – семена искусственно инфицировали возбудителем обыкновенной гнили *Bipolaris sorokiniana* Shoem. (*B. sorokiniana*). Инфицирование проросших семян осуществляли конидиальной суспензией смеси среднепатогенных изолятов *B. sorokiniana*, приготовленной на 0.1 % водном агаре (5000 конидий на 1 зерно). Для каждого сорта в вариантах закладывали по 100 зерен с учетом их всхожести. Инфекционную нагрузку наносили в капле суспензии возбудителя после подсчета конидий в камере Тома–Горяева. Отобранное, без внешних признаков поражения и повреждения зерно предварительно стерилизовали 90 % этиловым спиртом в течение 2 мин с последующим троекратным промыванием дистиллированной водой.

Проростки выращивали в рулонной культуре на водопроводной воде в камере искусственного микроклимата «Биотрон-7» (разработка Сибирского физико-технического института (Сибирский федеральный научный центр агробиотехнологий Российской академии наук)) до фазы 1–2 листа при 16-часовом фотопериоде с освещенностью 20000 лк (день), температура днем 22 °С, ночью – 18 °С, влажность – 60 % (Гурова и др., 2017). Для проведения съемки с помощью гиперспектральной камеры использовали побеги проростков пшеницы с признаками поражения корневой гнилью (штрихи и полосы темно-бурого цвета).

Образцы клубней картофеля. В исследовании использованы 82 образца картофеля (*S. tuberosum* L.) из коллекции ИЦиГ «ГенАгро» (Новосибирск). Все сорта и гибриды, анализируемые в ходе эксперимента, были выращены в полевых условиях (в пределах одной локации) с июня по начало сентября 2018 г. Все образцы были посажены одновременно или с разницей в один день и выкопаны таким же образом. Клубни всех образцов были посажены в два ряда с расстоянием между рядами в 0.75 м и между растениями – в 0.3 м. Длина каждого ряда составляла 3 м, выращивалось по 10 растений. После извлечения клубней из почвы их направляли на хранение в течение 3 нед при температуре 4 °С. По прошествии указанного времени отбирали только визуально здоровые, типичные по форме и размеру клубни для каждой линии. Клубни (два на образец) были вымыты водопроводной водой и оставлены на ночь для испарения водопроводной воды с поверхности клубней в комнатных условиях.

Для проведения съемки с помощью гиперспектральной камеры использовали срезы клубней. Выполняли поперечные разрезы клубней по центру с помощью ножа на две приблизительно равные доли, от одной из них проводился срез, максимально однородный по толщине в различных частях среза. Все срезы имели толщину в пределах 2–3 см. В случаях невозможности выполнения поперечного среза по центру клубня (выявляемые дефекты только при разрезании клубня, например, такие, как результат поражения различными инфекциями) делали продольный срез по центру доли клубня, полученной при поперечном разрезе. Срезы в пределах изучаемого сорта или гибрида выполняли непосредственно перед съемкой.

Получение гиперспектральных изображений. Спектральные характеристики проростков пшеницы и клубней картофеля для анализа были собраны с помощью гиперспектральной камеры Specim IQ (Spectral Imaging Ltd., https://www.specim.fi/iq/). Эта камера позволяет оценивать спектры отражения в интервале 400–1000 нм. Спектральное разрешение камеры составляет 7 нм и включает 204 полосы, пространственное разрешение сенсора – 512 × 512 пикселей. Технические характеристики камеры более подробно приведены в работе (Bohnenkamp et al., 2018). Камера была любезна предоставлена ООО «Компания «Азимут Фотоникс» (Москва). Камера была установлена на штативе над столом, на расстоянии 15–20 см от образца, расположенного на белом листе бумаги. Образцы освещались тремя галогенными лампами: две мощностью 500 Вт, одна – 700 Вт, как это рекомендовано в инструкции к камере. Перед съемкой проводилась калибровка камеры с помощью калибровочной панели, после этого при получении серий снимков панель удаляли из кадра.

Обработка спектральных изображений. Предварительный визуальный контроль качества гиперспектральных изображений осуществлялся с помощью программы Specim IQ Studio software. Для массовой обработки изображений использовали библиотеки языка Python. Извлечение данных по интенсивности спектральных линий из выходных файлов Specim IQ в формате envi проводили с помощью пакета spectral (http://www.spectralpython.net/). Сглаживание спектров было выполнено с использованием фильтра Савицкого–Голая (Savitzky, Golay, 1964), программа savgol_filter – из пакета scipy (https://www.scipy.org/).

Выделение областей проростков и срезов клубней осуществляли на основе анализа интенсивности сигнала по различным линиям спектра (белый фон имел практически одинаковую интенсивность отражения по всем линиям спектра).

Для исследования были взяты от трех до пяти гиперспектральных изображений здоровых и зараженных проростков для каждого сорта пшеницы. При получении спектральных кривых проводили сегментацию изображений и использовали средние значения спектральных яркостей выделенных сегментов по нескольким изображениям. При анализе проростков рассчитывали серию вегетационных индексов, как это было описано ранее в работе (Дубровская и др., 2018).

При анализе клубней для каждого изображения мы выбирали случайным образом 6000 пикселей (выборка с возвращением), принадлежащих области мякоти клубня, и по ним усредняли значение интенсивностей для каждой линии спектра. Полученные таким образом средние значения характеризовали спектры каждого из 82 образцов мякоти картофеля.

Для предсказания содержания крахмала и мезги в клубнях картофеля по гиперспекральным данным применен метод частных наименьших квадратов (PLS), в качестве зависимых переменных рассматривали значения содержания крахмала или мезги, независимыми переменными были значения первой производной интенсивностей спектра (дифференциальные кривые).

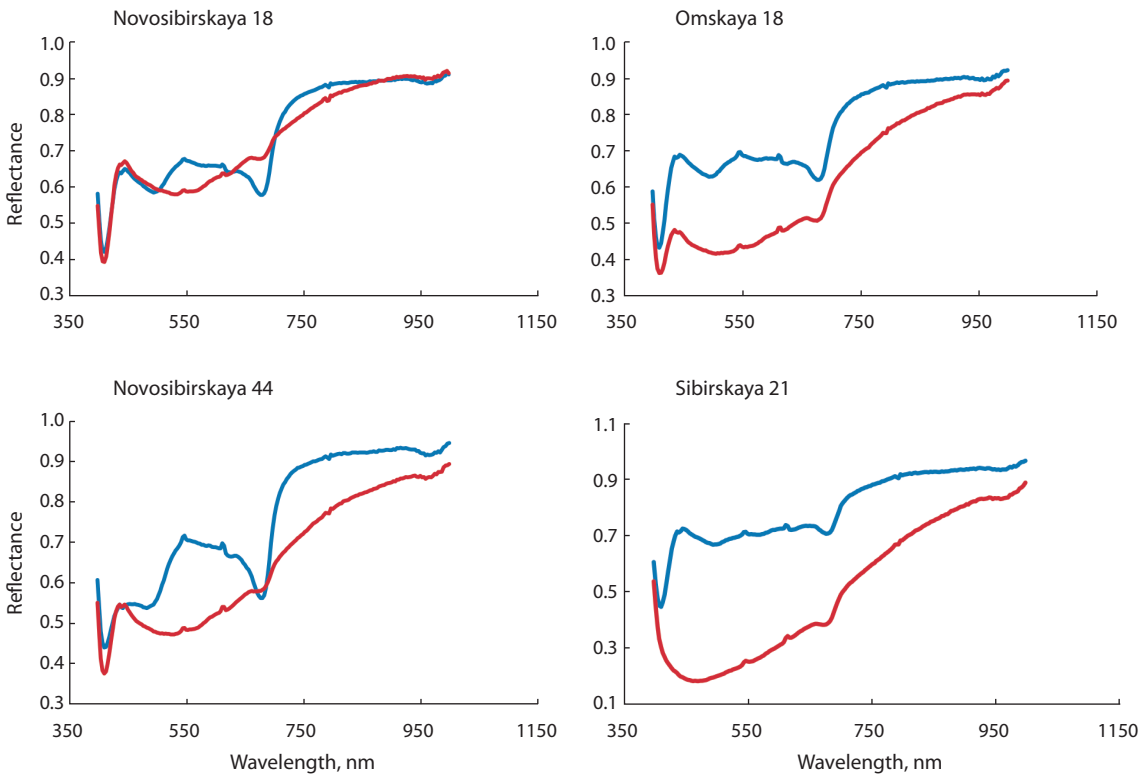

Fig. 1. Spectra of wheat varieties: blue curve, healthy seedlings; red curve, seedlings damaged by wheat scab (*B. sorokiniana*).

Результаты

Анализ данных по корневой гнили пшеницы. Анализ спектральных кривых, полученных для проростков сортов Новосибирская 18, Омская 18, Новосибирская 44 и Сибирская 21, показал, что отражательные характеристики здоровых проростков пшеницы и инфицированных возбудителем обыкновенной корневой гнили злаков отличаются в исследуемых частях спектра – видимой (400–700 нм) и ближней инфракрасной области (700–900 нм) (рис. 1). У проростков контрольных вариантов в видимой части спектра наблюдается возрастание отражательной способности с небольшим пиком в зеленой области (около 550 нм), затем идет понижение из-за поглощения света пигментами растений с экстремумом при длине волны около 680 нм. В ближней инфракрасной области отражательная способность проростков контрольных и опытных вариантов повышается, что связано с внутренним рассеянием света мезофиллом (Behmann et al., 2014).

Различия отражательных характеристик в определенных зонах спектра послужили основой для применения вегетационных индексов для обнаружения и диагностики корневой гнили на посевах и распознавания особенностей здоровых и пораженных заболеванием всходов пшеницы. В результате анализа различных вегетационных индексов, ранее используемых при диагностике и мониторинге развития других заболеваний пшеницы (Дубровская и др., 2018), а также на основе анализа спектральных характеристик, полученных при лабораторном эксперименте, было выбрано 13 вегетационных индексов для идентификации корневой гнили (*B. sorokiniana*) (табл. 1). Детальное описание индексов приведено в работе (Дубровская и др., 2018).

Анализ гистограмм значений вегетационных индексов показал (рис. 2), что индексы TVI и MCARI наиболее информативны для обнаружения патогена на проростках пшеницы, по данным гиперспектральной съемки.

Анализ мякоти клубней картофеля. Спектральные кривые мякоти клубней 82 образцов картофеля представлены на рис. 3, *a*.

Из диаграмм видны две характерные спектральные области, в которых наблюдаются существенные различия в интенсивности спектров отражения различных сортов: 420–470 и 860–980 нм. Для области 420–470 нм наблюдаются два характерных типа значений интенсивности: больше 0.4 (53 образца, 64 %) и меньше этого значения (см. рис. 3, *a*; 30 образцов, 36 %). Для области 860–980 нм также наблюдаются два типа значений: больше 0.8 (см. рис. 3, *a*; 63 образца, 75 %) и меньше этого значения (20 образцов, 25 %). Более детальный анализ показал, что все образцы в выборке можно разделить на три группы по значению величин интенсивности спектра в этих двух областях. К первой группе (А) относятся образцы, у которых значение интенсивности в интервале 420–470 нм больше 0.4, а в интервале 860–980 нм – больше 0.8. Таких образцов оказалось 53 (на рис. 3 они показаны синим цветом). Списки названий сортов картофеля, которые относятся к этой и двум другим группам, приведены в Приложении 1)[1]. К группе В относятся образцы, интенсивность

[1] Приложения 1 и 2 см. по адресу:
http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx2.pdf

Table 1. Vegetation indices used for wheat scab identification on wheat seedlings

Index	Index name	Expression*
mSR_{705}	Modified Red Edge Simple Ratio Index	$(R_{750} - R_{445})/(R_{750} + R_{445})$
$NDVI_{705}$	Red Edge Normalized Difference Vegetation Index	$(R_{750} - R_{705})/(R_{750} + R_{705})$
$mNDVI_{705}$	Modified Red Edge Normalized Difference Vegetation Index	$(R_{750} - R_{705})/(R_{750} + R_{705} - 2R_{445})$
NBNDVI	Narrow-Band Normalized Difference Vegetation Index	$(R_{850} - R_{680})/(R_{850} + R_{680})$
RVSI	Red-Edge Vegetation Stress Index	$[(R_{712} + R_{752})/2] - R_{732}$
PSRI	Plant Senescence Reflectance Index	$(R_{Red} - R_G)/R_{Nir}$ $(R_{678} - R_{500})/R_{750}$
ARI	Anthocyanin Reflectance Index	$(R_{550})^{-1} - (R_{700})^{-1}$
PRI	Photochemical/Physiological Reflectance Index	$(R_{531} - R_{570})/(R_{531} + R_{570})$
SIPI	Structural Independent Pigment Index	$(R_{800} - R_{445})/(R_{800} + R_{680})$
PhRI	Physiological Reflectance Index	$(R_{550} - R_{531})/(R_{550} + R_{531})$
NPCI	Normalized Pigment Chlorophyll Index	$(R_{680} - R_{430})/(R_{680} + R_{430})$
MCARI	Modified Chlorophyll Absorption Ratio Index	$3 \cdot \left[(R_{701} - R_{671}) - 0.2 \cdot (R_{701} - R_{549}) \cdot \left[\frac{R_{701}}{R_{671}} \right] \right]$
TVI	Triangular Vegetation Index	$0.5 \cdot [120 \cdot (R_{Nir} - R_G) - 200 \cdot (R_{Red} - R_G)]$ $0.5 \cdot [120 \cdot (R_{750} - R_{550}) - 200 \cdot (R_{670} - R_{550})]$

* R_X – is reflectance at the corresponding wavelength or in the corresponding spectral range: *G* (green – 520–600 nm), *Red* (630–690 nm), *Nir* (760–900 nm).

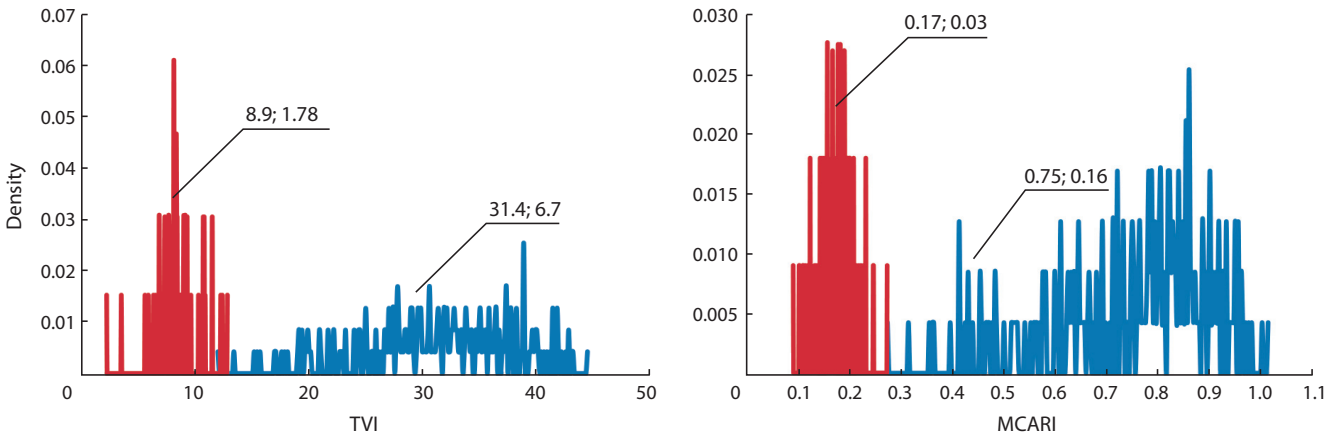

Fig. 2. Imputed distribution densities of the indices TVI (left) and MCARI (right) for classes of seedlings (cv. Novosibirskaya 18): blue bars, healthy plants; red bars, plants damaged by wheat scab.

Callouts indicate sample means and standard deviations of indices.

поглощения которых в интервале 420–470 нм меньше 0.4, а в интервале 860–980 нм – меньше 0.8. Таких образцов оказалось 20 (на рис. 3 они выделены оранжевым цветом). К группе С относятся образцы, у которых интенсивность поглощения в области 420–470 нм меньше 0.4, а в области 860–980 нм – больше 0.8. Таких образцов было 9 (на рис. 3 они показаны сиреневым цветом). Эти три кластера оказались хорошо различимы на графике рассеяния для двух главных компонент, полученных при анализе производных от спектров (рис. 4). Образцов, у которых в об-

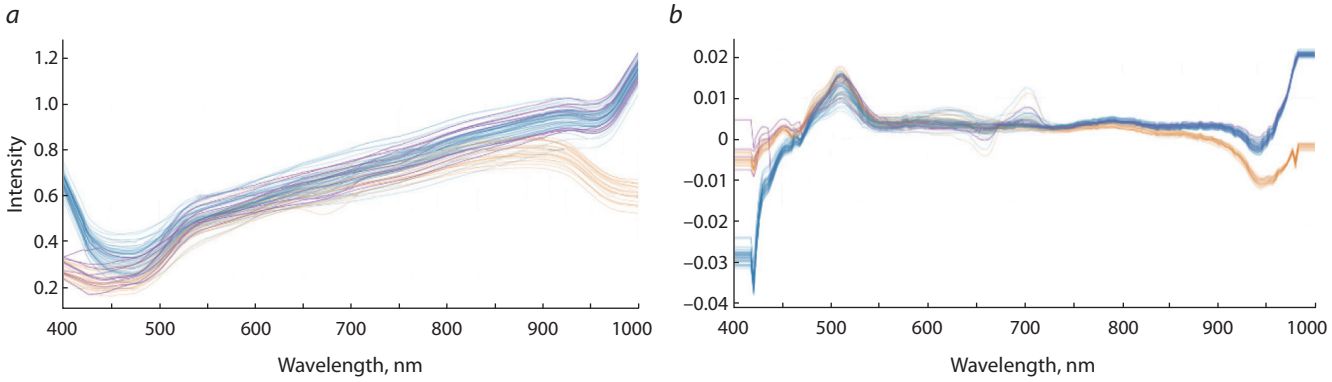

Fig. 3. Spectral features of potato tuber flesh in 82 potato genotypes:

a – mean reflection intensities for 204 spectral lines within 400–1000 nm; *b* – differential curves. Colors of the curves correspond to three types of spectral lines (see text).

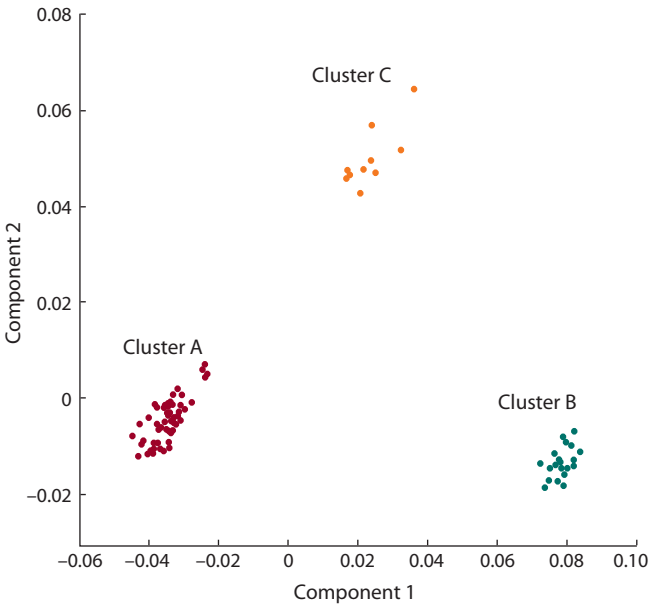

Fig. 4. Scattering of two principal components in 82 specimens obtained by principal component analysis of differential curves.

X-axis: component 1 (79.9 % of variation); *Y*-axis: component 2 (12.9 % of variation).

ласти спектра 420–470 нм интенсивность больше 0.4, а в области 860–980 нм – меньше 0.8, не обнаружено.

Необходимо отметить, что эти кластеры также можно идентифицировать на основе анализа диаграммы рассеяния в пространстве двух главных компонент для интенсивностей спектральных линий пикселей, соответствующих областям мякоти клубней (Приложение 2). На этой диаграмме также видно, что пиксели клубней одного сорта формируют компактные облака, которые хорошо отделяются от областей, соответствующих другим генотипам картофеля.

В спектрах некоторых образцов наблюдаются менее выраженные различия в области 640–730 нм. Этот интервал длин волн соответствует области поглощения света хлорофиллом (Guidi et al., 2017).

Обсуждение

Проведенные нами исследования показали возможность применения камеры Specim IQ для анализа спектров отражения растительных образцов (проростков пшеницы и срезов клубней картофеля). В первом случае удалось выявить значимые различия в спектрах проростков здоровых и пораженных корневой гнилью. У здоровых проростков минимумы отражения наблюдаются в полосах сильного поглощения хлорофиллов в красной области спектра (680 нм) и в полосе совместного поглощения хлорофиллов и каротиноидов в синей области (~500 нм).

У пораженных проростков трех сортов пшеницы (кроме сорта Новосибирская 18) коэффициенты отражения практически во всем диапазоне значительно ниже, чем у здоровых. Снижение отражательной способности у пораженных проростков, наиболее выраженное у сорта Сибирская 21, возможно, обусловлено особенностями протекания инфекционного процесса и формирования защитно-приспособительных реакций при патогенезе.

Резкое изменение спектральных характеристик растений на границе видимой красной и ближней инфракрасной частей спектра в диапазоне 690…740 нм (положение «красных краев») имеет большое значение для диагностики стрессовых воздействий. Подобную особенность отмечали A. Lowe с коллегами (2017). Она может быть объяснена тем, что хлорофилл сильно поглощает длины волн вплоть до 700 нм, и, следовательно, растительный материал в этом диапазоне имеет низкую отражательную способность, которая резко возрастает в ближней инфракрасной области спектра (~720 нм). Обнаружено, что различия в спектрах можно описать за счет изменения ряда известных индексов, в частности TVI и MCARI, связанных с содержанием хлорофилла.

При анализе срезов клубней картофеля мы выделили две области спектра (400–450 и 900–1000 нм), в которых наблюдаются наибольшие различия в нашей выборке образцов. Их можно связать с областями поглощения ряда растительных пигментов, а также молекул воды и групп OH (табл. 2).

Обнаруженные полосы поглощения позволяют предположить наличие или образование в ходе эксперимента (регистрации спектра) поглощающих в видимой или

Table 2. Absorbance ranges of some metabolites

Metabolite	Absorbance ranges, nm	Reference
Melanin	400	Busch, 1999
Chlorophyll	640–730, 400	Guidi et al., 2017
Water, OH groups	~ 970	López-Maestresalas et al., 2016

ближней инфракрасной области спектра метаболитов. Так, известно, что в овощах при взаимодействии производных тирозина с кислородом воздуха в присутствии оксидаз может происходить образование меланина – олигомерного окрашенного соединения (Busch, 1999), имеющего в спектре полосу поглощения при 400 нм. Возможно, в процессе экспонирования некоторые сорта картофеля более легко подвергались каталитическому окислению с образованием коричневой окраски.

Поглощение в области 640–730 нм может соответствовать наличию зеленого пигмента – хлорофилла – в краевых областях срезов в некоторых рассмотренных образцах картофеля (Tanios et al., 2018). Обе распространенные в растениях разновидности хлорофилла – *a* и *b* – поглощают свет 640–730 нм. Кроме того, хлорофилл *a* имеет полосу поглощения при 400 нм (Guidi et al., 2017). Возможно, именно сорта с хлорофиллом в краевых участках срезов клубней относятся к группе С по РСА (см. рис. 4).

Поглощение при 860–980 нм относится к водородным связям воды (López-Maestresalas et al., 2016) и, возможно, связанных с ней полярных полимеров (крахмала, пектинов, целлюлозы), присутствующих в мякоти картофеля. Различием в связывании воды и групп ОН можно объяснить наличие двух групп сортов картофеля.

Оценка скорости потемнения среза клубня (образования мелатонина) имеет практическое значение при производстве чипсов и полупродуктов для жареного картофеля. Анализ наличия хлорофилла в картофеле позволяет судить о том, экспонировался ли картофель на свету и, соответственно, есть ли риск присутствия в нем ядовитого алкалоида соланина. Таким образом, использование гиперспектральной камеры имеет значительный потенциал для оценки наличия метаболитов в картофеле по его срезу и автоматизации процессов переработки картофеля в продукты с высокой добавленной стоимостью.

Заключение

С помощью гиперспектральной камеры Specim IQ проведен анализ двух типов биологических образцов: проростков пшеницы четырех сортов с поражением корневой гнилью (возбудитель – *B. sorokiniana* Shoem.) и здоровых. Выявлено наличие различий в спектрах здоровых и пораженных растений, определены индексы TVI и MCARI, которые в наибольшей степени различаются для здоровых и больных проростков. Анализ мякоти клубней картофеля показал выраженные группы образцов, которые различаются по интенсивности спектров отражения в областях 400–450 и 900–1000 нм, что может быть связано с областями поглощения меланина в первом случае (почернение мякоти) и молекул воды и групп ОН – во втором (содержание влаги).

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Acknowledgements. The records of plant sample spectra and spectrum analysis were supported by the integrated program of basic research for the Siberian Branch of the Russian Academy of Sciences "Interdisciplinary Integrated Research" for 2018–2020, project "Development of numerical technologies for early detection and localization of damage of crops in the field". Potato growth and sample preparation were supported by the integrated program of research "Potato breeding and seed industry". The study was supported by the Russian Foundation for Basic Research, project 17-29-08006. We are grateful to A.M. Genaev for discussion and valuable remarks and to I.V. Totskiy for help in the annotation of potato samples.

Conflict of interest. The authors declare no conflict of interest.

Received November 27, 2019. Revised March 21, 2020. Accepted March 23, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):267-275
DOI 10.18699/VJ20.619

A comparison of statistical methods for assessing winter wheat grain yield stability

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Abstract. The multitude of existing methods for assessing the phenotypic stability of plants makes breeders be faced with the problem of choosing an appropriate variant. The purpose of this study was to compare different methods of analyzing the genotype × environment interaction and, on their basis, assess the stability of the yield of 7 varieties of winter wheat. The article compares 17 stability statistics by applying them to data obtained from agrotechnical experiments carried in 2009–2011 for evaluating the grain yield of 7 varieties of winter common wheat of Siberian selection (Novosibirskaya 32, Novosibirskaya 40, Novosibirskaya 51, Novosibirskaya 3, Novosibirskaya 2, Obskaya winter, Omskaya 6). Analysis of variance revealed a significant ($p < 0.001$) genotype × environment interaction in the experiments, which indicates a different reaction of genotypes to changes in environmental conditions. Genotypes were ranked according to the level of stability. Based on the analysis of the rank correlation matrix, the stability statistics were categorized in five groups. Recommendations were made on which group of methods to use depending on the objectives of the study. In the case when the goal of breeding research is the selection of the most biologically stable varieties with the minimum variance across a range of environments, one should use the methods of the static concept. If it is necessary to choose a genotype with a predictable reaction to changes of environmental conditions, corresponding to the calculated level or forecast, the regression approach is the most appropriate. The stability statistics generally identified Novosibirskaya 32 as the most stable variety from a biological point of view. The regression approach showed that Novosibirskaya 3 was the genotype with the smallest deviation from mean yield in all environments, while methods accessing the contribution of each genotype to the genotype × environment interaction defined Novosibirskaya 51 as the most stable variety.
Key words: phenotypic stability; genotype × environment interaction; plant breeding; statistical methods; winter wheat.

For citation: Cheshkova A.F., Stepochkin P.I., Aleynikov A.F., Grebennikova I.G., Ponomarenko V.I. A comparison of statistical methods for assessing winter wheat grain yield stability. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):267-275. DOI 10.18699/VJ20.619

Сравнение статистических методов оценки стабильности урожайности озимой пшеницы

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Аннотация. Многообразие существующих методов оценки фенотипической стабильности растений ставит перед селекционерами проблему выбора подходящего варианта. Целью настоящего исследования было сравнение различных методов анализа взаимодействия генотип × среда и оценка на их основе стабильности урожайности семи сортов озимой пшеницы. Проанализированы 17 статистических показателей стабильности на примере данных полевого опыта 2009–2011 гг. по оценке урожайности зерна семи сортов озимой мягкой пшеницы сибирской селекции (Новосибирская 32, Новосибирская 40, Новосибирская 51, Новосибирская 3, Новосибирская 2, Обская озимая, Омская 6) в шести вариантах сред. Дисперсионный анализ выявил значимое ($p < 0.001$) взаимодействие генотип × среда в опыте, что говорит о различной реакции генотипов на изменение условий среды. Выполнено ранжирование сортов по уровню стабильности и рассчитаны корреляционные связи между параметрами стабильности. На основе анализа корреляционной матрицы рангов проведена классификация методов, разделяющая их на пять групп. Предложены рекомендации по выбору способа определения стабильности генотипов в зависимости от целей исследования. В случае, когда целью селекционных исследований является выбор наиболее стабильных в биологическом смысле сортов, обладающих наименьшей вариацией признака, независимо от меняющихся условий среды, следует использовать методы статической концепции. Если среди набора сортов необходимо выбрать генотип с предсказуемой реакцией на изменения условий среды, соответ-

ствующей расчетному уровню или прогнозу, наиболее оптимален регрессионный подход. В результате оценки методами статической концепции сорт Новосибирская 32 был определен как наиболее стабильный с биологической точки зрения. Сорт Новосибирская 3, имевший наименьшее отклонение от средней урожайности для всех сред, оказался наиболее стабильным при оценке регрессионными методами. Методы, оценивающие вклад генотипа во взаимодействие генотип × среда, определили сорт Новосибирская 51 как самый стабильный.
Ключевые слова: фенотипическая стабильность; взаимодействие генотип × среда; селекция растений; статистические методы; озимая пшеница.

Introduction

Genotype × environment (GE) interaction means that varieties react differently to changes in growing conditions. Relative ranking of the productivity of a group of varieties in different environmental conditions gives different results. Even among specific, regionally adapted breeding forms, it is difficult to choose the best genotype, due to the variability of yields in different years of observations and under different environmental conditions.

Currently, many different ways of evaluating the GE interaction are developed, which are based on statistical methods for calculating certain parameters characterizing the degree of genotype response to changing environmental conditions. Different terms are used: adaptability, homeostaticity, plasticity, stability, etc.

Becker and Leon (1988) consider that, depending on the purpose of the research, there are two main differing concepts of stability: static (biological) and dynamic (agronomic).

The static concept implies a stable genotype possesses an unchanged performance, regardless of variation of the environmental conditions. "This concept of stability is useful for traits the levels of which have to be maintained at all costs, e. g. for quality traits, for resistance against diseases, or for stress characters like winter hardiness". The concept may include methods described by Roemer (1917, in: Becker, Leon, 1988), Francis and Kannenberg (1978), Lin and Binns (1988).

Unlike the static concept, the dynamic concept permits a predictable reaction of the genotype to changes in environmental conditions. The stable genotype, in accordance with this concept, does not deviate from the average values of this reaction. For each environment, the productivity of a stable genotype corresponds exactly to the predicted level. Among the methods of the dynamic concept used to assess stability, the following groups can be distinguished:

- methods that assess the stability of the genotype, based on the contribution to the variation of genotype × environment interaction are presented in the articles of Wricke (1962), Shukla (1972);
- a regression approach to assessing the stability of a trait is considered in the articles of Finlay and Wilkinson (1963), Eberhart and Russell (1966), Tai (1971);
- non-parametric methods based on relative data rankings were proposed by Nassar and Huehn (1987), Fox et al. (1990), Kang and Pham (1991);
- multidimensional methods that use the results of a cluster or a component analysis to assess stability were described in Fox and Rosielle (1982), Zobel et al. (1988), Lin and Binns (1991), Purchase (2000), Gauch (2006).

Comparison of different stability statistics and classification of methods was made by Lin and Binns (Lin et al., 1986), Becker and Leon (1988), Flores et al. (1998), Adugna and Labuschagne (2003), Mohammadi and Amri (2008), Mohammadi et al. (2016). Kang (1997) made a wide analysis of the GE interaction phenomenon and gave recommendations how to use this interaction for crop cultivar development.

But some methods for stability assessing suggested by Khangildin et al. (1979), Dragavtsev (Dragavtsev, Averyanova, 1983), Udachin (1990), Martynov (1990), Kilchevsky and Khotyleva (1989), were not mentioned and compared in that works.

The purpose of this study is to compare 17 different methods of analyzing the GE interaction and, on their basis, assess the stability of the yield of 7 varieties of Siberian winter wheat.

Materials and methods

Experimental data

All analyses were performed on the grain yield data obtained from 7 varieties of winter wheat *T. aestivum* L.: Novosibirskaya 32, Novosibirskaya 40, Novosibirskaya 51, Novosibirskaya 3, Novosibirskaya 2, Obskaya winter, Omskaya 6. The trials were conducted at the experimental fields of the Siberian Research Institute of Plant Production and Breeding (SibNIIRS) – Branch of the Institute of Cytology and Genetics of SB RAS during three cropping seasons (2009–2011). The experiment was carried out in two variants each year: with use of mustard protection coulisses and without them. The meteorological conditions during the years of the experiments were different both in temperature and precipitation. Coulisses contributed to a greater accumulation of snow, as compared with no-coulisse plots, which allowed a long period to retain soil moisture in spring and early summer periods. Thus, contrasting growth conditions were provided, which made it possible to take into account six variants of environments for stability analysis.

The trials were evaluated using randomized complete block design with three replications. Sowing was carried out on the fallow precursor on plots of 25 m^2. Seeding rate was 600 seeds/m^2, sowing date was August 20–24. The grain yield was measured for each plot and then converted into tons/ha for further statistical analysis.

Statistical methods

The yield data were subjected to statistical analyses using the R (R Development Core Team, 2014) software. A model of analysis of variance (ANOVA) for v genotypes, n environments, r replications of the following form was considered:

$$Y_{ij} = \mu + d_i + \epsilon_j + g_{ij} + \bar{e}_{ij}, \quad (1)$$

where Y_{ij} is the mean grain yield of the i-th genotype in the j-th environment ($i = 1, \ldots v$; $j = 1, \ldots n$); μ is the grand mean; d_i is the main effect of the i-th genotype; ϵ_j is the main effect of the j-th environment; g_{ij} is the effect of genotype × envi-

ronment interaction; $\bar{e}_{ij}$ is the GE interaction residual of the *i*-th genotype in the *j*-th environment.

Normality of model residuals was assessed by Shapiro–Wilk test (Shapiro, Wilk, 1965), and homogeneity of variance was estimated based on the criterion of Levene (1960).

The following methods were applied to experimental data (coded abbreviation was used for convenience).

1. (*EV*). Roemer (1917, in: Becker, Leon, 1988) used the "environmental variance of genotypes" to determine the stability of a genotype:

$$S_{yi}^2 = \frac{\Sigma_j (Y_{ij} - \bar{Y}_{i.})^2}{n-1}, \quad (2)$$

where Y_{ij} is the mean value of the trait for the *i*-th genotype in the *j*-th environment ($i = 1, \dots v; j = 1, \dots n$); $\bar{Y}_{i.}$ is the mean value of the trait for the *i*-th genotype in all environments. The most stable genotype has the lowest value of environmental variance. In fact S_{yi}^2 is unbiased estimation of genotype variation.

2. (*CV*). Francis and Kannenberg (1978) used a combination of mean yield and standard deviation as a measure of stability. The coefficient of variation of the *i*-th genotype is estimated by the standard formula:

$$CV_i = \frac{\sigma_i}{\bar{Y}_{i.}} \cdot 100\ \%, \quad (3)$$

where σ_i is the standard deviation of the trait for the *i*-th genotype; $\bar{Y}_{i.}$ is the mean value of the trait for the *i*-th genotype in all environments.

Genotypes with yield above overall mean yield and CV_i below overall coefficient of variation are considered more stable than the others.

3. (*HOM*). Khangildin (Khangildin et al., 1979) uses the "coefficient of homeostaticity" as stability index:

$$Hom_i = \frac{\bar{Y}_{i.}^2}{\sigma_i (\bar{Y}_{i(opt)} - \bar{Y}_{i(lim)})}, \quad (4)$$

where σ_i is the standard deviation of the trait for the *i*-th genotype; $\bar{Y}_{i.}$, $\bar{Y}_{i(opt)}$, $\bar{Y}_{i(lim)}$ are the mean values of the trait for the *i*-th genotype in all environments, in the optimal and limited environment, respectively. The higher the coefficient of homeostasis is, the more stable is the genotype.

4. (*Pi*). Lin and Binns (1988) proposed to use the "superiority measure" P_i as a parameter of stability:

$$P_i = \Sigma_{j=1}^{n} (Y_{ij} - M_j)^2 / 2n, \quad (5)$$

where M_j is the maximal value Y_{ij} for all genotypes in *j*-th environment. Genotypes with a lower P_i value are considered to be more stable.

5. (*UST*). Udachin (1990) modified the method of Khangildin, proposing a new indicator – "steadiness of stability index":

$$U_i = \left(1 - \frac{I_{i(opt)} - I_{i(lim)}}{\bar{I}}\right) \cdot 100\ \%. \quad (6)$$

Here $I_{i(opt)}$, $I_{i(lim)}$, $\bar{I}$ are stability indexes calculated by formulas:

$$I_{i(opt)} = \frac{\bar{Y}_{i(opt)}^2}{\sigma_{i(opt)}},\ I_{i(lim)} = \frac{\bar{Y}_{i(lim)}^2}{\sigma_{i(lim)}},\ \bar{I} = \frac{\Sigma_{ij} \bar{Y}_{ij}^2 / \sigma_{ij}}{v \cdot n}, \quad (7)$$

where σ_{ij}, $\sigma_{i(opt)}$, $\sigma_{i(lim)}$ are standard deviations of the trait for *i*-th genotype in *j*-th, optimal and limited environments, respectively; $\bar{Y}_{ij}$, $\bar{Y}_{i(opt)}$, $\bar{Y}_{i(lim)}$ are average values of the trait for *i*-th genotype in *j*-th, optimal and limited environments; *v* is a number of genotypes, *n* is a number of environments. The higher this indicator is the more stable is the genotype.

6. (*MAR*). Martynov (1990) proposes to use a "weighted homeostacity index" for stability evaluation:

$$H_i = \Sigma_i a_{j(k)} (Y_{ij} - \bar{Y}_{.j}) / \sigma_j, \quad (8)$$

where $a_{j(k)}$ is the calculated weighting factor determined for the *j*-th environment as follows.

All environments are divided into three groups with similar conditions: favorable ($\bar{Y}_{.j} > \bar{Y}_{..} + LSD$), medium ($\bar{Y}_{..} - LSD < \bar{Y}_{.j} < \bar{Y}_{..} + LSD$) and unfavorable ($\bar{Y}_{.j} < \bar{Y}_{..} - LSD$). The value of *LSD* (least significant difference) is determined by the formula:

$$LSD = t_\alpha \sqrt{MS_{\text{GE}}(n+1)/(nv)}, \quad (9)$$

where t_α is the value of the Student's *t*-test (for selected significance level α and $df = (v-1)(n-1)$); MS_{GE} is the mean square of G × E interaction.

Let's $N_k\,(k = 1, 2, 3)$ – number of elements within *k*-th group of environments, then the weighting coefficients $a_{j(k)}$ for each environment in *k*-th group are the same:

$$a_{j(k)} = \frac{n}{3} N_k \quad (k = 1, 2, 3). \quad (10)$$

The higher the H_i value, the more homeostatic the genotype.

7. (*CAC*). Kilchevsky and Khotyleva (1989) call the sum of the effects of the *j*-th environment and the interaction of genotype × environment as a "specific adaptive ability" of the *i*-th genotype in the *j*-th environment.

$$CAC_{ij} = d_j + (vd)_{ij} = \bar{Y}_{.j} - \bar{Y}_{..} + (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..}) = Y_{ij} - \bar{Y}_{i.}. \quad (11)$$

As a measure of the stability of the *i*-th genotype, they use the CAC_i variance:

$$\sigma_{CAC_i}^2 = \frac{1}{n-1} \Sigma_j (d_j + (vd)_{ij})^2 - \frac{n-1}{1} \sigma^2 = \frac{\Sigma_j (Y_{ij} - \bar{Y}_{i.})^2}{n-1} - \frac{n-1}{n} \sigma^2. \quad (12)$$

8. (*Wi*). Wricke (1962) proposed to use the sum of squares of environmental effects as a measure of genotype stability. This parameter is called "ecovalence" and is calculated by the formula:

$$W_i = \Sigma_j (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..})^2. \quad (13)$$

The more stable genotype is, the smaller its ecovalence value will be.

9. (*SIGMA*). Shukla (1972) developed an unbiased estimate using "stability variance" of genotypes:

$$\hat{\sigma}_i^2 = \frac{v(v-1)\Sigma_j (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..})^2 - \Sigma_{ij} (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..})^2}{(n-1)(v-1)(v-2)}. \quad (14)$$

10–11. (*Bi*, *S2di*). Eberhart and Russell (1966) used a regression approach to assess the stability. The following model is considered:

$$Y_{ij} = \mu_i + \beta_i I_j + \delta_{ij}, \quad (15)$$

where Y_{ij} is the variety mean of the *i*-th variety in the *j*-th environment ($i = 1, \dots v; j = 1, \dots n$); μ_i is the mean of the *i*-th

variety over all environments; β_i is the regression coefficient, that measures the response of the *i*-th variety to varying environments; δ_{ij} is the deviation from regression of the *i*-th variety at the *j*-th environment; I_j is the environmental index of the *j*-th environment, calculated by the formula:

$$I_j = \frac{\Sigma_i Y_{ij}}{v} - \frac{\Sigma_i \Sigma_j Y_{ij}}{vn}. \quad (16)$$

The estimation of the regression coefficient b_i is the first parameter of genotype stability.

$$b_i = \frac{\Sigma_j Y_{ij} I_j}{\Sigma_j I_j^2}. \quad (17)$$

The second parameter of stability is the variance of deviation from the regression line.

$$s^2_{d_i} = \frac{\Sigma_j \hat{\delta}^2_{ij}}{n-2} - \frac{s^2_e}{r}, \quad (18)$$

where s^2_e is the estimate of the pooled error; r – number of replications. The sum of the squares of deviations from the regression is calculated by the formula:

$$\Sigma_j \hat{\delta}^2_{ij} = \left(\Sigma_j Y^2_{ij} - (\Sigma_j Y_{ij})^2/n\right) - (\Sigma_j Y_{ij} I_j)^2/\Sigma_j I_j^2. \quad (19)$$

A variety with $b_i = 1$, $s^2_{d_i} = 0$ are referred to stable ones.

12–13. (*Alpha*, *Lambda*). The regression approach is also used by Tai (Tai, 1971; Tai, Young, 1972). A model of analysis of variance with random effects of the environment is considered:

$$y_{ijk} = \mu + d_i + \epsilon_j + \gamma_{k(j)} + g_{ij} + e_{ijk}, \quad (20)$$

where y_{ijk} ($i = 1, \dots v$, $j = 1, \dots n$, $k = 1, \dots r$) is the value of the trait of the *i*-th genotype in the *j*-th environment in the *k*-th replication; μ is the overall mean; d_i is the effect of the *i*-th genotype; ϵ_j is effect of the *j*-th environment; $\gamma_{k(j)}$ is the effect of replicates within environments; g_{ij} is the effect of genotype × environment interaction; e_{ijk} is the residual variation due to replications.

Tai and Young (1972) proposed to determine the linear response of the genotype to the effect of the environment α_i and the deviation from the linear response λ_i. They are related to the parameters of Eberhart and Russell in the following way:

$$\alpha_i = \frac{MSL(b_i - 1)}{MSL - MSB}, \quad (21)$$

$$\lambda_i = \left(\frac{v}{v-1}\right)\left(\frac{n-2}{n-1}\right)\frac{s^2_{d_i}}{MSE/r} - \alpha_i\left(\frac{b_i - 1}{m-1}\right)\frac{MSB}{MSE}, \quad (22)$$

where *MSL*, *MSB*, *MSE* are mean squares, due to environments, replicates within environments and error, respectively. Tai considers that in terms of agricultural usage the preferred variety has ($\alpha = 0$, $\lambda = 1$) and a mean performance which is above-average of all varieties over a series of environments.

14. (*Ai*). The regression coefficient in the model of Eberhart and Russell depends on the average value of the trait. Varieties with a high average value will have a higher coefficient (scale effect). To eliminate this drawback Dragavtsev (Dragavtsev, Averyanova, 1983) proposed to use the dimensionless "coefficient of multiplicativity":

$$a_i = \frac{\overline{Y}_{i.} - b_i \overline{Y}_{..}}{\overline{Y}_{i.}}. \quad (23)$$

15–16. (*S1*, *S2*). Nassar and Huehn (1987) ranked genotypes in the *j*-th environment ($j = 1, \dots N$), assigning the minimum rank 1 to the genotype with the lowest corrected mean value $Y^*_{ij} = \left(Y_{ij} - (\overline{Y}_{i.} - \overline{Y}_{..})\right)$ ($i = 1, \dots K$), and the maximum rank K to the genotype with the highest corrected mean value of the trait. A genotype is considered stable if its ranks are close for different environments. The most stable genotype has a constant rank. The following parameters are proposed as a measure of stability:

$$S_i^{(1)} = 2 \cdot \Sigma_{j=1}^{N-1} \Sigma_{j'=j+1}^{N} |r_{ij} - r_{ij'}|/[N(N-1)], \quad (24)$$

$$S_i^{(2)} = \Sigma_{j=1}^{N} (r_{ij} - \bar{r}_{i.})^2/(N-1), \text{ where } \bar{r}_{i.} = \Sigma_{j=1}^{N} r_{ij}/N. \quad (25)$$

For the most stable genotypes $S_i^{(1)} = 0$, $S_i^{(2)} = 0$.

17. (*ASVI*). Zobel, Wright and Gauch (1988) suggested to use the principal component method and a graphical representation of stability based on the AMMI model (Additive Main effects and Multiplicative Interaction). The model has the form:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \Sigma_{k=1}^{N} \lambda_k \zeta_{ik} \eta_{jk} + \theta_{ij}, \quad (26)$$

where Y_{ij} is the mean value of the trait of *i*-th genotype in *j*-th environment ($i = 1, \dots v$; $j = 1, \dots n$); μ is an overall mean; α_i is the genotype mean deviation; β_j is the environment mean deviation; λ_k is the eigenvalue of the PCA axis; ζ_{ik}, η_{jk} are the genotype and environment PCA scores; N is a number of PCA axes, retained in the model; θ_{ij} is the residual.

Based on this model, Purchase (Purchase et al., 2000) proposed to use the ASV (AMMI Stability Value) parameter for ranking genotypes by stability:

$$ASV_i = \sqrt{\left[\frac{SS_{IPCA1}}{SS_{IPCA2}}(IPCA1score)\right]^2 + (IPCA2score)^2}, \quad (27)$$

where SS_{IPCA1}, SS_{IPCA2} are sums of squares of the first and second principal components; *IPCA*1*score*, *IPCA*2*score* are projections of the *i*-th genotype on the first and second PCA axis. The smaller the value of the ASV parameter is, the more stable the genotype is considered.

18. (*MeanY*). In addition, values of mean yield were calculated for each genotype using standard formulas.

For comparison of various methods for assessing stability, 17 statistical indexes described above were calculated. Then all varieties were ranked according each of the stability statistics. The ranks were assigned in such a way that the rank 1 received the most desirable variety:

- for statistics *MeanY*, *HOM*, *UST*, *MAR*, *Ai*, rank 1 received varieties with the highest value of the index;
- for statistics *ASVI*, *S1*, *S2*, *Lambda*, *S2di*, *SIGMA*, *Wi*, *CAC*, *Pi*, *CV*, *EV*, rank 1 received varieties with the lowest value of the index;
- for regression coefficient *Bi*, rank 1 was assigned to the variety with the smallest (in modulo) coefficient's deviation from one;
- for *Alpha* coefficient, rank 1 was assigned to the variety with the smallest (in modulo) coefficient's deviation from zero.

Results

Mean grain yield and rank of 7 genotypes across 6 environments are shown in Table 1. The fact that genotypes switch ranks from one environment to another means the presence of a crossover GE interaction.

The result of analysis of variance (Table 2) shows that GE interaction is significant ($p < 0.001$), which indicates a significant difference between the responses of genotypes to changes in environmental conditions. Shapiro–Wilk test showed the normality of model residuals ($W = 0.99$, p-value = 0.31). The Levene test proved the homogeneity of variance ($F = 0.57$, p-value = 0.97).

Table 3 provides the estimates of mean grain yield, 17 stability statistics and the result of ranking of the seven winter wheat varieties according these statistics.

Taking mean yield as a first parameter for evaluating the genotypes, Novosibirskaya 3 gave the best mean yield while Novosibirskaya 32 had the lowest mean yield across environments. The highest variation of grain yield had Novosibirskaya 2, the lowest – Novosibirskaya 32. Genotypes Novosibirskaya 2, Novosibirskaya 3, Novosibirskaya 40, Obskaya winter, and Omskaya 6, with regression coefficients b_i higher than one were able to take advantage of favorable environments, whereas Novosibirskaya 32, and Novosibirskaya 51 with $b_i < 1$ were less responsive to environmental changes. According to Shukla's statistic, Novosibirskaya 51 provided the lowest contribution to total GE interaction, while Novosibirskaya 2 had the largest stability variance.

One can see the significant difference between methods in genotypes ranking. Such is the variety Novosibirskaya 32, which has the lowest mean yield and the lowest variance in the experiment. It is defined as the most stable one by methods focusing on the variation of the yield (*EV*, *CAC*, *CV*, *HOM*, *UST*, *Ai*), and vice versa, as the most unstable, by methods focusing on the mean value of the yield (*Pi*, *MAR*, *Bi*, *Alpha*, *S1*).

For further determination of the interrelationships of the methods, paired correlations of ranks defined by the stability statistics were calculated by Kendall's method. The results are presented in Table 4 and graphically in the Figure.

High level of paired correlations was found between statistics *UST*, *CV*, *CAC*, *HOM*, *Ai* and *EV*. *Pi* and *MAR* were strongly correlated with the average yield of genotype *MeanY*. Another group of methods with significant paired correlations included *Wi*, *SIGMA*, *S2di*, *Lambda*, and *ASVI.*

Some of the considered methods were ranking genotypes identically: *CAC* and *EV*; *Bi* and *Alpha*; *S2di* and *Lambda*; *Wi* and *SIGMA*.

Discussion

According the rank correlation matrix the considered methods can be divided into five groups determined by a high level of paired rank correlations ($r > 0.8$).

Group 1. Includes the regression coefficient of Eberhart and Russell b_i and the similar coefficient of Tai α_i. In fact α_i is equal to $b_i - 1$ when the effect of replicate in environment is not considered. Becker and Leon (1988) pointed a strong correlation between b_i and Roemer's environmental variance S^2_{yi}. But when we proceeded to consideration of ranks then none correlation between these parameters was found. It is explained by the fact that for environmental variance rank 1 received the variety with S^2_{yi} closest to zero, and for regression coefficient b_i, rank 1 was assigned to the variety with the

Table 1. Mean grain yield (t/ha) and range of 7 winter wheat varieties tested across 6 environments

Variety	2009				2010				2011			
	No-coulisses		In coulisses		No-coulisses		In coulisses		No-coulisses		In coulisses	
	Yield	R	Yield	R	Yield	R	Yield	R	Yield	R	Yield	R
Novosibirskaya 2	5.66	4	5.70	5	3.42	3	3.65	7	5.49	1	5.84	1
Novosibirskaya 3	5.86	2	6.50	1	3.74	1	4.84	1	4.67	4	5.62	2
Novosibirskaya 32	4.93	7	4.71	7	3.21	7	3.96	5	3.78	7	4.41	7
Novosibirskaya 40	5.64	5	6.08	4	3.36	4	3.86	6	4.65	5	5.28	4
Novosibirskaya 51	5.54	6	5.45	6	3.34	5	4.00	4	4.46	6	5.18	6
Obskaya winter	5.90	1	6.34	2	3.72	2	4.35	3	4.72	3	5.20	5
Omskaya 6	5.76	3	6.14	3	3.30	6	4.47	2	5.44	2	5.61	3

Table 2. The results of the variance analysis for grain yield of 7 winter wheat varieties tested across 6 environments

Source of variation	Df	Sum of squares	Mean square	F
Genotype	6	13.51	2.251	171.892***
Environment	5	89.30	17.860	1363.969***
Genotype × environment	30	9.18	0.306	23.378***
Error	84	1.10	0.013	

*** $p < 0.001$.

Table 3. Stability statistics and ranks of 7 varieties of winter wheat tested across 6 environments

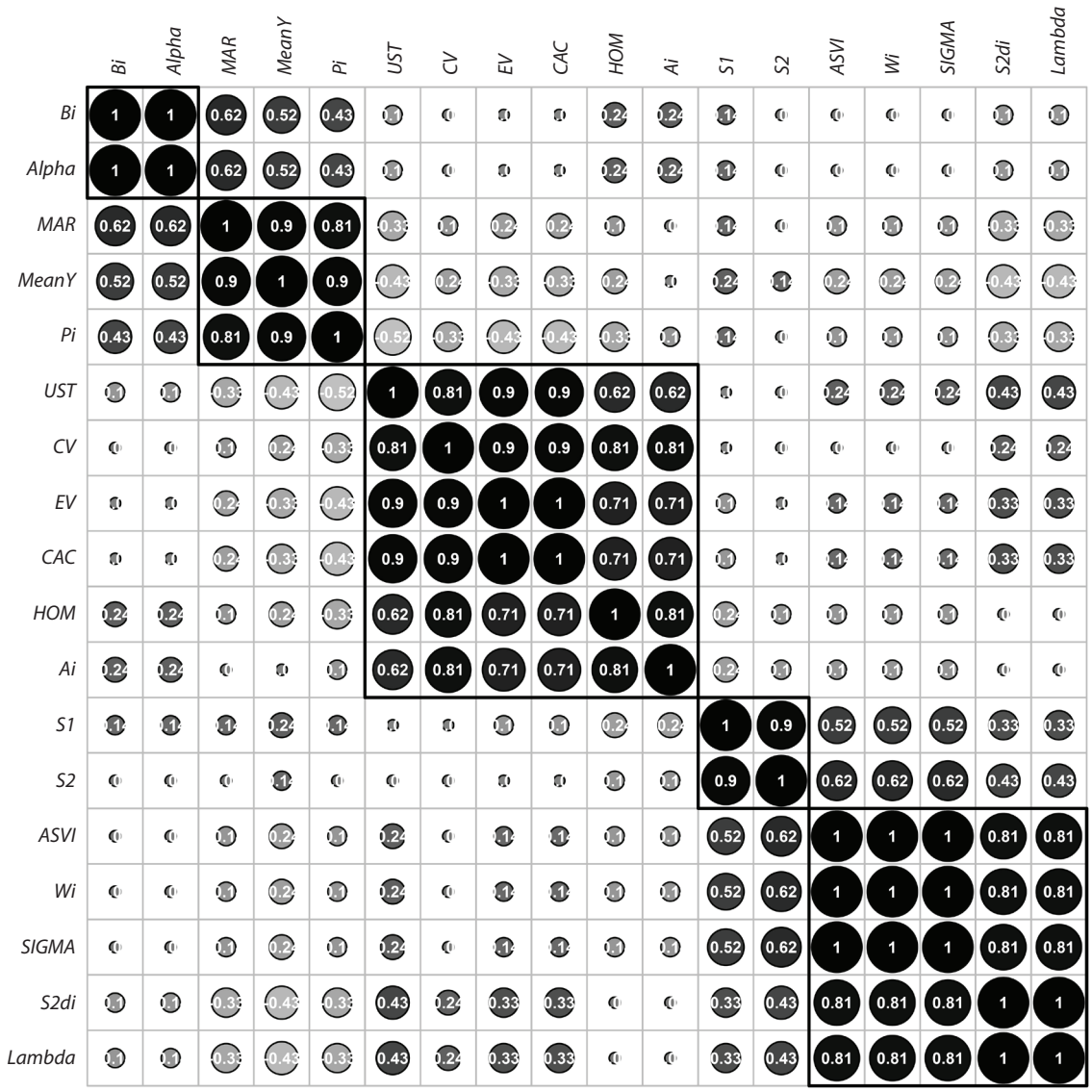
Method	Novosibirskaya 2		Novosibirskaya 3		Novosibirskaya 32		Novosibirskaya 40		Novosibirskaya 51		Obskaya winter		Omskaya 6	
	Val	R	Val	R	Val	R	Val	R	Val	R	Val	R	Val	R
MeanY	4.96	4	5.20	1	4.17	7	4.81	5	4.66	6	5.04	3	5.12	2
EV	1.24	7	0.97	4	0.41	1	1.11	6	0.78	2	0.96	3	1.10	5
CAC	1.24	7	0.97	4	0.41	1	1.11	6	0.77	2	0.95	3	1.10	5
Pi	0.18	4	0.06	2	0.85	7	0.20	5	0.30	6	0.11	3	0.04	1
CV	21.15	7	17.92	3	14.55	1	20.68	6	17.88	2	18.32	4	19.36	5
HOM	9.68	5	10.52	3	16.66	1	8.56	7	11.88	2	10.50	4	9.33	6
UST	–4.61	7	0.70	4	3.64	1	–0.98	5	1.01	2	0.85	3	–3.31	6
MAR	0.98	4	5.44	1	–9.10	7	–0.89	5	–2.52	6	3.23	2	2.85	3
Wi	1.06	7	0.39	5	0.76	6	0.15	2	0.08	1	0.25	3	0.37	4
SIGMA	0.05	7	0.01	5	0.03	6	0.00	2	0.00	1	0.01	3	0.01	4
Bi	1.10	4	1.02	1	0.65	7	1.14	6	0.95	3	1.03	2	1.11	5
S2di	0.25	7	0.09	6	0.06	4	0.01	2	0.01	1	0.06	3	0.08	5
Ai	–0.08	6	0.05	2	0.24	1	–0.15	7	0.01	3	0.00	4	–0.05	5
Alpha	0.10	4	0.02	1	–0.35	7	0.14	6	–0.05	3	0.03	2	0.11	5
Lambda	71.24	7	27.06	6	16.46	4	4.40	2	3.68	1	16.44	3	22.12	5
S1	3.07	6	2.47	3	3.13	7	1.27	1	1.80	2	2.80	5	2.53	4
S2	7.07	7	4.30	3	6.97	6	1.37	1	2.17	2	5.47	5	4.67	4
ASVI	2.20	7	1.11	5	1.56	6	0.42	2	0.29	1	0.75	3	0.90	4

Note: Refer to text for details of methods. Val is the value of the stability statistic, corresponding to the method; R is the genotype rank, corresponding to the method.

Table 4. Correlations of ranks between mean yield and stability measures for 7 varieties of winter wheat

Parameter	*MeanY*	*EV*	*CAC*	*Pi*	*CV*	*HOM*	*UST*	*MAR*	*Wi*	*SIGMA*	*Bi*	*S2di*	*Ai*	*Alpha*	*Lambda*	*S1*	*S2*
EV	–0.33																
CAC	–0.33	1.00															
Pi	0.90	–0.43	–0.43														
CV	–0.24	0.90	0.90	–0.33													
HOM	–0.24	0.71	0.71	–0.33	0.81												
UST	–0.43	0.90	0.90	–0.52	0.81	0.62											
MAR	0.90	–0.24	–0.24	0.81	–0.14	–0.14	–0.33										
Wi	–0.24	0.14	0.14	–0.14	0.05	–0.14	0.24	–0.14									
SIGMA	–0.24	0.14	0.14	–0.14	0.05	–0.14	0.24	–0.14	1.00								
Bi	0.52	–0.05	–0.05	0.43	0.05	0.24	–0.14	0.62	0.05	0.05							
S2di	–0.43	0.33	0.33	–0.33	0.24	0.05	0.43	–0.33	0.81	0.81	–0.14						
Ai	–0.05	0.71	0.71	–0.14	0.81	0.81	0.62	0.05	–0.14	–0.14	0.24	0.05					
Alpha	0.52	–0.05	–0.05	0.43	0.05	0.24	–0.14	0.62	0.05	0.05	1.00	–0.14	0.24				
Lambda	–0.43	0.33	0.33	–0.33	0.24	0.05	0.43	–0.33	0.81	0.81	–0.14	1.00	0.05	–0.14			
S1	0.24	–0.14	–0.14	0.14	–0.05	–0.24	–0.05	0.14	0.52	0.52	0.14	0.33	–0.24	0.14	0.33		
S2	0.14	–0.05	–0.05	0.05	0.05	–0.14	0.05	0.05	0.62	0.62	0.05	0.43	–0.14	0.05	0.43	0.90	
ASVI	–0.24	0.14	0.14	–0.14	0.05	–0.14	0.24	–0.14	1.00	1.00	0.05	0.81	–0.14	0.05	0.81	0.52	0.62

Note: Refer to text for details of methods.



Graphic representation of the rank correlation matrix ordered by the cluster method.

smallest (in modulo) coefficient's deviation from one. And so we consider b_i not as a measure of stability but as additional information on the average response of a genotype to advantageousness of environmental conditions.

Group 2. Includes the superiority index *Pi* of Lin and Binns and Martynov's weighted homeostaticity index H_i. These statistics were strongly correlated with yield. Breeding based on these methods would favor breeding for yield, as Kang and Pham (1991) and Flores (Flores et al., 1998) found.

Group 3. Includes steadiness of stability index of Udachin U_i, coefficient of variation of Francis and Kannenberg CV_i, variance of the specific adaptive ability of Kilchevsky and Khotyleva $\sigma^2_{CAC_i}$, Roemer's environmental variance S^2_{yi}, Khangildin's coefficient of homeostaticity Hom_i, Dragavtsev's coefficient of multiplicativity a_i. All these parameters are connected to genotype variance. The identicall genotypes ranking was received for $\sigma^2_{CAC_i}$ and S^2_{yi}, because the variance of specific adaptive ability of Kilchevsky and Khotyleva is nothing more than the Roemer's environmental variance, reduced by error of experiment.

Group 4. Includes non-parametric estimates of stability of Nassar and Huehn $S_i^{(1)}$, $S_i^{(2)}$. Becker and Leon (1988), Adugna and Labuschagne (2003), Mohammadi and Amri (2008), reported that these statistics were highly correlated with Shukla's stability variance $\hat{\sigma}^2_i$ and with Eberhart and Russell's $s^2_{d_i}$. But we received only medium correlation of ranks for them.

Group 5. Includes Purchase's parameter based on AMMI model ASV_i, ecovalence of Wricke W_i, Shukla's stability variance $\hat{\sigma}^2_i$, Eberhart and Russell's variance of deviation from the regression $s^2_{d_i}$, and Tai's λ_i variance. The W_i and $\hat{\sigma}^2_i$ parameters produced the same ranking of genotypes wich is in agreement with Kang et al. (1987). The ranks established by W_i and $s^2_{d_i}$ were highly correlated because the ecovalence and the deviation from regression are mathematically linked (Flores et al., 1998). Also, $s^2_{d_i}$ and λ_i identically ranged the genotypes because they are mathematically linked. As expected, the statistics

$\hat{\sigma}_i^2$ and $s_{d_i}^2$ were highly rank-correlated too. The multivariate parameter ASV_i produced the same ranging of genotypes as W_i and $\hat{\sigma}_i^2$ in our experiment, which is in agreement with strong correlation of these parameters demonstrated by Purchase et al. (2000).

According to Becker and Leon's (1988) classification, the Group 3 presents methods related to the static (biological) concept of stability, and all other groups of methods refer to dynamic (agronomic) concept of stability.

Lin et al. (1986) suggested the classification of methods which contains three concepts of stability:

Type 1: A genotype is considered to be stable if its among-environment variance is small.

Type 2: A genotype is considered to be stable if its response to environments is parallel to the mean response of all genotypes in the trial.

Type 3: A genotype is considered to be stable if the residual MS from the regression model on the environmental index is small.

According to this classification the statistics of Group 1 corresponds to Type 2 stability, those of Group 3 to Type 1, and those of Group 4 and Group 5 to Type 3 stability.

The choice of the appropriate method for evaluation the stability of genotypes depends on the objectives of the study. In the case when the goal of breeding research is the selection of the most biologically stable varieties with the minimum variance across a range of environments, the stability is defined in the sense of homeostasis, one should use the methods of the static concept from Group 3. It should be remembered that the most stable varieties in this concept may have relatively low yields. Among the varieties of winter wheat under study, the variety Novosibirskaya 32 was the most stable from the biological point of view. This variety has the smallest variance, the smallest coefficient of variation, and the greatest coefficient of homeostaticity. At the same time, this variety showed the lowest yield in the experiment.

If it is necessary to choose a genotype with a predictable reaction to changes of environmental conditions, corresponding to the calculated level or forecast, the regression approach (Group 1) is the most appropriate. The most stable will be the genotype, which has the smallest deviation from the mean yield in all environments. The variety Novosibirskaya 3 with the smallest deviation of b_i from one fits this definition.

To compare the contributions of each genotype to the GE interaction, one should use the methods presented in Group 5. In these methods, the genotype that makes the least contribution to the interaction is considered the most stable. In our experiments the variety Novosibirskaya 51 was defined as the most stable from this point of view.

In the nonparametric methods of Group 4, the concept of stability is the same as in the methods of Group 5. Nonparametric methods should be used in the case when the initial data do not meet the requirements of normal distribution and homogeneity of variance. The most suitable genotype for this group was Novosibirskaya 40.

The methods from Group 2 cannot be recommended for stability evaluation, because they are strongly correlated with the mean yield and breeding based on these methods would favor breeding for yield.

Conclusions

The considered methods reflect different aspects of GE interaction. As Flores et al. (1998) mentioned: "…all the methods examined here to study the stability of genotypes are valid, although they are based on very different concepts of stability". The breeder can choose the appropriate method depending on whether the breeding is to be based primarily on yield, primarily on stability, or simultaneously on yield and yield stability.

In our experiment the methods were applied to a small number of unique cultivars and environments. In order to find stable genotypes of winter wheat for breeding purpose farther field experiments need to be conducted.

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Acknowledgements. This work was supported by IC&G budget project No. 0324-2019-0039 in part of field trial and experimental measurements and by SFSCAT budget project No. 0778-2019-0001 in part of statistical analysis.

Conflict of interest. The authors declare no conflict of interest.

Received August 7, 2019. Revised October 4, 2019. Accepted October 7, 2019.

Вавиловский журнал генетики и селекции. 2020;24(3):276-283
DOI 10.18699/VJ20.620

Concentration of viruses and electron microscopy

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Abstract. Nearly all lethal viral outbreaks in the past two decades were caused by newly emerging viruses. Viruses are often studied by electron microscopy (EM), which provides new high-resolution data on the structure of viral particles relevant to both fundamental virology and practical pharmaceutical nanobiotechnology. Electron microscopy is also applied to ecological studies to detect viruses in the environment, to analysis of technological processes in the production of vaccines and other biotechnological components, and to diagnostics. Despite the advances in more sensitive methods, electron microscopy is still in active use for diagnostics. The main advantage of EM is the lack of specificity to any group of viruses, which allows working with unknown materials. However, the main limitation of the method is the relatively high detection limit (10^7 particles/mL), requiring viral material to be concentrated. There is no most effective universal method to concentrate viruses. Various combinations of methods and approaches are used depending on the virus and the goal. A modern virus concentration protocol involves precipitation, centrifugation, filtration, and chromatography. Here we describe the main concentrating techniques exemplified for different viruses. Effective elution techniques are required to disrupt the bonds between filter media and viruses in order to increase recovery. The paper reviews studies on unique traps, magnetic beads, and composite polyaniline and carbon nanotubes, including those of changeable size to concentrate viral particles. It also describes centrifugal concentrators to concentrate viruses on a polyethersulfone membrane. Our review suggests that the method to concentrate viruses and other nanoparticles should be chosen with regard to objectives of the study and the equipment status of the laboratory.

Key words: virus concentration; electron microscopy; virus purification; virus detection; review.

For citation: Petrova I.D., Zaitsev B.N., Taranov O.S. Concentration of viruses and electron microscopy. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):276-283. DOI 10.18699/VJ20.620

Концентрирование вирусов и электронная микроскопия

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Аннотация. Почти все смертельные вирусные вспышки в последние два десятилетия были вызваны вновь появляющимися вирусами. Для изучения вирусов часто используют электронную микроскопию (ЭМ). Она позволяет получить новые данные о структуре вирусных частиц с высоким разрешением, что представляет интерес как для фундаментальной вирусологии, так и для практической фармацевтической нанобиотехнологии. Кроме того, ЭМ применяется в экологических исследованиях для определения наличия вирусов в окружающей среде, при анализе технологических процессов для производства вакцин и других биотехнологических компонентов, а также в диагностических целях. Несмотря на развитие более чувствительных методов, электронная микроскопия в диагностике остается рабочим методом. Главное преимущество ЭМ – отсутствие специфичности к какой-либо определенной группе вирусов, что способствует работе с неизвестным материалом. Однако основное ограничение метода – относительно высокий предел обнаружения (10^7 частиц/мл), в связи с чем необходимо концентрировать вирусный материал. Не существует какого-то одного наиболее эффективного метода. В зависимости от самого вируса и поставленной цели используются различные комбинации методов и подходов. В настоящее время концентрирование вируса включает операции осаждения, центрифугирования, фильтрации и хроматографии. В обзоре на примере разных вирусов описаны эти основные методы. Существует необходимость в разработке эффективных методик элюирования, которые могут нарушить связь между фильтрующими материалами и вирусами, чтобы повысить степень восстановления. Рассмотрены работы по созданию уникальных ловушек, магнитных шариков, композитных полианилиновых и углеродных нанотрубок и нанотрубок с изменяемым размером для концентрирования вирусных частиц. Приведен

пример применения центрифужных концентраторов, в которых вирус осаждается на мембране из полиэфирсульфона. Проанализированные данные указывают на то, что способ концентрирования вирусов или других наночастиц выбирается в каждом конкретном случае в зависимости от поставленной цели и оснащенности лаборатории.
Ключевые слова: концентрирование вирусов; электронная микроскопия; очистка вирусов; выявление вирусов; обзор.

Introduction

Electron microscopy (EM) is often used to study viruses. This method makes it possible to determine the shapes and sizes of viral particles and their localization within cells, as well as to identify cytological changes in cells. The main advantage of EM is the lack of specificity to any group of viruses, as opposed to immunological and molecular tests. With the present advances in genetic engineering and the use of a wide range of pseudovirus-based assays in vaccine development, the list of possible research targets for EM and the scope of problems solved have expanded significantly. A valid EM study requires that the sample should contain enough viral particles. It was shown earlier (Reid et al., 2003) that EM visualization requires virion contents no lower than 10^7 particles/mL. Thus, the necessity for purification and concentration of viral material arises rather often. It is noteworthy that EM is also necessary for the refinement of virus concentration methods. Moreover, in the age of nanotechnology we cannot ignore the fact that transmission EM (TEM) is used to control the fabrication of nanoparticles and their application (Zajtsev et al., 2016).

In addition to the classical application of the method, i. e. studying structures of viruses, infiltration processes, and virus transmission in organisms, EM is also used in at least three fields of applied research: diagnostic EM (DEM); ecological EM (detection of viruses in the environment), and problems associated with technological processes (manufacturing of vaccines and other components) that use biotechnological methods to yield the products and require that the product should be proven to be virus-free.

Diagnostic electron microscopy

Despite the advances in more sensitive methods, such as polymerase chain reaction (PCR) or immunoenzyme analysis (IEA), EM still remains a widely applicable diagnostic tool (Goldsmith, Miller, 2009; Gentile, Gelderblom, 2014). The main advantage of EM in diagnostics of infectious diseases is its versatility, i. e. the ability to observe 'anything' in a single sample without prior knowledge of possible microorganism identities present in the sample (Hazelton, Gelderblom, 2003). Another advantage of EM is its fastness, as it takes as little as 10–15 minutes for a wide range of object sizes to be studied. EM makes it possible to analyze both liquid and tissue samples and requires no additional information, unlike the diagnostic methods using nucleic acids (PCR) or antibodies (IEA).

The main limitation of DEM is the virion content in samples. To overcome this limitation, various concentration techniques are used, such as ultracentrifugation, ultrafiltration, examination of ultrathin sections, etc. In (Beniac et al., 2014), the authors describe virus concentration by filtration straight onto EM support grid with holey carbon substrate Quantifoil R1/4 (Quantifoil Micro Tools GmbH, Großlöbichau, Germany). This technique combined with scanning EM (SEM) improves the virus detection threshold to contents of 10^2 viral particles per 1 mL.

Thus, DEM is a valuable method for observing newly emerging diseases and potential bioterrorism agents. Finally, ultrastructural studies performed using EM provide detailed examination of virus morphogenesis, virus–cell interactions, and pathogenetic aspects of viral infections including the development of preventives and treatments.

Electron microscopy in ecological studies

The use of EM for ecological purposes (detection of viruses in the environment) appears to be an obvious solution, but it is rarely used in practice. When it is, its most common target is the pollution of water resources, which supplies almost illimited volumes of research material. One of the few papers dealing with seawater as a research subject was published in 1999 (Alonso et al., 1999). The described concentration process included two stages: concentration of a vast amount of seawater using a tangential flow filtration system and ultrafiltration using a centrifugal concentrator with further visualization by means of TEM. The same methods were used later in (Sun et al., 2014). The authors studied over 150 L of seawater from the Yangshan Deep-Water Port area (South East Shanghai, China). However, these days EM is quite rarely used for such analytical tasks, as they are mostly solved by more sensitive methods, first of all, PCR. The reason is the limited range of possible targets, which are being typically presupposed or known beforehand, so PCR is often substituted for EM at the stage when an infectious agent has to be detected.

Analysis of biotechnological processes

Vaccines are among the most effective pharmaceutical products in public healthcare showing great results in terms of safety. Since biological materials are involved in vaccine production, the process must be protected from sporadical contamination. The necessity for controlling biotechnologically produced materials is broadly discussed (Sheets, 2013). The production of virus vaccines and other cellular biopharmaceutical products is a complex technological process, which involves various biological materials (e. g., various cell substrates, such as chicken eggs with embryos; primary cell cultures; and continuous cell lines). The

whole process is potentially vulnerable to contamination by foreign agents that may be unintentionally brought in with the materials used or from environmental sources. An example of this type of study may be found in (Reid et al., 2003), where three methods for retrovirus contamination assessment in mouse culture supernatants and CHO cell lines were compared: direct count of viral particles in a solution with latex beads (detection limit 10^7 particles/mL), ultracentrifuge concentration in a discontinuous sucrose density gradient with EM (10^5 particles/mL), and the method involving a thin section of a homogenous matrix of viral particles, cell debris, agar medium (sensitivity 10^5 particles/mL). Routine EM is obviously not suitable for registering virus contamination.

Various biotechnological fields, such as development of vaccines and adjuvants, manufacturing of biologically active complexes, development of contrast media, targeted delivery, and microelectronics use virus-like particles studied with EM. For instance, preparation of spherical particles (SP) from tobacco mosaic virus was controlled by TEM. It was shown that SP could be visualized via TEM without contrasting agents (Trifonova et al., 2015).

Virus concentration methods

There is no most effective universal method to concentrate viruses. Various combinations of methods and approaches are used depending on the virus and the problem set. Virus concentration processes currently include sedimentation, centrifugation, filtration, and chromatography (Transfiguracion et al., 2007; Vicente et al., 2011).

Precipitation

Virus precipitation is commonly achieved with polyethylene glycol (PEG), ammonium sulfate, or calcium phosphate. It is a convenient tool for obtaining viruses in both large and small quantities, while also providing the advantage of virus separation from most heterologous proteins. For example, the precipitation and purification method using PEG 6000 with further ultracentrifugation and clarification widely used for influenza viruses was successfully applied for concentration and purification of avian influenza Type A virus (ten different strains) from virus-containing suspensions (Ismagambetov et al., 2017). Another paper (Ryabinnikova et al., 2015) presented data on purification and concentration of horse rhinopneumonia (HRP) virus using various techniques. It was found that concentration of HRP virus using PEG-6000 with 0.5 M sodium chloride with subsequent dialysis yields concentrates with high infectious and antigen activity. It was shown that adsorption in presence of PEG produced virus preparations suitable for manufacturing inactivated HRP vaccine.

Polyethylene polyamine (PEPA) with a mass of 16,000 D was shown to be more suitable for concentration of FMD virus and porcine enterovirus (serotype 7) by precipitation than PEG-6000 (Bahutashvili et al., 2002). It was found that the most complete precipitation of FMD virus was achieved at PEPA contents of 0.1 and 0.2 %. There, virus losses were 0.74 and 0.11 %, respectively. It was also shown that vaccines produced using the preparations obtained via two different precipitation techniques were different in terms of their efficacy. The PEPA-based technique was preferable since the respective vaccine was three times as effective.

Centrifugation and density gradient

Centrifugation is an easy-to-use large-scale separation method based on differences in density. Due to small sizes of viral particles, high speed and centrifugal force are required, which, however, can make the particles noninfectious (Burova, Loffe, 2005). Centrifugation in density gradient using sucrose, cesium chloride (CsCl), or iodixanol is a viable alternative. The use of sucrose provides a viscous hyperosmotic solution. Density gradient of iodixanol is a low-viscosity system capable of producing isotonic solution and maintaining virus functionality (Gias et al., 2008). It was shown (Segura et al., 2006) that the use of iodixanol gradient provides 37 % retrovirus recovery with a promising purity of 95 %. To separate horse influenza virus from virus-containing allantoic fluid, ultracentrifugation in sucrose density gradient and DEAE cellulose-based ion exchange chromatography were used (Tajlakova et al., 2011). It was found that complete virus adsorption by ion exchanger occurred in a buffer solution with pH 7.0, whereas elution required a buffer with 0.5 M sodium chloride solution pH 7.4. As a result, virus preparations were obtained with ballast protein removal of 98 %.

Filtration

To concentrate enteroviruses from various water bodies, a unified membrane filtration method was developed using the microfiltration (MF) mode of the MFM 0142 module equipped with MMK1 membrane (Sanamyan et al., 2006). The method demonstrated high efficacy in enterovirus concentration from water with various degrees of contamination (potable, underground, river, and waste within the permissible limits) with the concentration time shortened to 42 min. The MMK-type filtration membrane modified with 0.5 % amino compounds proved most effective among the studied membranes. Hollow fiber MF membranes ($d = 0.2$ μm) were used to study the process of human adenovirus 2 (HAdV-2) concentration (Lu et al., 2016) depending on the virus content varying between 1.3×10^7 and 3.4×10^8 copies/mL.

To optimize the separation of hepatitis A virus from water, membranes based on polyamide, cellulose nitrate, cellulose acetate, and polyethersulfone were studied (Zalesskikh, Bystrova, 2018). Glass fiber, cardboard, and polypropylene were used as pre-filters. It was shown that the most effective membrane combination for filtration of highly impure water includes a cardboard or polypropylene filter for preliminary filtration and a polyamide filter for

the main stage of the process. A pressure filtration device (AF-142K, Vladisart, Vladimir, Russia) was used for filtration of samples with water volumes up to 10 L carried out at the pressure of 2 atm.

Epidemiological surveillance over human enteroviruses requires virus concentration from wastewater. This typically involves processing of large volumes (100–1000 L) of water, the most common method being the VIRADEL process with microporous filters. Positively charged filters do not require sample pretreatment, and they can concentrate viruses from water with a wider pH range than electronegative filters due to the virus surface charge typically being negative. Virosorb 1MDS is one of the most common electropositive filters. In recent years, it has been joined by positively charged NanoCeram filters (Soto-Beltran et al., 2013). Virus elution from filters after concentration was performed using organic (beef extract) or inorganic (sodium polyphosphates) solutions. Then, the eluates were repeatedly concentrated to reduce sample volumes and improve virus detection. Most filters proved highly effective in capturing the virus, while the elution and repeated concentration methods showed varying degrees of success due to the biological variation of viruses present in water (Ikner et al., 2012).

Nanofiltration (NF) was used in the filtration of serum protein solutions, production of serum derivatives, and manufacturing of a wide range of biopharmaceutical products. Depending on the material used, NF membranes fall into two main groups: ceramic and polymeric ones. Hybrid NF membranes are also of interest due to their performance under suboptimal conditions. Various polymers, such as polyethersulfone (Maximous et al., 2009), polydimethylsiloxane (Yousefi et al., 2017), polyvinylidene fluoride (Dong et al., 2013), and polysulfone (Ghaee et al., 2017) were tested as top layer materials for hybrid membranes. The use of polymers for obtaining hybrid NF membranes makes it possible to change pore sizes, chemical properties, and charges of membrane surfaces. Nanofiltration increasingly becomes a common step in pharmaceutical production, since it does not raise toxicity issues (Doodeji, Zerafat, 2018). Obtaining a purified virus concentrate is one of the key steps in vaccine production. Polyethersulfone membrane 300 kD combined with weak anionic detergent solution appears to be the most suitable for influenza virus concentration and removal of small molecule impurities (Kyzin et al., 2014).

Elution and repeated concentration methods were reported in the literature (Falman et al., 2019). First, wastewater was concentrated with ViroCap cartridge filters, and then Celite beef extract, ViroCap flat disc filters, InnovaPrep concentrating pipettes, PEG/NaCl precipitation, and skimmed milk flocculation were used. PEG/NaCl sedimentation and skimmed milk flocculation turned out to be the most effective methods among the five methods tested in experiments with poliovirus Type 1 (PV 1). Optimization of the skimmed milk flocculation method increased PV 1 recovery nearly twofold as compared to PEG/NaCl precipitation.

Tangential flow filtration is a common method of filtration by size (Wickramasinghe et al., 2005). Influenza virus was used to test a two-step purification and concentration protocol for viral particles.

Chromatography

Chromatography is a separation method based on the interaction between the target virus and column matrix. In general, separation functionality depends on charge, size, hydropathicity, and affinity.

Ion exchange chromatography utilizes the phenomenon of ion exchange between an immobile solid phase and a mobile liquid phase, which makes it possible to separate structurally and morphologically similar viral particles provided they have different total charges and/or charge distributions over their outer surfaces (Ruščić et al., 2015). Virus elution may require different salt concentrations, and the method can be applied to any mixed infection if viruses have different isoelectric points. A simple separation and concentration technique for mixed infection by cowpea chlorotic mottle virus (CCMV) and cucumber mosaic virus (CMV) is proposed in (Ali, Roossinck, 2008).

Monolithic supports made it possible to use chromatography for purification and concentration of various viruses (Svec et al., 2011; Krajačić et al., 2017). Unlike the classical chromatographic supports with diffusion-based mass transfer and relatively small pores, monoliths are characterized by significantly enhanced mass transfer due to convection and channel sizes of several microns. I. Gutiérrez-Aguirre et al. (2009) showed that CIM QA supports effectively retained rotaviruses present in stool samples, as well as in wastewater and river water samples. Concentration of rotaviruses was achieved by elution of the retained viruses with 1 M NaCl solution. The obtained viruses preserved their integrity, as confirmed by electron microscopy.

Size exclusion chromatography (or gel filtration in case of aqueous liquid phase) is a fluid chromatography based on varying capabilities of different-sized molecules to infiltrate the pores of non-ionogenic gel acting as a stationary phase. In this separation method, a matrix of densely packed silica gel or agarose gel is used (Barth et al., 1994). The main advantages of this method are its simplicity and the low cost of resins. Nevertheless, the method suffers from the lack of selectivity, requires low flow velocity, and shows low overall performance. Tick-borne encephalitis virus was used to refine the gel filtration method for the Superdex 200 column (Havlik et al., 2014). The samples obtained were tested by the following methods: (1) the immunological (dot blot) technique for testing biological activity, (2) gas-phase electrophoretic mobility macromolecular analysis (GEMMA) for determining particle sizes, and (3) atomic force microscopy (AFM) or TEM for obtaining information

on shapes and sizes of viral particles. The mean diameter of inactivated tick-borne encephalitis viral particles determined using GEMMA was 46.6 ± 0.5 nm, as opposed to AFM and TEM images, which showed the diameters of about 58 ± 4 and 52 ± 5 nm, respectively.

Hydrophobic interaction chromatography is based on particle adsorption at weakly hydrophobic surface at high salt contents with subsequent elution with descending salt gradient. Virus surfaces include hydrophobic areas, which are linked to immobilized hydrophobic ligands at chromatographic carriers (Roettger et al., 1989). The limitation of the method is illustrated by an example with adenovirus (Schagen et al., 2000). It was found that large salt concentrations could not only reduce the virus immunogenicity but could also favor viral particle aggregation.

Polyvinyl alcohol cryogel with pore sizes of 0.04–2.0 μm was used in a concentration method with virus capturing by bioaffine sorbent (Lozinskiy et al., 2013). The use of the sorbent was tested in the concentration process for influenza Type A viruses H1N1 and H3N2, parainfluenza virus type 6, smallpox virus, and FMD viruses. Bioaffine sorbent based on viscoelastic, non-brittle, and hydrolytically stable PVA cryogel allows virus concentration not only in a chromatographic column, but in reactors with sorbent mixing, which significantly intensifies mass transfer. The advantage of the method is that it provides the possibility of concentration not only for small but also for the largest (0.5 μm and above) viruses. According to the invention description, the bioaffine sorbent may include not only antibodies but also immobilized enzymes capable of modifying the virus.

Other methods

To monitor the presence of influenza virus in open water bodies, a custom device was designed (Levchenko et al., 2007). It employed magnetic immunosorbents in which antibodies were immobilized on a solid magnetic support.

Sorption of influenza viruses onto polyaniline (PANI) and carbon nanotubes, as well as on PANI composites (nanotubes and grains) with or without silver, was studied (Ivanova et al., 2015). It was found that inclusion of silver increased the sorption capability of PANI tubes in case of allantoic influenza Type A viruses. PANI nanotube composites with 30 % silver appear to be the most promising virus sorption material in water solutions with regard to the combination of properties.

Hepatitis A virus (HAV) was used as a model for comparison of various carbohydrate-binding lectins, including concanavalin A (Con A), wheat germ agglutinin, and soybean agglutinin, based on their binding affinity to the virus (Ko et al., 2018). Con A showed higher binding affinity than other lectins. Con A-bound immunomagnetic separation combined with RT-PCR made it possible to detect HAV at 10^{-4} dilution of the initial virus concentration (the titer being equal to 10^4 infectious doses for cell culture per mL). It shows that Con A may be a promising candidate for HAV concentration.

It was shown in several papers (Flavigny et al., 2004; Sakudo et al., 2009b) that magnetic beads coated with anionic polymers, such as poly-(methyl vinyl ether-co-maleic anhydride) may be used for effective capture of various types of viruses, which include human immunodeficiency virus Type 1 (Sakudo, Ikuta, 2012), respiratory-syncytial virus (Sakudo et al., 2009a), Borna disease virus (Sakudo et al., 2011b), influenza virus (Sakudo et al., 2008), and Dengue fever virus (Sakudo et al., 2011a). The method was later applied to adenovirus as well (Sakudo et al., 2016).

Japanese researchers (Mogi et al., 2016) worked on a new virus concentration method. They proposed and experimentally demonstrated a virus concentration device, which employed the ion depletion zone generated by ion polarization. The efficacy was assessed with fluorescent nanoparticles, Baculovirus, albumin, and dextran. All samples were successfully concentrated.

A new virus concentration strategy using the expression of human poliovirus receptor gene (hPVR) on *Escherichia coli* cell surfaces was developed (Abbaszadegan et al., 2011). The ability of modified bacterial cells to capture viral particles was confirmed by TEM. This approach provides new opportunities for effective capture and concentration of water-transmitted viruses.

To process field samples, unique microdevices capable of effective virus enrichment and concentration were designed using carbon nanotubes with variable sizes (CNT-STEM) (Yeh et al., 2016). The intertube distance between CNTs could be set between 17 and 325 nm to precisely match the sizes of specific viruses. With this device, the authors managed to identify two new strains.

The paper recently published by B.N. Zajtsev et al. (2019) described the use of a Vivaspin centrifugal concentrator. Viral particles were concentrated on a double vertical polyethersulfone membrane. Following centrifugation, the membrane was retrieved from the concentrator to assess the number of particles sedimented on the membrane by EM examination of ultrathin sections.

Conclusions

The desired research goal plays a major part in choosing the virus concentration method. For instance, ecological, pharmaceutical, and medical problems require different approaches. For a water contamination test to be successful, one should filter hundreds of liters of water from a specific area and then collect the virus material sedimented on the surface and inside charged filters. This procedure is typically followed by the second filtration step using different filters with adjusted pore sizes (Tarasov et al., 2012). A series of gains have been achieved in designing materials for filtration membranes in recent years. However, much is to be done for complete virus recovery from membranes and membrane surfaces.

To improve the virus concentration technology, one should focus on surface interactions between viruses and filter materials. In particular, effective elution methods should be developed to disrupt the bonds between filter materials and viruses to increase recovery. In diagnostic studies in specific patients, the amount of material is often limited to few milliliters. In many cases, especially when it comes to mixed infections, electron microscopy turns out to be one of the most reliable virus detection tools. Certainly, ultrathin sections can only be obtained at well-equipped EM laboratories, where EM diagnostics of virus infections is a routine process.

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Acknowledgements. The study was performed as part of the Government Assignment GZ-7/18 for the State Research Center of Virology and Biotechnology "Vector", Federal Service for Surveillance on Consumer Rights Protection and Human Well-Being.

Conflicts of interest. The authors declare no conflict of interest.

Received July 10, 2019. Revised November 18, 2019. Accepted November 20, 2019.

Вавиловский журнал генетики и селекции. 2020;24(3):284-291
DOI 10.18699/VJ20.621

Оценка *in vitro* биологической активности отечественного препарата макрофаг-активирующего фактора (GcMAF-RF)

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Аннотация. В статье сообщается о разработанном оригинальном способе получения витамин D_3-связывающего белка (DBP) и его конвертации в макрофаг-активирующий фактор GcMAF-RF. Согласно разработанному регламенту, DBP получали из плазмы крови человека, применяя аффинную колоночную хроматографию, очищали и модифицировали до GcMAF-RF с использованием цитоиммобилизованных гликозидаз (бета-галактозидаза и нейраминидаза). Принадлежность полученного полипептида к Gc-группе глобулинов плазмы крови подтверждали вестерн-блотом с использованием специфических антител. Полученный полипептид по своим молекулярным свойствам соответствует описанному в литературе белку GcMAF, находящемуся на стадии клинических испытаний в США, Британии, Израиле и Японии (Saisei Mirai, Reno Integrative Medical Center, Immuno Biotech Ltd, Efranat, Catalytic Longevity). Биологическую активность препарата GcMAF-RF определяли по индукции у перитонеальных макрофагов мыши фагоцитарной активности и способности продуцировать монооксид азота (NO) *in vitro*. Фагоцитарную активность макрофагов оценивали по эффективности захвата магнитных шариков. Степень активации макрофагов рассчитывали по отношению числа захваченных шариков к общему числу макрофагов. Уровень продукции NO оценивали по накоплению монооксида азота в культуральных супернатантах перитонеальных макрофагов колориметрическим методом с использованием реактива Грисса. Показано, что GcMAF-RF кратно увеличивает фагоцитарную активность макрофагов и достоверно увеличивает продукцию ими монооксида азота. Выделенный оригинальным способом активатор макрофагов GcMAF-RF по своим характеристикам (согласно материалам, опубликованным в печати) соответствует препаратам GcMAF, представляемым на рынке зарубежными компаниями, и может рассматриваться как новый отечественный биологически активный препарат с широким спектром действия. Наибольший интерес вызывает его способность через активацию макрофагов усиливать адаптивный иммунитет организма. В этой связи предполагаются два направления терапевтического применения препарата GcMAF-RF. Препарат может быть востребован в области лечения онкологических заболеваний и, кроме того, может быть использован при лечении ряда нейродегенеративных патологий и иммунодефицитных состояний.

Ключевые слова: макрофаг-активирующий фактор (GcMAF); витамин D_3-связывающий белок (DBP); фагоцитоз; монооксид азота (NO); перитонеальные макрофаги.

Для цитирования: Левитес Е.В., Кирикович С.С., Долгова Е.В., Проскурина А.С., Риттер Г.С., Останин А.А., Черных Е.Р., Богачев С.С. Оценка *in vitro* биологической активности отечественного препарата макрофаг-активирующего фактора (GcMAF-RF). Вавиловский журнал генетики и селекции. 2020;24(3):284-291. DOI 10.18699/VJ20.621

In vitro assay of biological activity of a national preparation of macrophage activating factor (GcMAF-RF)

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Abstract. The article reports an original method for producing vitamin D_3-binding protein (DBP) and its conversion into macrophage-activating factor GcMAF-RF. According to an original protocol, DBPs were obtained from human blood plasma using affinity chromatography, purified and modified to GcMAF-RF using cytoimmobilized glycosidases (beta-galactosidase and neuraminidase). The presence of the polypeptide obtained in the Gc group of blood plasma globulins was confirmed by Western blot using specific antibodies. The molecular properties of this polypeptide put it in correspondence with the GcMAF protein described in the literature, which is undergoing clinical trials in the USA, Britain, Israel and Japan (at Saisei Mirai; Reno Integrative Medical Center; Immuno Biotech Ltd; Efranat; and Catalytic

Е.В. Левитес и С.С. Кирикович внесли равный вклад в работу

Longevity). The biological activity of the GcMAF-RF preparation was detected by the induction of phagocytic activity of macrophages and their ability to produce nitrogen monoxide (NO) *in vitro*. The phagocytic activity of macrophages was evaluated by their ability to uptake magnetic beads. The degree of activation of macrophages was calculated by the ratio of trapped beads to the total number of macrophages. The level of NO production was estimated by the accumulation of nitrogen monoxide in the culture supernatants of peritoneal macrophages by the colorimetric method using the Griess reagent. It was shown that GcMAF-RF multiplies the phagocytic activity of macrophages and significantly increases their production of nitrogen monoxide. The macrophage activator GcMAF-RF, according to its characteristics, corresponds to similar preparations which are made available to the market by foreign companies, and can be considered as a new biologically active preparation with a wide spectrum of action. Of greatest interest is its ability – through the activation of macrophages – to enhance the adaptive immunity. In this regard, two areas of therapeutic use of the GcMAF-RF are proposed. The preparation will be in demand in the field of cancer treatment, and, in addition, it can be used in the treatment of a number of neurodegenerative pathologies.
Key words: Gc protein-derived macrophage activating factor (GcMAF); vitamin D_3-binding protein (DBP); phagocytosis; nitrogen monoxide (NO); peritoneal macrophages.

For citation: Levites E.V., Kirikovich S.S., Dolgova E.V., Proskurina A.S., Ritter G.S., Ostanin A.A., Chernykh E.R., Bogachev S.S. *In vitro* assay of biological activity of a national preparation of macrophage activating factor (GcMAF-RF). Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):284-291. DOI 10.18699/VJ20.621 (in Russian)

Введение

GcMAF (group-specific component protein-derived macrophage activating factor), важный компонент системы активации макрофагов, образуется в результате сайт-специфического дегликозилирования витамин D_3-связывающего белка (DBP), который присутствует в плазме крови человека в большом количестве (300–600 мг/л) (Malik et al., 2013; Delanghe et al., 2015). Нативный DBP содержит один трисахарид, ковалентно связанный с треонином в позиции 420 и состоящий из *N*-ацетилгалактозамина (GalNAc) с присоединенными к нему галактозой и сиаловой кислотой. Преобразование DBP в GcMAF происходит путем отсоединения от GalNAc галактозы и сиаловой кислоты под действием β-галактозидазы и сиалидазы, локализованных на клеточных мембранах активированных B- и T-лимфоцитов соответственно (Yamamoto, Homma, 1991; Yamamoto, Kumashiro, 1993). В результате такого селективного дегликозилирования образуется активный белок GcMAF. Считается, что именно GalNAc, входящий в состав активного центра GcMAF, обеспечивает активацию макрофагов (Naraparaju, Yamamoto, 1994; Mohamad et al., 2002; Saburi et al., 2017a, b).

Исследовательский и практический интерес к GcMAF определяется его способностью через активацию макрофагов участвовать в защитных реакциях организма: в защите от патогенов, в элиминации стареющих, опухолевых и поврежденных клеток, а также в процессах заживления. Широта биологических эффектов макрофагов, имеющих в ряде случаев оппозитную направленность, обеспечивается высокой функциональной гетерогенностью макрофагов (Gordon, 2003; Cassetta et al., 2011). Наиболее четко выделяются два субтипа макрофагов, которые обозначаются как M1- и M2-клетки с про- и противовоспалительной активностью соответственно.

M1-макрофаги играют важную роль в элиминации опухолевых клеток. Они способны проявлять цитотоксическую, микробицидную и антипролиферативную активности, опосредованные продукцией активных метаболитов кислорода (например, H_2O_2), монооксида азота (NO) и провоспалительных цитокинов. M2-макрофаги, напротив, проявляя противовоспалительную активность, ограничивают воспалительный/иммунный ответ. Повышенная активность M2-макрофагов сопряжена с развитием иммуносупрессии, приводящей к опухолевому росту (Lamagna et al., 2006; Sica, Bronte, 2007; Murray, Wynn, 2011).

Установлено, что функциональный тип макрофагов во многом определяется условиями их активации и инактивации (Korbelik et al., 1998; Mosser, 2003; Saburi et al., 2017a, b). Одним из факторов, препятствующих активации макрофагов, является ингибирование продукции GcMAF, осуществляемое ферментом нагалазой (α-*N*-ацетилгалактозаминидазой), секретируемым опухолевыми клетками (Korbelik et al., 1998; Rehder et al., 2009; Saburi et al., 2017a, b). Сывороточная нагалаза у больных раком способна полностью дегликозилировать предшественник MAF (DBP), осуществляя гидролиз по GalNAc-остатку. Лишенный активного сайта, полипептид теряет способность активировать инфильтрующие опухоль макрофаги, что в клинических наблюдениях характеризуется как иммуносупрессия, связанная с потерей макрофагами специфических активностей (Yamamoto et al., 1996; Mohamad et al., 2002; Matsuura et al., 2004; Thyer et al., 2013a). У здоровых людей уровень нагалазы в несколько раз ниже, чем у онкологических больных, и нагалаза в отсутствие патологии не дегликозилирует трисахарид DBP (Ioannou et al., 1992; Nagasawa et al., 2005).

В пионерных работах N. Yamamoto (Yamamoto, Homma, 1991; Yamamoto, Kumashiro, 1993; Yamamoto et al., 1996) было сделано предположение, что инъекции очищенного экзогенного GcMAF могут компенсировать дефектный фактор, активировать систему макрофагов и их противораковую активность. Проведенные клинические исследования свидетельствовали об эффективном воздействии GcMAF на опухоль, приводящем к значительной редукции опухолевого очага или полному уходу опухоли с продолжительным (несколько лет) безрецидивным периодом (Yamamoto et al., 2008; Rehder et al., 2009; Inui et al., 2013; Thyer et al., 2013a, b).

Со времени опубликования N. Yamamoto своих результатов относительно принадлежности выделенного полипептида к групп-специфическому активатору макрофагов прошло более 15 лет. Исследованием полипептида в на-

правлении поиска мишеней его клинического применения занимались разные лаборатории. Получены многочисленные, противоречивые данные о его функциональных возможностях в качестве активатора иммунных реакций при лечении злокачественных новообразований, аутизма, различных нарушений в работе иммунной системы. Противоречивые результаты, касающиеся эффективности клинических возможностей GcMAF, вызвали немалую долю скептицизма в научном сообществе (Rehder et al., 2009; Ugarte et al., 2014; Borges, Rehder, 2016; Ruggiero et al., 2016). Такое состояние вопроса связано еще и с тем, что препарат невозможно сделать предметом промышленной собственности, а можно только патентовать различные способы его получения и различные композиции, в составе которых он может применяется. Именно по такому пути идут все производители GcMAF (Saisei Mirai, Reno Integrative Medical Center, Immuno Biotech Ltd, Efranat, Catalytic Longevity). Тем не менее о перспективности возможного практического использования препарата GcMAF свидетельствуют многочисленные данные, полученные на экспериментальных животных, а также данные доклинических исследований и накопленный положительный опыт его клинического применения (Korbelik et al., 1997; Kisker et al., 2003; Yamamoto et al., 2008; Toyohara et al., 2011; Pacini et al., 2012; Inui et al., 2013, 2016a, b; Kuchiike et al., 2013; Thyer et al., 2013a, b; Klokol, Teppone, 2016; Saburi et al., 2017a, b; Moya et al., 2018; Păduraru et al., 2019; Greilberger, Herwig, 2020).

Проведенный нами анализ литературных источников продемонстрировал большой интерес к препарату GcMAF в мире, несмотря на имеющиеся различные точки зрения (Останин и др., 2019), и определил направление его исследования в нашей лаборатории. Поскольку практически во всех исследованиях процедура получения и активации препарата переписывается с одной-двух пионерных работ (Link et al., 1986; Yamamoto, Homma, 1991; Yamamoto, Kumashiro, 1993), что связано с его коммерческой перспективностью и нежеланием раскрывать детали выделения больших количеств активатора, мы решили разработать независимый регламент получения и активации фактора и экспериментально определить его возможную «клиническую мишень».

В настоящей работе, представляющей собой первую статью цикла из трех статей, описывающих действие препарата на экспериментальные биологические системы, оценивается способность полученного оригинальным способом препарата GcMAF-RF (GcMAF-Related Factor) активировать фагоцитарную функцию макрофагов и продуцировать монооксид азота *in vitro*.

Оригинальный способ выделения DBP и процедура его конвертации в GcMAF-RF цитоэнзиматическим способом были разработаны в ООО «Активатор MAF» совместно с лабораторией индуцированных клеточных процессов Федерального исследовательского центра Институт цитологии и генетики (ФИЦ ИЦиГ) СО РАН. Способ получения препарата GcMAF-RF, в связи с его статусом «предмета промышленной собственности» компании ООО «Активатор MAF», охарактеризован здесь без указания деталей процедур. Тем не менее общая характеристика способа, представленная в разделе «Результаты», дает достаточно полную информацию, демонстрирующую оригинальность подхода. По своим молекулярным свойствам полученный полипептид соответствует описанному в литературе белку GcMAF, находящемуся на стадии клинических испытаний в США, Британии, Израиле и Японии (Saisei Mirai, Reno Integrative Medical Center, Immuno Biotech Ltd, Efranat, Catalytic Longevity).

Материалы и методы

В экспериментах использовали по две-три месячных мыши линии СВА разведения вивария № 2 ФИЦ ИЦиГ СО РАН (стандартное содержание). Перитонеальные макрофаги (5×10^5 клеток/лунку) культивировали в 12-луночных планшетах в среде RPMI-1640 (Biolot), содержащей 10 % FBS (HyClone) и 40 мкг/мл гентамицина в течение 12 ч. Затем адгезивную фракцию макрофагов три раза отмывали забуференным физиологическим раствором (PBS) для удаления неприкрепленных клеток. Полученные макрофаги использовали в дальнейшем для анализа их биологической активности.

Фагоцитарную функцию макрофагов оценивали согласно методике, представленной в работе (Ishikawa et al., 2014). Перитонеальные макрофаги выделяли из брюшной полости двух-трех мышей, объединяли, распределяли по лункам планшета в равном количестве и культивировали в бессывороточной среде RPMI-1640 в течение 2 ч. Затем среду меняли на RPMI-1640, содержащую 10 % FBS в отсутствие (контроль) или в присутствии следующих активаторов (позитивный контроль): липополисахарида (LPS, Sigma, 10 мкг/мл, *E. coli* 0114:B4) либо полученных нами DBP (5 мкг/мл) или GcMAF-RF (5 мкг/мл). В каждую лунку добавляли также магнитные шарики (Dynabeads M-280, Invitrogen) в дозе 60 мкг/лунку. После трехчасовой инкубации макрофаги три раза отмывали PBS для удаления неинтернализованных шариков, затем фотографировали в проходящем свете с использованием инвертированного микроскопа AxioObserver Z1 (Zeiss) и подсчитывали количество интернализованных гранул (IBN). Фагоцитарную активность макрофагов оценивали по формуле: IBN = количество интернализованных шариков/количество макрофагов. Для статистического анализа IBN учитывали данные четырех независимых экспериментов, в каждом эксперименте оценивали 300–500 клеток. Учет клеток был проведен из нескольких полей, расположенных в разных частях лунки планшета.

Продукцию NO определяли в пяти повторностях на семи мышах по накоплению нитритов после 3 ч инкубирования с активаторами в культуральных супернатантах перитонеальных макрофагов колориметрическим методом с использованием реактива Грисса (Green et al., 1982). Для этого 100 мкл каждого тестируемого супернатанта переносили в 96-луночный планшет, смешивали с равным объемом реактива Грисса и инкубировали при комнатной температуре в течение 15 мин. Оптическую плотность оценивали на многоканальном спектрофотометре при длине волны 540 нм. Результаты соотносили со стандартной калибровочной кривой, полученной на основе серийных разведений 3 мМ раствора нитрита натрия.

Статистический анализ проводили с использованием программного обеспечения Statistica 10. В каждом экс-

перименте было выполнено минимум четыре повторения. Существование статистически значимых различий между исследуемыми группами проанализировано при помощи критерия Краскела–Уоллиса. Для апостериорных сравнений между группами использовали U-критерий Манна–Уитни с учетом поправки Бонферрони (минимальный уровень значимости $p = 0.05$/число сравнений). Таким образом, в случае анализа IBN различия считали достоверными при уровне значимости $p < 0.017$ (три попарных сравнения), а в случае анализа продукции NO – при $p < 0.013$ (четыре попарных сравнения) (Гржибовский, 2008).

Результаты

Как сказано выше, витамин D_3-связывающий белок содержит три функциональных сайта: витамин D_3-связывающий домен, актин-связывающий сегмент полипептидной цепи и сайт гликозилирования. Соответственно, существуют два очевидных способа аффинного выделения специфического белка, за актин- и витамин D_3-связывающие домены (Haddad et al., 1984; Link et al., 1986; Swamy, Ray, 1995). В исследованиях других авторов описывается практически всегда один и тот же способ получения активатора макрофагов GcMAF. Витамин D_3 модифицируется в производную молекулу, содержащую гидроксил в положении 25, или химическим, или энзиматическим способом. Модифицированный витамин «пришивается» к активированной бромцианом сефарозе, и проводится аффинная хроматография. Далее белок активируется в GcMAF энзиматической конвертацией двумя гидролазами – салидазой и β-галактозидазой, ковалентно фиксированными на носителе (Yamamoto, Kumashiro, 1993; Yamamoto, 1996; Mohamad et al., 2002). Функциональная активность полученного GcMAF тестируется по его способности индуцировать у макрофагов способность фагоцитировать разнообразные внеклеточные частицы. Главным образом используются опсонированные эритроциты барана (Hammarstrom, Kabat, 1971; Yamamoto, Kumashiro, 1993). Наши многочисленные попытки выделить, активировать и оценить функциональную активность полученного полипептида способами, описанными в статьях, не увенчались успехом. Основными причинами неудач были: невозможность в достаточном количестве быстро получить составляющие компоненты всех сложных процедур, их высокая цена, постоянное утаивание авторами опубликованных работ принципиальных технических деталей той или иной процедуры.

В этой связи после тотальной проработки принципов получения GcMAF мы разработали следующий регламент выделения большого количества препарата витамин D_3-связывающего белка, его конвертации в GcMAF-RF и оценки его способности активировать фагоцитарную функцию макрофагов и продукцию ими NO.

Витамин D_3-связывающий белок был выделен из сыворотки крови здоровых доноров с использованием аффинной хроматографии на колонке с ковалентно пришитым актином. Данный подход состоял из двух процедур. Во-первых, актин, необходимый в качестве аффинного лиганда, получали самостоятельно из мышц кролика (Spudich, Watt, 1971), благодаря чему удалось быстро наладить аффинную хроматографию и на порядки сократить затраты. Одновременно из крабовых панцирей был выделен субстрат, необходимый для пришивки аффинного лиганда (de Souza et al., 2008). Этот способ также позволил не опираться на импортные дорогие реактивы и существенно ускорил время получения фактора. Для конвертации DBP в GcMAF-RF был разработан оригинальный способ с использованием активированных лизофосфатидилхолином (Lyso-Pc, Sigma) воспалительных лимфоцитов, получаемых от того же донора (Ngwenya, Yamamoto, 1986; Yamamoto, Homma, 1991; Asaoka et al., 1992). Суть подхода состоит в том, что на цитоплазматической мембране активированных к воспалению B- и T-лимфоцитов присутствуют оба необходимых для конвертации DBP в GcMAF фермента: β-галактозидаза и салидаза соответственно. Полученные от донора лимфоциты после воспалительной активации добавлялись к DBP. После инкубации полученный GcMAF-RF проверялся на способность активировать специфическую фагоцитарную активность макрофагов. Для этого был валидирован способ с использованием металлических

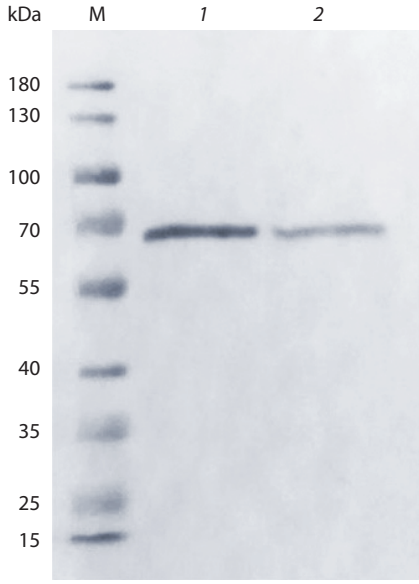

Fig. 1. Western blot analysis of DBP samples obtained by actin and sepharose (with 25-hydroxyvitamin D_3) chromatography.

Lanes: M, molecular marker "The Thermo Scientific™ Page Ruler™ Prestained protein Ladder" (Thermo Fisher Scientific Inc., USA); *1*, DBP obtained on a column with 25-hydroxyvitamin D_3-Sepharose; *2*, DBP obtained by actin-sepharose affinity chromatography.

бус (Ishikawa et al., 2014). Процедура хорошо стандартизируется, не требует многостадийного получения опсонированных эритроцитов барана и высокотехнологична. Дополнительно для оценки специфичности выделенного GcMAF-RF был разработан оригинальный метод получения лектина, позволяющий оценить эффективность отщепления хвостов сахаров и сохранения в сайте гликозилирования *N*-ацетилгалакозамина, который является основной молекулой, участвующей в активации макрофагов.

Для проверки соответствия DBP, выделенного с использованием в качестве аффинного лиганда актина, препарату DBP, выделяемому аффинно на 25-гидроксивитамин D_3-сефарозе, и характеристики принадлежности обоих полипептидов к группе специфических Gc-белков был проведен сравнительный вестерн-блот анализ. Прямой сравнительный вестерн-блот анализ образцов препарата с антителами против Gc-группы свидетельствует об идентичности упомянутых двух вариантов белков (рис. 1).

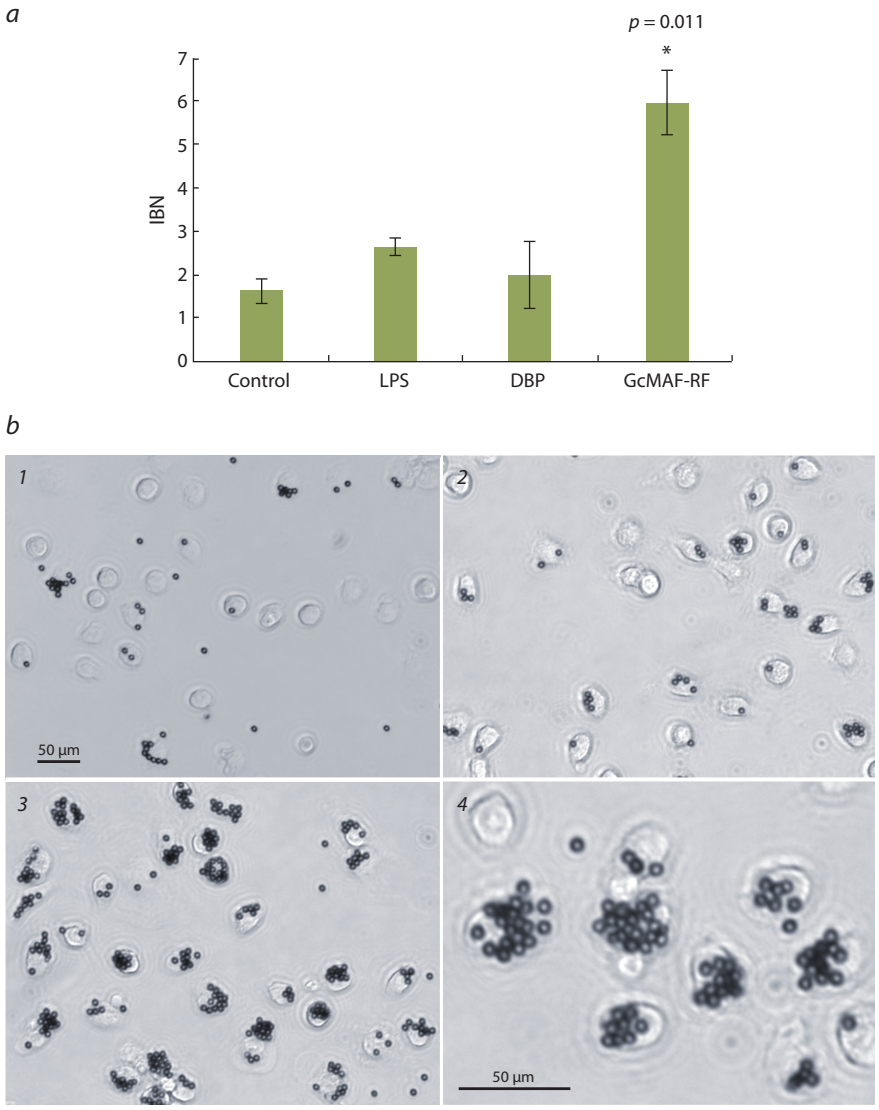

Fig. 2. Effect of GcMAF-RF on the phagocytic activity of peritoneal macrophages.

a, data are presented as M ± SEM ($n = 4$), * $p < 0.017$ – significance of differences compared with control (Bonferroni-corrected Mann–Whitney U-test); *b*, representative photographs of macrophages with internalized granules: *1*, control; *2*, after activation by DBP; *3*, *4*, after activation by GcMAF-RF.

После получения, конвертации и проверки специфической активности, способности активировать фагоцитарную активность макрофагов, препарат GcMAF-RF стерилизовали фильтрованием, а затем или замораживали и хранили при –70 °С, или лиофилизировали (см. рис. 1).

Биологическую активность GcMAF-RF оценивали по его влиянию на фагоцитарную функцию перитонеальных макрофагов (Ishikawa et al., 2014) (рис. 2). Было выполнено три апостериорных сравнения экспериментальных групп с контролем с использованием U-критерия Манна–Уитни с учетом поправки Бонферрони. Показано, что по сравнению с контролем только препарат GcMAF-RF статистически значимо усиливает способность макрофагов интернализировать магнитные шарики. В присутствии GcMAF-RF фагоцитарная активность макрофагов увеличивалась в 3.7 раза ($p = 0.011$), тогда как в ответ на DBP или ЛПС – в 1.2 и 1.6 раза соответственно. Репрезентативные фотографии макрофагов (см. рис. 2, *б*) четко демонстрируют, что при стимуляции препаратом GcMAF-RF (но не его предшественником DBP) в общей популяции перитонеальных макрофагов значительно возрастает число клеток с интернализованными магнитными шариками. В данном тесте оценивали биологическую активность образцов из каждой полученной партии препарата, при этом различные образцы GcMAF-RF демонстрировали трех-семикратное увеличение фагоцитарной функции макрофагов.

Препарат GcMAF-RF усиливал не только фагоцитарную активность перитонеальных макрофагов, но также их способность продуцировать NO (рис. 3). Было проведено четыре апостериорных сравнения (экспериментальные группы с контролем и ЛПС и GcMAF-RF между собой) с использованием U-критерия Манна–Уитни с учетом поправки Бонферрони. Оказалось, что GcMAF-RF (но не его предшественник DBP) статистически значимо ($p < 0.013$) повышал уровень продукции монооксида азота, при этом уровень индуцированной NO-продукции был даже выше, чем в ответ на стандартный активатор макрофагов ЛПС ($p = 0.008$).

Все перечисленные процедуры, валидирующие препарат как активатор макрофагов (GcMAF-RF), постоянно проводятся для характеристики каждого нового выделения препарата и доведения до состояния технологии, которая готовится к сертификации.

Обсуждение

В настоящем исследовании проведена оценка биологической активности первого отечественного препарата GcMAF-RF, который был выделен из плазмы крови человека в соответствии с новым технологическим регламентом, разработанным компанией ООО «Активатор MAF». Прямой сравнительный вестерн-блот анализ образцов препарата с антителами против Gc-группы показал (см. рис. 1), что молекулярная масса белков, выделенных двумя вариантами аффинной хроматографии, соответствует размеру GcMAF в 65–67 кДа, определенному другими авторами в аналогичной трис-глициновой электрофоретической системе (Smith et al., 2013). Полученный результат свидетельствует об идентичности полипептидов, выделяемых с использованием двух имеющихся в молекуле DBP доменов, различающихся по своей функциональной специфичности (актин-связывающий и витамин D_3-связывающий).

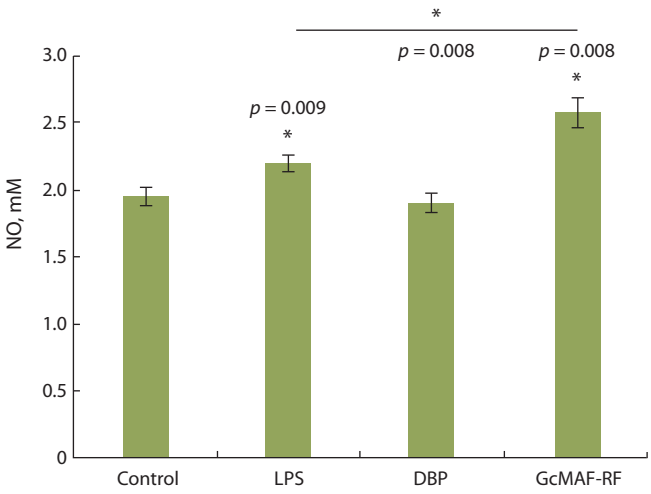

Fig. 3. Effect of the drug GcMAF-RF on the production of NO.

Data are presented as M ± SEM (n = 4). * Differences from the control and between the LPS and GcMAF-RF groups are significant at $p < 0.013$ (Bonferroni-corrected Mann–Whitney U-test).

В витральных тестах образцы GcMAF-RF из каждой полученной партии препарата проявляли свойства, характерные для макрофаг-активирующего фактора, а именно: кратно усиливали фагоцитарную функцию перитонеальных макрофагов мыши (см. рис. 2), а также статистически значимо стимулировали продукцию NO (см. рис. 3).

Выявленные в нашем исследовании факты стимулирующего влияния полученного препарата GcMAF-RF на свойства макрофагов согласуются с данными целого ряда исследователей (Mohamad et al., 2002; Thyer et al., 2013b; Ishikawa et al., 2014; Ruggiero et al., 2014; Saburi et al., 2017a, b). Это позволяет заключить, что препарат GcMAF-RF соответствует известным импортным аналогам не только по своим физико-химическим характеристикам, но и по проявляемой биологической активности.

Следующие два сообщения будут содержать результаты по влиянию препарата GcMAF-RF на культуру дендритных клеток и поляризацию М2-макрофагов. Также будет продемонстрирована способность активированных препаратом GcMAF-RF перитонеальных макрофагов лизировать клетки нескольких опухолевых культур и оценена его противораковая активность в биологических тестах на экспериментальных животных.

Заключение

Представлены первые экспериментальные данные, характеризующие способность препарата GcMAF-RF, полученного оригинальным способом, активировать перитонеальные макрофаги мыши, что проявляется кратным усилением их фагоцитарной функции и значимым повышением уровня продукции монооксида азота. Выделенный активатор макрофагов (GcMAF-RF) по своим характеристикам соответствует аналогичным препаратам, представляемым на рынке зарубежными компаниями, и может рассматриваться как новый отечественный биологически активный фактор разнонаправленного действия.

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Acknowledgements. The authors are grateful to Head of the Shared Access Center "Cell technologies", Institute of Cytology and Genetics, Novosibirsk, for access to the microscope. This work was supported by the companies Activator MAF and BA Pharma and by State Budgeted Project 0324-2019-0042 (registration ID AAAA-A17-117071240065-4).

Conflicts of interests. The co-authors of the paper A.S. Proskurina and S.S. Bogachev are directors of Activator MAF and BA Pharma, respectively.

Received December 10, 2019. Revised March 23, 2020. Accepted April 1, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):292-298
DOI 10.18699/VJ20.45-o

Ethnicity-specific distribution of *TRPM8* gene variants in Eurasian populations: signs of selection

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Abstract. The *TRPM8* gene encodes the ion channel, which is a cold receptor in afferent neurons of the mammalian somatosensory system. We studied the frequency of haplotype distribution from six SNPs in the *TRPM8* gene in Eurasian human populations, including Russians, Kazakhs and Chukchi. Four of the six SNPs are located in exon 7 (rs13004520, rs28901637, rs11562975, rs17868387), rs7593557 is in exon 11. These exons encode parts of the N-terminus, which is necessary for channel functioning in the plasma membrane of neurons. The rs11563071 is in exon 23 encoding part of the C-terminus. The primary difference in population distribution of haplotypes determines the SNP from exon 11 which leads to Ser419Asn substitution in protein. The most pronounced differences in the patterns of diversity and frequencies of haplotypes were observed between Chukchi and Russians. The frequency of major H1 haplotype encompassing the 419Ser gene variant differs in examined populations; 0.738 (Russians), 0.507 (Kazakhs) and 0.337 (Chukchi), $p < 0.001$. The *TRPM8* gene variants encoding 419Asn and carrying the minor alleles of rs28901637 (P249P) and rs11562975 (L250L) in exon 7 are characteristic of Asian populations. The frequency of all 419Asn variants in Chukchi is comparable to that in Africans, however, the minor allele frequencies of rs28901637, rs11562975 in Africans is low. Apparently in the process of human colonization of Eurasia, minor alleles of these SNPs diverged depending on rs7593557 structure in exon 11. We analyzed sequences of five *TRPM8* mRNA isoforms extracted by researchers from different tissues. Sequence analysis demonstrates that they are transcribed from major H1 variant of the *TRPM8* gene but contain different translation start codons, which are generated by alternative splicing from pro-mRNA.
Key words: *TRPM8* gene; haplotypes; Eurasian human populations; alternative start codons.

For citation: Potapova T.A., Romashchenko A.G., Yudin N.S., Voevoda M.I. Ethnicity-specific distribution of *TRPM8* gene variants in Eurasian populations: signs of selection. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):292-298. DOI 10.18699/VJ20.45-o

Этно-специфическое распределение вариантов гена *TRPM8* в евразийских популяциях: знаки отбора

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Аннотация. Ген *TRPM8* кодирует ионный канал, который является холодовым рецептором в афферентных нейронах соматосенсорной системы млекопитающих. Мы изучили распределение частот гаплотипов из шести ОНП гена *TRPM8* в евразийских популяциях человека, включая русских, казахов и чукчей. Четыре из шести ОНП расположены в экзоне 7 (rs13004520, rs28901637, rs11562975, rs17868387), ОНП rs7593557 находится в экзоне 11. Эти экзоны кодируют фрагменты N-терминального домена, необходимого для функционирования канала в плазматической мембране афферентных нейронов. ОНП rs11563071 расположен в экзоне 23, кодирующем фрагмент С-терминального домена канала. Основное различие в популяционном распределении гаплотипов определяет ОНП из экзона 11, обусловливающий Ser419Asn замещение в белке. Контрастные различия в многообразии и частотах гаплотипов наблюдали между популяциями чукчей и русских. Частоты основного гаплотипа H1, относящегося к 419Ser вариантам гена *TRPM8*, существенно различались в изученных популяциях: 0.738 у русских, 0.507 у казахов, 0.337 у чукчей ($p < 0.001$). Для азиатских популяций характерны варианты гена *TRPM8*, кодирующие 419Asn и содержащие минорные аллели ОНП rs28901637 (P249P) и rs11562975 (L250L) в экзоне 7. Суммарная частота таких гаплотипов у русских составляет 0.032, по сравнению с 0.142 у казахов и 0.358 у чукчей ($p < 10^{-3}$ для обоих сравнений). Частота всех 419Asn вариантов у чукчей сопоставима с таковой африканцев, однако частота минорных аллелей rs28901637 и rs11562975 у африканцев низкая. По-видимому, в процессе колонизации человеком Евразии минорные аллели этих ОНП дивергировали в зависимости от структуры rs7593557 в экзоне 11. Нами проанализированы

последовательности пяти изоформ мРНК гена *TRPM8*, выделенных исследователями из разных тканей. Показано, что они транскрибированы с основного варианта H1 гена *TRPM8*, но содержат различные старт-кодоны трансляции, генерированные альтернативным сплайсингом про-мРНК.
Ключевые слова: ген *TRPM8*; гаплотипы; евразийские популяции человека; альтернативные старт-кодоны.

Introduction

The gene *TRPM8* encodes a subunit of Ca^{2+}-permeable non-selective cation channel, belonging to the TRPM (transient receptor potential melastatin) subfamily of TRP domain-containing proteins (Tsavaler et al., 2001). TRP channels are formed by oligomerization of subunits sharing common structural features, including six putative transmembrane segments (S1–S6), a pore loop linking segments S5 and S6, and cytoplasmic N- and C-terminal (Ramsey et al., 2006). The majority of TRP proteins carry a conserved TRP box ('VWKFQR' in TRPM channels) in the C-terminal domain, adjacent to the S6 segment. Many of these proteins, including TRPM8, are involved in Ca^{2+} homeostasis in response to extracellular and intracellular physical and chemical factors. TRPM8 expression has been observed in somatic afferent neurons, myocytes, epithelial cells of the lung, bronchi, prostate, bladder and others (Sabnis et al., 2008; Babes et al., 2011). The modulation of TRPM8 protein activity is coupled with basic biochemical and physiological processes related to thermal sensitivity, proliferation, and apoptosis (Zhang, Barritt, 2006; Yee, 2015).

Three types of TRPM8 polypeptides potentially able to form Ca^{2+} channels by tetramerization have been described. The full-length variant of 1104 amino acids (aa) identified in sensory neurons contain the long N-terminal sequence (693 aa), six transmembrane segments and C-terminal domain with TRP box (Latorre et al., 2011). This channel is able to respond to changes in ambient temperature (threshold of ~22–34 °C). Another TRPM8 isoform of 1054 aa, with a rearranged N-terminal domain, was identified in prostate tumor cells (Lis et al., 2005). In addition, a truncated variant (304 aa), lacking the entire N-terminal sequence and the first two transmembrane segments (S1 and S2), but retaining parts of the voltage-dependent sensory module (S3 and S4), the pore-forming components (S5 and S6 and the loop between them), and the C-terminal domain, was identified in human epithelial cells of the bronchi and lung (Sabnis et al., 2008). Full-length TRPM8 is located to the plasma membrane, while the truncated variant is located in the endoplasmic reticulum membrane and is associated with the release of Ca^{2+} ions from intracellular stores (Bidaux et al., 2007).

Studies clarifying the contribution of the N-terminal domain to the TRPM8 channel function have shown that the first 40 amino acids of full-length TRPM8 modulate sensitivity of the protein to cold and menthol, and the region between residues 40 and 60 is involved in trafficking of the protein to the plasma membrane (Phelps, Gaudet, 2007; Bidaux et al., 2012; Pertusa et al., 2014). It is also an essential element in ensuring the proper folding and assembly of TRPM8.

In this study, we evaluated the distribution of the alleles at five SNPs located in *TRPM8* gene exons 7 and 11 in geographically dispersed Eurasian human populations in order to clear up the potential importance of the N-domain parts encoded by these modifiable exons. The composition and localization of the SNPs assayed in the *TRPM8* gene are unique. Four of six SNPs are densely clustered in exon 7; three in successive codons (encoding P249P, L250L, and Y251C), and the fourth in the codon but one upstream (encoding R247T). The SNP (S419N) in exon 11 and two SNPs (R247T and Y251C) from exon 7 can potentially influence on function of the TRPM8 channel by altering the protein structure and accessibility of these sites to post-translational modification. The two SNPs (P249P and L250L) in exon 7 and the SNP (encoding V1058V in the C-terminal domain) in exon 23 cannot directly influence on protein structure. However, recurrent emergence of the minor alleles of these SNPs in different *TRPM8* gene variants suggests that they may have influence on gene expression regulation.

Materials and methods

Human populations that have resided in geographically dispersed territories in Eurasia were examined, including Russians ($N = 170$, Novosibirsk), Kazakhs ($N = 119$, Kosh-Agach district, the Altai Republic) and Tundra Chukchi ($N = 80$, Kanchalan settlement, Chukotka Autonomous district). Ethnicity of individuals was determined by special questioning with elucidation of a nationality of the ancestors (at least in three generations). Blood samples were collected from unrelated representatives of the ethnic group. All subjects gave their informed consent for participation in the experiment. The work was approved by the Bioethical Committee of the Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences.

The general characteristics of the assayed SNPs are listed in Table 1. The information about variants and reference sequences (NM_024080.4 and BC143819.1) were extracted from dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and GenBank (https://www.ncbi.nih.gov/genbank/).

Genomic DNA from blood samples was isolated by phenol-chloroform extraction. DNA was genotyped by polymerase chain reaction. The details of the assay (primers and PCR parameters) developed for genotyping were described previously (Potapova et al., 2014). Primers for rs17868387 fragment

Table 1. *TRPM8* single nucleotide polymorphisms included in this study

SNP	Exon	Nucleotide substitution	Amino acid position in protein
rs13004520	7	G>C	R247T
rs28901637	7	A>T	P249P
rs11562975	7	G>C	L250L
rs17868387	7	A>G	Y251C
rs7593557	11	G>A	S419N
rs11563071	23	C>G	V1058V

Note. Amino acid positions are given to the full-length protein from neurons.

(200 nucleotides) was following: 5′-tgtgtggttgttgttcaggagat-3′ (A allele), 5′-tgtgtggttgttgttcaggagac-3′ (G allele), 5′-aaagctg ggaggaggaataatgt-3′ (total). The annealing temperature of the primers was 60 °C.

Haplotype variants, coefficients of linkage disequilibrium (D′) between the alleles of SNPs, fixation index F_{st} were determined using Arlequin ver. 3.5.2. The F_{st} was calculated on the basis of haplotype distributions. The level of significance (p_{χ^2}) of the differences between haplotype groups was assessed using SPSS 11.5 software.

Results

The distribution of haplotypes at six SNPs within the *TRPM8* gene in individuals from Russian (European), Kazakh (Central Asians) and Chukchi (Eastern Asians) populations, who have been resident in different climatic and geographical zones of Eurasia, was examined in this study. Twenty two haplotypes with frequencies exceeding 0.001 at least in one of the three populations were identified (Table 2).

The haplotypes can be categorized into two main groups, according to nucleotide substitutions of the rs7593557 SNP (exon 11) at position 5 in each of population samples. The G allele of this SNP leads to formation of the 419Ser codon in the mRNA. The 419Ser group includes the major H1 haplotype and H2–H5 with singleton substitutions at one of the five positions relative to the H1. The H6–H9 haplotypes differ by the allele combinations of the SNPs (P249P, L250L, V1058V) at positions 2, 3 and 6.

The founder haplotype H10 and its derivatives (H11–H22) carry the A allele (rs7593557, exon 11) at position 5 forming the 419Asn codon. The haplotypes constituting this group have additional substitutions at one or several positions. The H11–H17 subgroup includes haplotypes with minor allele combinations of rs28901637, rs11562975 and rs11563071. The H18–H22 haplotypes contain a substitution at position 4 (rs17868387, exon 7), which leads to a Tyr→Cys replacement. Presumably, this is a recurrent mutation, unrelated to haplotype H4, and variant H18 is the founder haplotype for H19–H22 subgroup. In additional, this haplotypes contain a substitution at position 1 (rs13004520, exon 7), leading to an Arg→Thr replacement. Thus, 419Asn H19-H22 haplotypes contain three substitutions that can affect the TRPM8 protein structure. The linkage disequilibrium (LD) between minor alleles of rs13004520 and rs17868387 SNPs was observed in all three populations (D′ = 1). The minor alleles of this SNPs was also linked to rs7593557 A allele from exon 11 (D′ = 1). In Kazakhs the rs17868387 G allele was observed in combination with both rs7593557 alleles (D′ = 0.9104). The presence of the nucleotide substitutions at positions 2, 3, and 6 (rs28901637, rs11562975, rs11563071, respectively) in different haplotype subgroups (H6–H9, H10–H17 and H19–H22) suggests that their appearance could be repeatedly.

Table 2. *TRPM8* haplotypes in Russian, Kazakh, and Chukchi populations

Haplotype designation	Haplotype structure[a]	Haplotype frequencies		
		Russians (*N* = 340)	Kazakhs (*N* = 238)	Chukchi (*N* = 160)
H1	GAGAGC	0.738 ± 0.024	0.508 ± 0.032	0.337 ± 0.037
H2	G**T**GAGC	0.006 ± 0.004	0.038 ± 0.012	0.025 ± 0.012
H3	GA**C**AGC	0.085 ± 0.015	0.097 ± 0.019	0.056 ± 0.018
H4	GAG**G**GC	0	0.004 ± 0.004	0
H5	GAGAG**G**	0.062 ± 0.013	0.021 ± 0.009	0.006 ± 0.006
H6	G**TC**AGC	0	0.004 ± 0.004	0.012 ± 0.009
H7	G**T**GAG**G**	0	0.008 ± 0.006	0
H8	GA**C**AG**G**	0.018 ± 0.007	0.050 ± 0.014	0.031 ± 0.014
H9	G**TC**AG**G**	0	0.013 ± 0.007	0
H10	GAGAAC	0.018 ± 0.007	0.064 ± 0.016	0.119 ± 0.026
H11	G**T**GAAC	0.011 ± 0.006	0.050 ± 0.014	0.113 ± 0.025
H12	GA**C**AAC	0.003 ± 0.003	0.021 ± 0.009	0.063 ± 0.019
H13	G**TC**AAC	0.006 ± 0.004	0.008 ± 0.006	0.062 ± 0.019
H14	GA**C**AA**G**	0.003 ± 0.003	0.004 ± 0.004	0.025 ± 0.012
H15	G**T**GAA**G**	0	0.030 ± 0.011	0.050 ± 0.017
H16	G**TC**AA**G**	0	0.017 ± 0.008	0.044 ± 0.016
H17	GAGAA**G**	0	0.004 ± 0.004	0.025 ± 0.012
H18	GAG**G**AC	0	0.004 ± 0.004	0
H19	**C**AG**G**AC	0.026 ± 0.009	0.043 ± 0.013	0.019 ± 0.011
H20	**C**A**C**G**A**C	0.006 ± 0.004	0.008 ± 0.006	0
H21	**CTGGAG**	0.003 ± 0.003	0.004 ± 0.004	0
H22	**C**AG**G**AG	0.015 ± 0.006	0	0.012 ± 0.009

Notes. [a]The SNP numeration in haplotypes are as follows: 1 – rs13004520: G>C, (R247T); 2 – rs28901637: A>T, (P249P); 3 – rs11562975: G>C, (L250L); 4 – rs17868387: A>G, (Y251C); 5 – rs7593557: G>A, (S419N); 6 – rs11563071: C>G, (V1058V). *N* – number of chromosomes.

Table 3. Frequencies of *TRPM8* haplotype groups in Russian, Kazakh and Chukchi populations and significance of the inter-population differences

Haplotype group	Frequency			P_{χ^2}		
	Russians[1]	Kazakhs[2]	Chukchi[3]	1–2	1–3	2–3
H1	0.738	0.508	0.337	0.000	0.000	0.001
H2–H5	0.153	0.160	0.087	n	0.044	0.036
H6–H9	0.018	0.075	0.043	0.001	n	n
H10–H17	0.041	0.198	0.501	0.000	0.000	0.000
H18–H22	0.050	0.059	0.031	n	n	n
H11–H16, H20, H21	0.032	0.142	0.357	0.000	0.000	0.000

Note: n – differences are unreliable.

Comparison among the populations revealed considerable differences in the frequencies of the H1 haplotype, with 0.738 in Russians versus 0.508 and 0.337 in Kazakhs and Chukchi, respectively (Table 3). The 419Ser H2–H5 singleton variants, except H3, have its own specific distribution in the examined populations. The H2 haplotype is present at very low frequency in the Russian population. The frequency of the haplotype H5 is higher in Russians in comparison with Asian populations, while haplotype H4 is absent in the Russian and Chukchi populations and present at a very low frequency in Kazakhs (0.004). The total frequency of the H2–H5 singleton variants is lower in Chukchi in comparison with Russian and Kazakh populations. The 419Ser H6-H9 subgroup demonstrates the more haplotype variety and total haplotype frequency (0.075 %) in Kazakhs compared with Russians (0.018).

It is evident that Russians differ drastically from Asian populations in relation to Asn *TRPM8* gene variants, with the exception of H19–H22, in which is observed similar frequencies in both European (0.050) and Asian (0.059 and 0.031) populations. Subgroup H10–H17 is the more heterogeneous (see Table 2) and demonstrates the most pronounced differences between populations in total frequency values: 0.041 in Russians versus 0.198 in Kazakhs, and 0.501 in Chukchi (see Table 3). In the Russian sample some variants in this subgroup are either absent or present with considerably lower frequencies compared to the Chukchi and Kazakh populations. The pattern of haplotype diversity observed in Chukchi is opposite to that in the Russian population; with relatively high frequencies of haplotypes H10 and H11 (0.119 and 0.113 respectively) and similar frequencies among the remaining haplotypes (H12–H17) in the range 0.025–0.063. The presence of minor alleles at positions 2 and 3 in the majority of haplotypes in this subgroup correlates with the relatively higher frequency of these variants in the Asian populations.

The total frequencies of haplotypes containing the minor alleles of the rs28901637 and rs11562975 SNPs (7 exon), which have no direct functional effect on the protein, show characteristic differences among the Russian and Asian populations. The total frequency of haplotypes containing the minor T allele at position 2 (rs28901637) in Russians is very low (0.026), relative to 0.306 and 0.172 in the Chukchi and Kazakh populations, respectively (see Table 2). The Russian population also has a very low frequency of variants containing a substitution at position 3 (rs11562975), except haplotype H3, with a total frequency (without H3) of 0.036, versus 0.125 in Kazakhs and 0.238 in Chukchi ($p < 10^{-3}$ for both comparisons). The total frequencies of 419Asn variants with minor rs28901637 and rs11562975 alleles in Russians was 0.032, compared with 0.142 in Kazakhs and 0.357 in Chukchi (see Table 3).

In addition Kazakh population shows a clear tendency to increase the frequency of H1 haplotype similar to the Russian population, and a decrease in the total frequency of haplotypes belonging to 419Asn subgroup H10–H17 in comparision with Chukchi. Possible, it is a consequence of their common evolutionary history in the territory of Eurasia. The highest inter-population difference observed between Russian and Chukchi populations ($F_{st} = 0.1442$, $p < 10^{-5}$). The estimated F_{st} values generated by comparisons of Russians versus Kazakhs and Kazakhs versus Chukchi were 0.0498 and 0.0242, respectively ($p < 10^{-5}$ for both comparisons).

Discussion

According to the current paradigm, anatomically modern humans have spread out of Africa over 60,000–80,000 years ago, following different routes to colonize Europe and Asia (Stoneking, Delfin, 2010; Stewart, Stringer, 2012). Respectively, to assist in interpretation of our results, we considered additional data describing the MAFs distribution of the six SNPs within the *TRPM8* gene included in this study in African, European, Chinese, and Japanese populations, extracted from the dbSNP (Table 4).

This analysis produced the following unambiguous inferences: (1) haplotypes comprising the rs7593557 A allele (exon 11) are widely distributed among Africans and Eastern Asians (Chinese, Japanese, and Chukchi), in comparison with Russians and Europeans. (2) There is absent the rs11562975 minor C allele (exon 7) in the African population and it's present at frequencies of 0.170–0.300 in Asian haplotypes. A similar situation is observed for the rs28901637 T allele (exon 7), which occurs at a relatively low frequency in African and European populations and at higher frequencies (0.142–0.306) in Asians. (3) No pronounced continental or ethnic specificity in the distribution of the haplotypes containing the rs11563071 minor G allele (exon 23) in the examined populations was detected. The exception is the Japanese population, in which the MAF at this SNP comprises 0.034, compared with 0.183–0.194 in Africans and Chukchi, and 0.100–0.141 in Russians and others Europeans. Consequently, it may be supposed that both of the Ser and Asn *TRPM8* gene variants were present in the ancestral population of anatomically modern humans emerging from Africa.

Table 4. Minor allele frequencies of *TRPM8* gene SNPs in African and Eurasian populations

Population[a]	MAFs[b]					
	rs13004520	rs28901637	rs11562975	rs17868387	rs7593557	rs11563071
	C (R247T)	T (P249P)	C (L250L)	G (Y251C)	A (S419N)	G (V1058V)
Russians	0.050 ± 0.008	0.026 ± 0.006	0.121 ± 0.012	0.050 ± 0.008	0.091 ± 0.011	0.100 ± 0.011
Europeans	0.058 ± 0.015	0.008 ± 0.006	0.092 ± 0.019	0.058 ± 0.015	0.067 ± 0.016	0.141 ± 0.022
Kazakhs	0.055 ± 0.010	0.172 ± 0.017	0.223 ± 0.019	0.063 ± 0.011	0.256 ± 0.020	0.151 ± 0.016
Chukchi	0.031 ± 0.010	0.306 ± 0.026	0.294 ± 0.025	0.031 ± 0.010	0.531 ± 0.028	0.194 ± 0.022
Chinese	0.100 ± 0.022	0.142 ± 0.022	0.300 ± 0.034	0.093 ± 0.022	0.456 ± 0.034	0.122 ± 0.024
Japanese	0.090 ± 0.021	0.142 ± 0.022	0.170 ± 0.028	0.088 ± 0.015	0.412 ± 0.027	0.034 ± 0.014
Africans	0.008 ± 0.006	0.050 ± 0.014	0	0.009 ± 0.006	0.650 ± 0.031	0.183 ± 0.025

Notes. [a] The MAFs in Europeans (CEU), Chinese (CHB), Japanese (YPT), and Africans (YRI) are extracted from dbSNP; [b] MAF – minor allele frequency.

Table 4 shows that 419Ser gene variants have spread in Eurasia compared with African population. The H1 haplotype is the most frequent among study populations. In Russians the majority haplotypes is H1 (~3/4). The total frequency of 419Ser haplotypes, together with H1, comprises 0.909. The European population also has high frequency 419Ser gene variants (0.933). It is likely that their ancestral population experienced a bottleneck after the Asian-European split. The lower haplotype diversity among Europeans is not only the result of purifying selection from the presumed African Asn haplotype variants, but is also due to the limited distribution of haplotypes containing the minor alleles of the rs28901637 and rs11562975 SNPs in exon 7.

The majority of Asian Asn variants contain minor alleles of the rs28901637 and rs11562975 SNPs. Specific patterns of these haplotypes are observed among Asian populations. The Asn haplotypes (H10–H17) are more frequent in Chukchi (0.501) compared with Russian (0.041) population (see Table 3). Apparently, the relatively large population differentiation, with an F_{st} value of 0.1420 ($p < 10^{-5}$) between the Russians (Europeans) and Chukchi (East Asians), emerged under the influence of the two factors: (1) the effect of purifying selection, with persistent fixation of the Ser H1 haplotype, among Russian and Kazakh ancestors in the West of Central Asia and (2) the displacement of the Asn gene variants outside of Africa by the novel Asn derivatives containing the minor rs28901637 and rs11562975 alleles of the SNPs (exon 7) among the ancestors of East Asians.

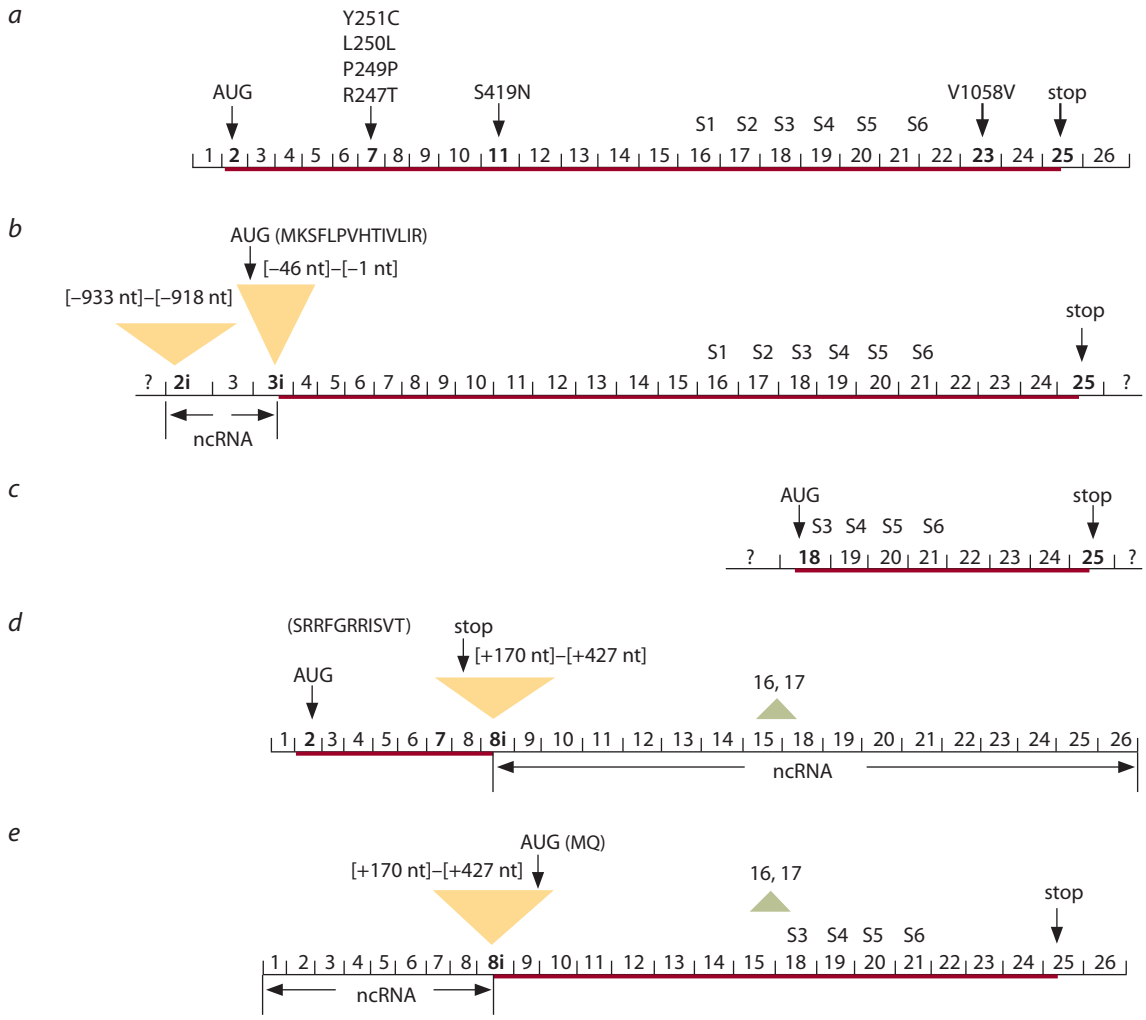
Interestingly, in all examined populations, haplotypes belonging to the H18–H22 subgroup occurred with similar frequencies (see Table 3). These gene variants include the minor alleles of three SNPs (positions 1, 4, and 5), which lead to amino acid replacements in protein. The strong LD between the minor alleles of the rs13004520 and rs17868387 SNPs (exon 7) and rs7593557 A allele (exon 11), detectable in all three populations, confirms the nonrandom character of their coevolution. Individuals carrying these haplotypes may potential produce functionally distinctive TRPM8 protein in comparison with H1 gene variant.

TRPM8 is a polymodal receptor responding to physical (cold and membrane potential) and chemical (phosphatidylinositol 4, 5-bisphosphate (PIP_2), menthol and icilin) factors (Babes et al., 2011; Latorre et al., 2011). In addition, this protein functions, not only on the cytoplasmic membrane, but also on the membranes of intracellular Ca^{2+} stores (Bidaux et al., 2007; Mahieu et al., 2007). We performed comparative nucleotide sequence analysis of the different *TRPM8* mRNA isoforms to ascertain the potential effects of their structure differences on protein function. The Figure schematically shows the structures of five *TRPM8* mRNA isoforms, including the variants that encode proteins described in the literature (Tsavaler et al., 2001; Lis et al., 2005; Sabnis et al., 2008) and mRNA isoforms extracted from GenBank.

The mRNA isoform (NM_024080.1) from thermosensitive neurons of the somatosensory system encodes the full-length TRPM8 protein, which is translated from the AUG codon in exon 2 (see the Figure, *a*) (Tsavaler et al., 2001). This isoform does not contain any of the SNP minor alleles in exon 7, and has G allele rs7593557 in exon 11, consequently, the processed transcript produces the 419Ser TRPM8 protein. Hence, the mRNA (NM_024080.1) has the nucleotide sequence corresponding to H1 gene variant.

The *TRPM8-b* mRNA has the 5′-terminal rearranged region by alternative splicing of *TRPM8* pro-mRNA (see the Figure, *b*). This may be an indication of a change in TRPM8 protein function in prostate epithelial cells (Lis et al., 2005), compared with the neurons. The rearranged initial region in the *TRPM8-b* mRNA isoform contains sequences from three gene parts, including 15 nucleotides (nt) of intron 2, exon 3 (which hasn't AUG codon), and 46 nts of the 3′-terminal sequence of intron 3 including a translation start codon. Together, these parts form a specific non-coding RNA (ncRNA) region, comprising a potential 5′-UTR, and potential coding sequence with the alternative AUG codon in a segment of intron 3. In consequence the *TRPM8-b* mRNA isoform may translates the polypeptide with truncated N-terminal domain without the peptide sequences encoded by exons 2 and 3.

The truncated $_{ER}$*TRPM8* mRNA isoform discovered in the bronchial epithelial cells (Sabnis et al., 2008) most likely encodes the minimum structural component required for a protein function (see the Figure, *c*). Exons 18 and 19 located at the 5′-end of this mRNA, encode the S3 and S4 transmembrane segments, which are necessary to open the pore and activate the channel (Latorre et al., 2011; Kühn et al., 2013). It is clear, the potential-dependent sensory module of this protein (transmembrane segments S1–S4) is represented the only two



Structural features of *TRPM8* mRNA isoforms.

a – the reference sequence NM_024080.1 comprises 26 exons and encodes the full-length protein (1104 aa) from sensory neurons translated from the AUG codon in exon 2. The location of the transmembrane segments (S1–S6) and the studied SNPs in the mRNA are shown; *b* – the *TRPM8-b* mRNA isoform with a rearranged 5′-terminal region contain the 15 nt fragment of intron 2 (2i), exon 3, and the 46 nt sequence from intron 3 end (3i). The AUG codon is located in the 3i insert. The rearranged RNA region without AUG codon is looked as a potential 5′-UTR. The *TRPM8-b* mRNA region, encoded by exons 4–25, corresponds to the neuronal mRNA structure; *c* – the truncated $_{ER}$TRPM8 mRNA isoform includes the second half of the exon 18, containing an AUG codon and the remaining part, up to exon 25, identical with neuronal mRNA isoform; *d*, *e* – the reference sequence BC143819.1 contain the 257 nt insert between positions [+171] and [+428] from the intron 8 and haven't two exons, 16 and 17, encoding the S1 and S2 segments; *d* – this mRNA translates the 325 aa protein from the AUG codon in exon 2 through exons 2–8 including the 33 nts in-frame initial region of the 257 nt insert, followed by a stop codon (UGA); *e* – this mRNA also translates the 682 aa protein from the terminal hexanucleotide in the 257 nt insert containing a putative alternative start codon (AUG) and glutamine codon (CAG), and then through exons 9–25, without exons 16 and 17, up to UGA codon. Thus, the BC143819.1 mRNA isoform contain non-protein-coding spliced exons from the opposite parts of the translated transcript and have different AUG start codons: one in exon 2 and the other in the 257 nt insert.
NcRNA – non-coding mRNA; nt – nucleotide.

transmembrane segments, S3 and S4, while the remaining S1 and S2, encoded by exons 16 and 17, are absent.

The absence of these exons is not unique, the another mRNA isoform (BC143819.1) with this feature has been identified (see the Figure, *d*, *e*). This mRNA after exon 8 contains the 257 nt insert from intron 8. This insert leads to the predicted production of two various proteins. The region comprising exons 2–8 is translated from the same start codon in exon 2 as neuronal mRNA, and ends the 33 nt sequence at the 5′-end of the 257 nt insert (see the Figure, *d*). Besides, the translation may starts from alternative AUG codon located in the terminal hexanucleotide of this insert (see the Figure, *e*). Unlike the truncated TRPM8 mRNA variant, this mRNA may potentially translates the protein having a greater extent the N-terminal domain with the peptide encoded by exon 11, including the rs7593557 SNP with the allele for serine codon. The absence of the minor alleles at rs28901637 and rs11562975 in exon 7 may indicate that both predicted proteins are alternative products of the H1 *TRPM8* gene variant. It is possible that the truncated protein isoform (325 aa) influence on protein activity by interacting with the full-length TRPM8 protein, for example, via its MHRs (Melastatin Homology Regions) (Phelps, Gaudet, 2007; Pedretti et al., 2009). The mechanisms of post-translational regulation of some TRPM8 protein iso-

forms by others have been described in previous reports (Bidaux et al., 2007, 2012). Thus, the *TRPM8* mRNAs produced by alternative splicing not only expand the protein diversity, but may also increase the range of post-translational regulation mechanisms.

The Figure illustrates a variety of *TRPM8* mRNA isoforms expressed only from the H1 gene variant; however, *TRPM8* mRNA isoform diversity may be far greater, given the functional coupling of the molecular machinery involved in transcription and alternative splicing (Montes et al., 2012; Kelemen et al., 2013). The results of this analysis indicate that mRNA isoforms generated by alternative splicing allow the potential synthesis of various proteins differing in the lengths of their N-terminal domains. Splicing may also generate alternative translation initiation zones, in addition to alternative mechanisms of post-translational regulation of *TRPM8* activity.

From the data in Tables 3 and 4, it follows that 419Asn variants of the *TRPM8* gene are more common in Asian populations compared with Russians. Their compositions are heterogeneous, probably, due to the recurrent emergence of the minor alleles of polymorphisms rs28901637 and rs11562975 (exon 7) in different 419Asn variants. It is possible that the minor alleles of these SNPs from exon 7 may influence on the features of alternative splicing of the 419Asn *TRPM8* pre-mRNA and, as a consequence, the composition of mRNA isoforms in Asians. These results demonstrate the need for research of *TRPM8* expression in individuals with different haplotype variants, to obtain direct confirmation of the forces underlying their selection.

Conclusion

In summary, it appears that the prevalent fixation of the 419Asn *TRPM8* gene variants carrying minor alleles SNPs in codons 249P and 250L (exon 7) in Asians is a Eurasian acquisition, which is presumably more characteristic for Eastern than Western Asians. The surrounding conditions probably favored this selection.

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Acknowledgements. Research supported by budget project No. 0324-2019-0041.

Conflict of interest. The authors declare no conflict of interest.

Received June 18, 2019. Revised October 24, 2019. Accepted December 14, 2019. Published online March 20, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):299-305
DOI 10.18699/VJ20.41-o

A rare splice site mutation in the gene encoding glucokinase/hexokinase 4 in a patient with MODY type 2

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Abstract. The article presents a variant of maturity onset diabetes of the young type 2, caused by a rare mutation in the *GCK* gene. Maturity onset diabetes of the young (MODY) is a hereditary form of diabetes with an autosomal dominant type of inheritance, an onset at a young age, and a primary defect in pancreatic β-cell function. This type of diabetes is different from classical types of diabetes mellitus (DM1 and DM2) in its clinical course, treatment strategies, and prognosis. Clinical manifestations of MODY are heterogeneous and may vary even among members of the same family, i. e., carriers of identical mutations. This phenotypic variation is due to the interaction of mutations with different genetic backgrounds and the influence of environmental factors (e. g., lifestyle). Using next-generation sequencing technology, the c.580–1G>A substitution (IVS5 –1G>A, rs1554335421) located in an acceptor splice site of intron 5 of the *GCK* gene was found in a proband. The identified variant cosegregated with a pathological phenotype in the examined family members. The *GCK* gene encodes glucokinase (hexokinase 4), which catalyzes the first step in a large number of glucose metabolic pathways such as glycolysis. Mutations in this gene are the cause of MODY2. The illness is characterized by an insignificant increase in the fasting glucose level, is a well-controlled disease without medication, and has a low prevalence of micro- and macrovascular complications of diabetes. The presented case of MODY2 reveals the clinical significance of a mutation in the splice site of the *GCK* gene. When nonclassical diabetes mellitus is being diagnosed in young people and pregnant women, genetic testing is needed to verify the diagnosis and to select the optimal treatment method.
Key words: human; maturity onset diabetes of the young; MODY2; glucokinase gene; next-generation sequencing; genetic analysis; bioinformatics.

For citation: Ivanoshchuk D.E., Shakhtshneider E.V., Ovsyannikova A.K., Mikhailova S.V., Rymar O.D., Oblaukhova V.I., Yurchenko A.A., Voevoda M.I. A rare splice site mutation in the gene encoding glucokinase/hexokinase 4 in a patient with MODY type 2. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3): 299-305. DOI 10.18699/VJ20.41-o

Редкий вариант мутации сайта сплайсинга гена, кодирующего глюкокиназу/гексокиназу 4, у пациента с MODY, подтип 2

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Аннотация. В статье рассмотрен вариант развития моногенной формы сахарного диабета (MODY), обусловленный редкой мутацией в гене *GCK*. Диабет MODY представляет собой сахарный диабет с аутосомно-доминантным типом наследования, возникающий в молодом возрасте и проявляющийся в дисфункции β-клеток поджелудочной железы. Этот тип отличается от классических типов сахарного диабета (СД1, СД2) клиническим течением, тактикой лечения и прогнозом для пациента. Клинические проявления MODY гетерогенны и могут различаться даже у представителей одной семьи, носителей одинаковых мутаций. Это обусловлено как сочетанием мутаций в различных генах у индивидуума, так и воздействием внешних факторов. Методом секвенирования нового поколения у пробанда была идентифицирована замена c.580 –1G>A (IVS5 –1G>A,

rs1554335421), локализующаяся в акцепторном сайте сплайсинга пятого интрона гена *GCK*. Обнаруженный вариант сегрегировал с патологическим фенотипом у обследованных членов семьи. Ген *GCK* кодирует глюкокиназу (гексокиназу 4), которая катализирует первый шаг в различных путях метаболизма глюкозы. Мутации в этом гене ассоциированы с сахарным диабетом взрослого типа у молодых, подтип 2 (MODY2). Заболевание характеризуется незначительным повышением глюкозы натощак, хорошо контролируется медикаментами и отличается низкой распространенностью микро- и макрососудистых осложнений. Представленный в исследовании случай MODY2 выявил клиническую значимость мутации в сайте сплайсинга гена *GCK*. При возникновении у молодых людей и беременных женщин неклассического сахарного диабета проведение генетического тестирования необходимо для подтверждения диагноза и оптимального выбора тактики и способа лечения.

Ключевые слова: человек; диабет взрослого типа у молодых; MODY2; ген глюкокиназы; секвенирование нового поколения; генетический анализ; биоинформатика.

Introduction

Maturity onset diabetes of the young (MODY) is a hereditary form of diabetes with autosomal dominant inheritance and is characterized by onset at a young age and by the presence of an initial defect in pancreatic β-cell function. This type of diabetes differs from classic types of diabetes mellitus-type 1 (DM1) and type 2 (DM2) in disease progression, in treatment strategies, and prognosis (Anık et al., 2015). Up to 80 % of MODY cases are not detected or are misdiagnosed as DM1 or DM2; therefore, patients with an incorrectly diagnosed type of diabetes are often prescribed inadequate therapy (Shields et al., 2010). On average, MODY is detected in 2–5 % of cases of diabetes (the rest being mostly DM1 and DM2) (Fajans et al., 2001). To reliably diagnose MODY in a patient, molecular genetic analysis should be carried out. To date, 14 types of MODY (MODY1 through MODY14) have been identified, each associated with mutations in a specific gene: *HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *KCNJ11*, *ABCC8* and *APPL1* (Thanabalasingham et al., 2011; Bonnefond et al., 2012; McDonald et al., 2013; Lachance, 2016; Ovsyannikova et al., 2016). Fourteen MODY-associated genes explain 70–85 % of the disease cases and are involved in various stages of glucose metabolism regulation (Thanabalasingham et al., 2011; Bonnefond et al., 2012; Lachance, 2016). According to various researchers, 11 to 30 % of MODY cases are caused by mutation in other genes (Edghill et al., 2010; Bonnefond et al., 2012). These forms of MODY are commonly referred to as MODY-X. Because the vast majority of pathogenic mutations are found in exons and adjacent splicing sites of genes (Stenson et al., 2017), it is reasonable to perform whole-exome sequencing on genomic DNA from individuals with MODY, with subsequent genetic testing of their relatives for the identified mutation. MODY verification allows for successful patient management and ensures healthy pregnancy and provision of genetic counseling to families (Lachance, 2016). Examination of relatives of MODY probands makes it possible to diagnose hyperglycemia in the preclinical phase.

In this report, we describe a clinical case of a family with MODY2 associated with a rare splice site mutation in the glucokinase (*GCK*) gene identified by the next-generation sequencing technology.

Materials and methods

The study protocol was approved by the local Ethics Committee of the Institute of Internal and Preventive Medicine (Branch of the Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia, approval # 22.06.2008). Written informed consent to be examined and to participate in the study was obtained from each patient. For individuals younger than 18 years, the informed consent form was signed by a parent or legal guardian.

Blood samples were collected from the ulnar vein for biochemical analysis in the morning on an empty stomach. Lipid levels (cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol) and glucose concentration were determined on a KoneLab 300i biochemical analyzer (Thermo Fisher Scientific, Waltham, MA, USA) with Thermo Fisher Scientific reagents.

Genomic DNA was isolated from leukocytes of venous blood by phenol-chloroform extraction (Sambrook, Russell, 2006). Quality of the extracted DNA was assessed on a capillary electrophoresis system, Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA). Sequencing of patients' DNA was carried out on an Illumina HiSeq1500 instrument (Illumina, San Diego, CA, USA). The enrichment and library preparation were performed with the SureSelectXT Human All Exon V5 + UTRs Kit (Agilent Technologies Inc., USA). Reads were mapped to the reference human genome (GRCh37) by means of the Burrow–Wheeler Alignment tool (BWA v.0.7.12) (Li, Durbin, 2009). Polymerase chain reaction (PCR)-generated duplicates were removed in the PICARD software (https://broadinstitute.github.io/picard/).

A search for single-nucleotide variants (SNVs) was conducted using the Genome Analysis Toolkit v.3.3 package by the procedure for local remapping of short insertions/deletions and recalibration of read quality (McKenna et al., 2010). The depth of coverage was 34× to 53×. SNVs with genotype quality scores <20 and coverage depth $<10\times$ were filtered out and excluded from further analysis. Annotation of the SNVs was performed in the ANNOVAR software (Wang et al., 2010) using the 1000 Genomes Project (The 1000 Genomes Project Consortium…, 2015) and The Genome Aggregation Database (gnomAD) (Karczewski et al., 2019) databases. We selected the spectrum of rare and novel sequence variants in MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *KCNJ11*, *ABCC8* and *APPL1*). Rare variants were selected if their minor allele frequency (MAF) was ≤ 0.5 in the 1000 Genomes Project and gnomAD. Heterozygous substitution c.580–1G>A (IVS5 –1G>A) at an acceptor splice site of intron 5 of the *GCK* gene was found

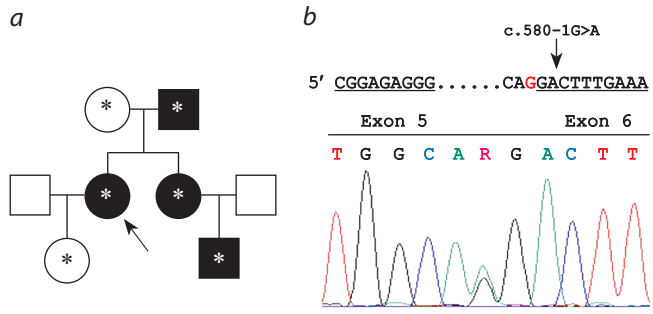
in the proband and her sister. To predict the possible effect of the SNV on splicing regulation, we employed the SPANR software (Xiong et al., 2015).

The substitution was corroborated by Sanger sequencing of the DNA fragment containing exons 5 and 6, intron 5, and parts of introns 4 and 6 using the following forward and reverse primers: 5′-CAGGGAGCCTCAGCAGTCTGGA-3′ and 5′-GCCACGAGGCCTATCTCTCCCC-3′. The oligonucleotides were designed in the Primer-Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized by the Biosset company (Russia, Novosibirsk). The sequencing reactions were carried out on an automated ABI 3500 DNA sequencer (Thermo Fisher Scientific, USA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). PCR was set up using BioMaster LR HS-PCR (2×) (BioLabMix, Russia), 1 μL of each primer, and 1 μL of DNA, with a total final volume of 25 μL. The thermocycling program consisted of initial denaturation at 94 °C for 3 min and then 35 cycles at 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 50 s. The PCR products were evaluated by electrophoresis in a 5 % polyacrylamide gel after visualization with an ethidium bromide solution. A 100 bp DNA Ladder (BioLabMix) was simultaneously run on the gel as molecular size markers. The amplicons were purified using Agencourt AMPure Xp beads (Beckman Coulter, USA). The sequencing reactions were conducted on an automated ABI 3500 DNA sequencer (Thermo Fisher Scientific, USA) via the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences were analyzed in the Vector NTI® Advance software (Thermo Fisher Scientific). The hg19 version of the human genome served as a reference sequence for the alignment.

Results

The white European 44-year-old female proband was under medical observation. When she underwent routine screening in 2012 (at age 40), hyperglycemia at 7.2 mmol/L was revealed. No complaints were registered. During subsequent glycemia control, maximal fasting glucose was 7.2 mmol/L, and the postprandial one was 8.9 mmol/L. C-peptide was 1.83 ng/mL (reference range 0.5–3.2 ng/mL), immunoreactive insulin was 7.9 μU/mL (reference range 2.0–25.0 μU/ mL), and glycated hemoglobin (HbA1c) was 7.1 %. Antibodies to insulin, to pancreatic islet cells, and to glutamic acid decarboxylase were absent. Blood biochemical analysis and determination of thyroid status did not reveal any abnormalities. Ultrasonography of internal organs, echocardiography, and a study of brachiocephalic vessels did not uncover any pathology. The body mass index (BMI) was 20.2 kg/m^2. DM2 was diagnosed in the patient, and sitagliptin was prescribed. At the age of 26, the patient spontaneously delivered a healthy girl at 39 weeks of gestation; hyperglycemia was not detected during the pregnancy.

The sister of the proband is a white European 35-year-old woman. At age 23, during tests before mastectomy for mastopathy, she got a diagnosis of fasting hyperglycemia (6.3 mmol/L). The patient did not have any complains, and a proper diet was recommended. At the age of 29, during additional examination before cholecystectomy for cholelithiasis, she received a diagnosis of DM2, and vildagliptin was



Mutation identified in *GCK* gene.

a – family history with inherited diabetes mellitus (DM). Asterisk indicates medically examined family members; *b* – schematic representation of the mutation in the splicing acceptor site and chromatogram of DNA sequence with mutated allele c.580 –1G>A (IVS5 –1G>A, rs1554335421) of the *GCK* gene.

prescribed at the dose of 50 mg twice a day. At age 31, the proband's sister visited an endocrinologist at the outpatient clinic of the Institute of Internal and Preventive Medicine (Branch of the Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia) with complaints of a failure to get pregnant within a year. On examination, BMI was 20.6 kg/m^2, and the objective status was unremarkable. Blood biochemical analysis revealed hypercalcemia (3.25 mmol/L), increased levels of high-density lipoprotein cholesterol (85 mg/dL), hypercholesterolemia (220 mg/dL), and hyperglycemia (6.8 mmol/L), but other analyzed parameters were within reference ranges. The HbA1c level was 7.1 %. Antibodies to insulin, to pancreatic islet cells, and to glutamic acid decarboxylase were absent. Thyroid-stimulating hormone concentration was 0.759 mU/mL (reference range 0.4–4.0), whereas the prolactin level was 216 ng/mL (reference range 1.2–19.5). Echocardiography, Doppler sonography of extracranial parts of cerebral vessels, and abdominal and renal ultrasonographic examination revealed no pathology. Cysts were found in both thyroid lobes during the ultrasonography. Given the existence of the proband's relatives with impaired glucose metabolism, persistence of normal C-peptide levels, the absence of diabetes-associated autoantibodies, normal BMIs of the proband and her sister, and stable mild hyperglycemia, MODY was assumed.

Exons and adjacent splice sites of MODY-associated genes were analyzed by whole-exome sequencing in the proband and her sister. As a result, heterozygous substitution c.580–1G>A (IVS5 –1G>A) at an acceptor splice site of intron 5 of the *GCK* gene was found in the proband and her sister. The IVS5 (–1G>A) polymorphism of *GCK* was submitted in ClinVar with an accession number of rs1554335421 (Landrum et al., 2018), but was absent in the 1000 Genomes Project (The 1000 Genomes Project Consortium…, 2015), in gnomAD project databases at the moment of publication. Subsequent genetic analysis by Sanger sequencing of the family members (mother, father, daughter, and nephew of the proband) uncovered segregation of the substitution with DM as an autosomal dominant trait (see the Figure). Our results, literature data, and databases suggest that this splice site mutation is likely pathogenic.

After confirmation of GCK-MODY in the proband and her sister, their relatives were screened for carbohydrate metabo-

lism disorders. The proband's mother and daughter did not have any abnormalities. The proband's father showed impaired fasting glucose. No complaints were registered, venous plasma fasting glucose was 6.3 mmol/L, and 2 h after the oral glucose tolerance test, it was 7.5 mmol/L. At present, the man does not take any medication. The same heterozygous substitution rs1554335421 (IVS5 –1G>A) in the proband's father's *GCK* gene was detected by genetic testing.

The proband's sister had her first pregnancy in 2014 (at age 31). In 2015, a boy weighing 3640 g was born by a caesarean section at 39 weeks of gestation. The pregnancy was complicated: premature rupture of membranes, weakness of labor, and fetal hypoxia. After delivery, due to stable glycemic indexes, it was decided that insulin therapy should be discontinued. In January 2018, during treatment with diet, the patient's HbA1c was 6.4 %.

The neonatal period of the proband's nephew was unremarkable. In 2017, his blood biochemical analysis resulted in a diagnosis of hyperglycemia (6.9 mmol/L). HbA1c was 6.3 %, and the C-peptide level was 0.54 ng/mL. Antibodies to insulin, pancreatic β-cells, and glutamic acid decarboxylase were undetectable. The same heterozygous substitution rs1554335421 (IVS5; –1G>A) in the *GCK* gene was identified by genetic testing. At present, the child is under medical observation at Almazov Federal Medical Research Centre (Saint Petersburg, Russia); because of GCK-MODY, a balanced diet was recommended.

Discussion

It is known that in young patients with impaired carbohydrate metabolism, DM1, DM2, or rarer monogenic forms of diabetes may be diagnosed. At the onset of the disease, the proband and her sister had no symptoms characteristic for the common types of diabetes, fasting hyperglycemia was not progressing, and carbohydrate metabolism disorders were detected during routine screening. The presence of DM in the proband's sister, persistence of normal C-peptide levels, a lack of autoantibodies, and a normal BMI in the proband and her sister pointed to MODY (Chakera et al., 2015).

Heterozygous splice site mutation c.580–1G>A (rs1554335421) in intron 5 of their *GCK* gene was identified by genetic testing. Mutations in this gene are associated with DM2, MODY, and neonatal DM (Plengvidhya et al., 2009; Lachance, 2016). More than 600 variants of the *GCK* gene associated with MODY have been described, and the list of the mutations is constantly growing. The vast majority of the mutations are missense substitutions, but splice site mutations, deletions, and insertions are reported too (Stenson et al., 2017).

The *GCK* gene is located in chromosomal region 7p15.3-p15.1 and consists of 12 exons that encode a 465-amino-acid protein, glucokinase (Osbak et al., 2009), which is one of four members of the hexokinase family of enzymes. In 1992, *GCK* was the first gene to be linked to MODY. It plays an important regulatory role in glucose metabolism. Glucokinase catalyzes phosphorylation of glucose to produce glucose-6-phosphate as the first step of glycolysis in pancreatic β-cells (Matschinsky et al., 1993; Iynedjian, 2009). Most individuals with heterozygous *GCK* mutations show fasting plasma glucose levels between 5.5 and 8.0 mmol/L and a small increase in plasma glucose (< 3 mmol/L in 70 % of the patients) 2 h after the oral glucose test (Stride et al., 2002). This feature also explains asymptomatic fasting hyperglycemia (HbA1c range 5.8–7.6 % (40–60 mmol/mol)) and rare microvascular and macrovascular complications in patients with GCK-MODY (Caetano et al., 2012; Steele et al., 2014). Most patients have an aberrant fasting glucose level or impaired glucose tolerance, and less than 50 % of the affected individuals have diabetes, which is diagnosed during childhood, adolescence, or pregnancy (Caetano et al., 2012). In a study on Italian patients under 18 years of age with incidental hyperglycemia, it was estimated that 15 % of these cases are caused by *GCK* mutations (Lorini et al., 2009).

It was found here that the proband and her sister carry a heterozygous substitution, c.580–1G>A (IVS5 –1G>A, rs1554335421), at an acceptor splice site of *GCK* intron 5. This allelic variant is of interest because consensus donor (GT dinucleotide) and acceptor (AG dinucleotide) splice sites are highly conserved. Point mutations at these loci can lead to cryptic splice site activation and synthesis of aberrant protein isoforms.

In silico analysis of the functional significance of this substitution suggested that the inclusion of exons 5, 6, and 7 in gene transcripts will be reduced in case of the detected variant (see the Table).

Furthermore, rs1554335421 (IVS5 –1G>A) of *GCK* is in the HGMD database (Stenson et al., 2017). This mutation was described in a German family, where it segregated with

The predicted (by SPANR) effect of c.580 –1G>A (IVS5 –1G>A, rs1554335421) of the *GCK* gene on splicing

Transcript	dPSI*	dPSI_percentile**	PSI_WT***
chr7:GCK – NM_000162:Exon6	–7.14	0.40	82.49
chr7:GCK – NM_000162:Exon5	–1.45	5.20	80.01
chr7:GCK – NM_033508:Exon7	–7.14	0.40	82.49
chr7:GCK – NM_033508:Exon6	–1.45	5.20	80.01
chr7:GCK – NM_033507:Exon6	–7.14	0.40	82.49
chr7:GCK – NM_033507:Exon5	–1.45	5.20	80.01

Note. PSI – percentage of transcripts with the exon spliced in: * dPSI: a maximal difference in PSI across 16 tissues; ** dPSI_percentile: percentile of mutant dPSI among dPSIs of common SNPs; *** PSI_WT: predicted PSI in the wild type.

DM and a family history of gestational diabetes. Experiments on lymphoblastoid cells indicate that rs1554335421 (IVS5 –1G>A) of *GCK* can activate a cryptic splice site in intron 5 and cause retention of 27 bp of the intron (Toaima et al., 2005). Information about rs1554335421 (IVS5 –1G>A) of *GCK* is absent in the 1000 Genomes Project, The Exome Aggregation Consortium, and GNOMAD (https://gnomad.broadinstitute.org/) databases; however, taking into account the previous study and the data we obtained here, carriage of the A allele at position –1 of intron 5 is most likely a causative dominant variant of the *GCK* gene in MODY-affected people.

Cryptic splice site activation and formation of several alternative transcripts with intron 7 fragments' retention were demonstrated in a system of model *GCK* minigenes with acceptor site mutation IVS7 (–1G>C) (Igudin et al., 2014).

Model mice with the homozygous mutation in the splice site of β-cell-specific exon 1 IVS1A (–1G>T) show hyperglycemia, glucosuria, and growth retardation and die within the first week after birth. This phenotype can be explained by exon skipping or intron retention (Inoue et al., 2004). Splicing sites affected by mutations have been described for many pathological phenotypes: neurofibromatosis type 1 (Jang et al., 2016), familial hypercholesterolemia (Shakhtshneider et al., 2017), Wiskott–Aldrich syndrome and chronic colitis (Esmaeilzadeh et al., 2018), hypophosphatemic rickets (Ma et al., 2015), and others. Mutations affecting splicing have been found not only in canonical splicing sites but also in introns and exons and may have a tissue-specific effect, as in familial dysautonomia (Slaugenhaupt et al., 2001; Abramowicz, Gos, 2018). That analysis indicated that the donor splice site mutations were more prevalent than the acceptor splice site variants (ratio 1.5:1.0) (Abramowicz, Gos, 2018). Because the mutations in the *GCK* gene can cause a mild clinical phenotype, which can vary under the influence of many genetic and lifestyle factors, research on the carriers of these mutations is essential for identifying additional risk factors.

GCK-MODY is inherited as an autosomal dominant trait manifested throughout the lifespan as stable, mild fasting hyperglycemia usually reaching 6.7 mmol/L and higher only in middle age (Wedrychowicz et al., 2017). A similar pattern was observed here in the proband and her sister. Nonetheless, the metabolic disturbances in the carriers of *GCK* mutations are present from birth and can be identified already in the first years of life, almost all of them after puberty (Steele et al., 2014). The proband's nephew, who is a heterozygous mutation carrier, developed carbohydrate metabolism disorders at two years of age.

It has been reported that carriers of *GCK* gene mutations with a long history of hyperglycemia (48.6 years on average) usually have micro- and macrovascular complications of diabetes and are at a risk of cardiovascular diseases that is identical to that in the general population (Pruhova et al., 2013).

Patients with GCK-MODY in childhood and adolescence can be treated only with diet in most cases, and glucose-lowering therapy should be considered during pregnancy (Lachance, 2016). The present subtype of MODY2 (in the proband and her sister) currently is treated with diet resulting in sufficient glycemic control.

The presence or absence of a *GCK* mutation in the fetus affects its sensitivity to maternal hyperglycemia (Chakera et al., 2012). If the fetus does not have the mutation, then it will secrete insulin excessively and as a result have a risk of macrosomia (Spyer et al., 2009). In that case, low doses of insulin should be prescribed during pregnancy (Chakera et al., 2014). During her pregnancy, the proband's sister was given insulin injections in small doses. After delivery, insulin therapy was discontinued. Subsequently, the child was found to carry the same substitution.

Conclusion

The presented subtype of MODY2 reveals the clinical significance of the mutation in a splice site of the *GCK* gene. When nonclassical diabetes mellitus is being diagnosed in young people and pregnant women, genetic testing is needed to verify the diagnosis and to select the optimal treatment method.

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Acknowledgements. Collection of the samples and clinical data, clinical examination, high-throughput sequencing, and Sanger sequencing have been supported by the Russian Science Foundation (grant No. 14-15-00496-P). Bioinformatic analyses have been supported as part of the budget topic in state assignment No. AAAA-A19-119100990053-4. The English language was corrected and certified by shevchuk-editing.com. The authors thank the patients for participation in this study.

Conflict of interest. The authors declare no conflict of interest.

Received October 16, 2019. Revised December 6, 2019. Accepted December 13, 2019. Published online February 26, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):306-314
DOI 10.18699/VJ20.622

Plant genetic resources in India: management and utilization

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Abstract. Plant genetic resources (PGR) are the foundation of agriculture as well as food and nutritional security. The ICAR-NBPGR is the nodal institution at national level for management of PGR in India under the umbrella of Indian Council of Agricultural Research (ICAR), New Delhi. India being one of the gene-rich countries faces a unique challenge of protecting its natural heritage while evolving mutually beneficial strategies for germplasm exchange with other countries. The Bureaus activities include PGR exploration, collection, exchange, characterization, evaluation, conservation and documentation. It also has the responsibility to carry out quarantine of all imported PGR including transgenics meant for research purposes. The multifarious activities are carried out from ICAR-NBPGR headquarters and its 10 regional stations located in different agro-climatic zones of India. It has linkages with international organizations of the Consultative Group on International Agricultural Research (CGIAR) and national crop-based institutes to accomplish its mandated activities. NBPGR collects and acquires germplasm from various sources, conserves it in the Genebank, characterizes and evaluates it for different traits and provides ready material for breeders to develop varieties for farmers. ICAR-NBPGR encompasses the National Genebank Network and at present, the National Genebank conserves more than 0.40 million accessions. NBPGR works in service-mode for effective utilization of PGR in crop improvement programmes which depends mainly on its systematic characterization and evaluation, and identification of potentially useful germplasm. NBPGR is responsible for identifying trait-specific pre-adapted climate resilient genotypes, promising material with disease resistance and quality traits which the breeders use for various crop improvement programmes. The system has contributed immensely towards safeguarding the indigenous and introducing useful exotic PGR for enhancing the agricultural production. Presently, our focus is on characterization of *ex situ* conserved germplasm and detailed evaluation of prioritized crops for enhanced utilization; assessment of impact of on-farm conservation practices on genetic diversity; genome-wide association mapping for identification of novel genes and alleles for enhanced utilization of PGR; identification and deployment of germplasm/landraces using climate analog data; validation of trait-specific introduced germplasm for enhanced utilization.
Key words: plant genetic resources; gene banks; wild relatives; biotic and abiotic stresses; marker-assisted selection.

For citation: Singh K., Gupta K., Tyagi V., Rajkumar S. Plant genetic resources in India: management and utilization. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):306-314. DOI 10.18699/VJ20.622

Генетические ресурсы растений Индии: контроль и использование

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Аннотация. Генетические ресурсы растений – основа сельского хозяйства и главный фактор, определяющий качество потребляемой пищи. В Индии на национальном уровне этой проблемой занимается Национальное бюро генетических ресурсов растений (NBPGR), действующее под эгидой Индийского совета по сельскохозяйственным исследованиям (ICAR), со штаб-квартирой в Нью-Дели. Обладая богатыми растительными ресурсами, Индия должна учитывать интересы безопасности своего природного наследия при выработке даже самых выгодных стратегий обмена генетическим материалом со своими международными партнерами. В задачи Бюро входят исследование, сбор, обмен, описание, оценка, сохранение и учет генетических ресурсов растений, а также обеспечение карантинных мер для всего ввозимого из-за рубежа материала, включая трансгенные растения, предназначенные для исследовательских целей. Бюро и десять его региональных отделений, расположенных в разных агроклиматических зонах страны, осуществляют деятельность в нескольких направлениях. Поддерживают связи с международными организациями, входящими в состав Консультативной группы по международным сельскохозяйственным исследованиям (CGIAR), и национальными институтами, занимающимися проблемами сельскохозяйственных культур. Об-

разцы генофонда из самых разных источников пополняют генбанк, где проводится их описание и оценка по заданным признакам. На основе этого материала выводятся сорта сельскохозяйственных культур. Существующий при Бюро Национальный генетический банк (National Genebank Network) насчитывает более 400 тысяч образцов. Бюро работает в сервисном режиме, обеспечивая эффективное использование генетических ресурсов растений в программах улучшения сельскохозяйственных культур, что стало возможным во многом благодаря последовательному подходу к описанию и оценке этих ресурсов, а также отбору потенциально полезного генетического материала. Другими задачами являются определение генотипов с теми или иными признаками, специфичными к изменению климата, а также отбор перспективного материала, обладающего устойчивостью к заболеваниям и признаками качества, на которые ориентируются селекционеры при работе над улучшением сельскохозяйственных культур. Действующая таким образом система сыграла важнейшую роль в выработке столь необходимого стране баланса в отношении генетических ресурсов растений: интродукция ценного экзотического генофонда в целях интенсификации производства сельскохозяйственной продукции ведется без ущерба для местных ресурсов. В настоящее время основными направлениями работы являются: описание генетического материала, сохраненного путем консервации *ex situ*, и всесторонняя оценка приоритетных сельскохозяйственных культур для более эффективного их использования; оценка влияния различных методов мелиорации земель на генетическое разнообразие; полногеномное ассоциативное картирование с целью выявления ранее неизвестных генов и аллелей для более эффективного использования генетических ресурсов растений; отбор генетического материала и/или местных разновидностей и определение оптимальных районов выращивания на основе аналоговых данных наблюдений за климатом; проверка соответствия интродуцированного генетического материала заданным критериям.
Ключевые слова: генетические ресурсы растений; генетические банки; дикорастущие родственники культурных растений; биотический и абиотический стресс; отбор с помощью маркеров.

Introduction

Plant genetic resources (PGR) are one of essential components of agro-biodiversity and defined as the genetic material of plants having value as a resource for present and future generations. PGR hold the key to the very foundation of agriculture as well as food and nutritional security for the world. Agricultural biodiversity, agri- or agro-biodiversity (a subset of biodiversity) is defined as 'all crops and livestocks, their wild relatives, and all interacting species of pollinators, symbionts, pests, parasites, predators and competitors' (Qualset et al., 1995). Indian subcontinent has a rich and varied heritage of biodiversity, encompassing a wide spectrum of habitats from tropical rainforests to alpine vegetation and from temperate forests to coastal wetlands. It is one of the eight centres of origin (Vavilov, 1951) and is one of the 12 mega gene centres of the world. It possesses 11.9 % of world flora, and about 33 % of the country's recorded flora are endemic to the region and are concentrated mainly in the North-East, Western Ghats, North West Himalayas and the Andaman and Nicobar islands. Of the 49,219 higher plant species, 5,725 are endemic and belong to 141 genera under 47 families (Nayar, 1980). Of these 3,500 are found in the Himalayas and adjoining regions and 1,600 in the Western Ghats alone (Arora, 1991). The concept of biodiversity hotspots was originated by Dr. Norman Myers in two articles in "The Environmentalist" (1988), revised after thorough analysis by Myers and others in "Hotspots: Earth's biologically Richest and Most Endangered Terrestrial Ecoregions". The hotspots idea was also promoted by Russell Mittermeier in the popular book "Hotspots revisited". Around the world, 34 biodiversity hotspots exists as of today. These sites support nearly 60 % of the world's plant, bird, mammal, reptile, and amphibian species, with a very high share of endemic species (Myers et al., 2000).

From centuries, tribal or traditional farming communities have continuously adapted and shapened the dimensions of rich genetic material available with them. These resources or traditional varieties or landrace populations often bear specific traits – early or late maturing, adaptability to a particular soil type, uses and usually have local names which has enabled them to survive so long under various biotic and abiotic stresses in the centers of diversity along with wild progenitors of crop plants, wild and weedy relatives. Besides these resources, potential domesticates (also called wild economic species) are involved in the PGR spectrum; they are those wild species, which are not yet domesticated but are extensively used. Some of them grow widely, though genetically and culturally in a near wild state. With richness of plant genetic resources, the tribal regions have therefore been identified as "Hot Spots" of agri-biodiversty. However, they are different from the biodiversity hot-spots (as per definition of Myers et al., 2000) which include all entire endemic biodiversity as a priority for defining hot-spots of a region.

During the process of crop evolution crops originated in its centre of origin have changed from the wild progenitors in morphological, physiological and agronomic traits to newer types through selection for desired traits. In this whole process, agriculture (broadly the process of rearing plants and animals, wild or tamed), cultivation (physical activities which are relevant to and associated with agriculture) and domestication (process of genetic shift in domesticated population to adapt them to better/changed or artificial environment, created by cultivation conditions) have shapened them for appropriate PGR. PGRs are exchanged and searched continuously for specific traits to improve crops in terms of yield and nutritional value, and their interdependence plays a very important role in international collection and exchange of germplasm. Every nation is concerned with acquisition of diverse and superior germplasm for conservation and utilization.

Over the years, India has developed sound and scientific management regimes for *ex situ* conservation and access to its genetic resources (Dhillon, Saxena, 2003). Groups of institutions, scientific societies, non-governmental organizations are

addressing the task with ICAR-NBPGR, New Delhi, as the nodal agency for its coordination. It aims at efficient management of plant genetic resources by providing convenience of access to the various crop improvement programmes. It also encompasses the National Genebank Network. At present, the genebank holds more than 4.4 lakhs accessions. The Cryobank Facility in the National Genebank has accessions of varied germplasm of orthodox, intermediate and recalcitrant seed species and also of pollen samples. The *in vitro* genebank conserves various priority crops which are maintained under short- to medium-term storage periods. These include tuberous and bulbous crops, tropical fruits species, spices and industrial crops, medicinal and aromatic plants species.

Centre of origin and diversity of crop plants are concepts relating to the patterns of distribution and build-up in regions and habitats, consequent to use and domestication by man in areas representing independent agricultural systems and separated by major geographical barriers. Various terms have been used to designate these 'geobotanical' diversity patterns in crop plants viz. primary and secondary centres of diversity, gene centres, cradles/subcradles of agriculture, megacentres, regions (Vavilov, 1926; Zhukovsky, 1968), non-centres, microcentres (Harlan, 1975), cradles of angiosperm diversity (Takhtajan, 1969). However, within centres of diversity, geobotanical patterns of variation, add a practical connotation to the use of these concepts in PGR. The significant point, as stated above is that centres of diversity are the consequence of continuing processes of evolution and domestication, and hence subject to change. The concept of 'ecological passports' in different sites where different crops show parallelism in characters by Vavilov, form the basis for understanding the diversity within the crop species, and in relation to the crop genepool (Harlan, de Wet, 1971).

PGR conservation

The National Bureau of Plant Genetic Resources (ICAR-NBPGR) was established by the Indian Council of Agricultural Research (ICAR) in 1976 with its headquarters at New Delhi. The chronology of events leading to the present day ICAR-NBPGR dates back to 1905 when Botany Division was established under the then Imperial Agricultural Research Institute. ICAR-NBPGR has been given the mandate to act as a nodal institute at the national level for acquisition and management of indigenous and exotic PGR for agriculture, and to carry out related research and human resources development for sustainable growth of agriculture. The Bureau is also vested with the authority to issue Import Permit and Phytosanitary Certificate and conduct quarantine checks in seed material and vegetative propagules (including transgenic material) introduced from abroad or exported for research purposes. Besides having a 40 ha experimental farm at Issapur village (about 45 km west of Pusa Campus), the Bureau has a strong national network comprising Regional Stations/Base Centers and ICAR Institutes/SAUs that provide access to representative agro-ecological situations in the country.

The major components of the National Genebank include the seed genebank, field genebank, cryo-bank and the *in vitro* genebank. The focus of seed genebank of NGB is long term conservation of orthodox seeds. The basic parameters that are assessed for conservation are:

(i) Uniqueness of the accession. Redundancy is a major issue in all genebanks and best efforts are made to avoid duplication of accessions.
(ii) Seed quality. The global genebank standards recommends a minimum of 2000 seeds in self-pollinated crops and 4000 seeds in cross pollinated crops. In wild germplasm accessions, the minimum number has been relaxed to 500 seeds.
(iii) Seed viability. A minimum viability of 85 % is essential for seed samples. However in those cases where the Indian Minimum Seed Certification Standards have approved a lower level of standard germination, the viability requirements are accordingly modified in gene bank also.
(iv) Seed health. Pest free conservation is a priority at NGB. There is a strong collaboration between the Division of Germplasm Conservation and Division of Plant Quarantine, wherein all accessions are tested for any form of pest infestation prior to their processing.
(v) Availability of passport information. The utilization of the conserved accessions can be facilitated only if all relevant passport information is available in the database. Hence, only those accessions having basic information on parameters like biological status, collection details if acquired through exploration, pedigree details if it's a breeding line/genetic stock or cultivar and any other unique trait if applicable, is accepted for long term conservation.

The qualified accessions are then subjected to drying, which is the most crucial step in genebank processing. A walk-in-drying chamber functioning at 15 % RH and 15 °C is used for the drying purpose. For species with hard seed coats and which require longer drying duration are shifted to batch dryers, after preliminary drying in chamber. The standard moisture testing method used in gene banks is the hot-air oven method and the procedure recommended for each crop by the International Seed Testing Association is duly followed. Once the desired moisture is achieved (as mentioned below), the seeds are packed in aluminum foil packets. They have the advantage that they can be resealed and also occupy less space than other containers.

Conservation of genetic resources is carried out through two types of collections:

(a) Collection of seed samples for long term conservation, which is known as base collection. Base collections are maintained at –18 to –20 °C, to ensure seed viability for maximum possible time period. The moisture content of seed to be stored as base collections should be between 3 and 7 % depending on the species.
(b) Collection of seed samples for immediate use, termed as active collection. Active collection are maintained in conditions that ensure at least 65 % viability for 10–20 years. The moisture content of seeds to be stored as active collections should be between 3 and 8 % for seeds having poor storability and between 7 and 11 % for seeds having good storability, depending on the temperature used for storage.

These collections are conserved in different types of storage facilities. Depending on the duration of storage, three basic types of storages are recognized:

(a) Short term storage. The period for short term storage of seeds is from one year upto 18 months. It requires a cool

and dry atmosphere (20–22 °C and 45–50 % RH) where the seeds can be conveniently stored for one to two years without much loss in their viability.

(b) Medium term storage. The active collection, which are generally larger than those meant for base collection are conserved in the medium term storage. The accessions are for regular distribution and therefore the time period for storage is not more than 5 yrs. The active collections are stored at temperatures ranging from 0–10 °C and relative humidity of 20–30 %.

(c) Long term storage. This is the storage facility for base collection, where the seeds that meet all the above mentioned criteria for NGB conservation are maintained at –18 to –20 °C.

The *ex situ* genebank at NBPGR comprises 12 long term modules holding the base collection. The active collections are distributed in 22 medium term modules maintained at 4 °C for storing germplasm at active sites. The genebank is currently being upgraded with new infrastructure and all efforts are being made to bring the NGB to global standards.

Crop wild relatives

Crop wild relatives (CWR) are wild taxa closely related to crop plants, including wild progenitors and/or wild forms of crops. Maxted et al. (2006) defined a CWR as a wild plant taxon that has an indirect use derived from its close genetic relationship to a crop. The closer the species related, the more the possibility/practicality to get their traits incorporated. They form an important source of useful traits such as agronomic, quality, biotic and abiotic stresses, which are identified as critical component for food security and environmental sustainability in the 21st century. CWRs are often associated with disturbed habitats and neither these habitats are offered adequate protection by ecosystem conservation agencies (Maxted, Kell, 2009) nor their diversity properly conserved *ex situ*. CWR diversity, like that for many species, is at a declining stage; which is associated with the loss of genetic diversity (Hopkins, Maxted, 2010). This necessitates the need to establish CWR inventories which is also an indispensable tool for exploration, surveys and collection of CWR. Therefore, the need for novel genes for developing climate resilient varieties, increasing pressure on wild species populations and habitats and the present meagre *ex situ* collections, all accentuate the importance of locating and collecting germplasm of wild relatives.

Arora and Nayar (1984) reported the occurrence of over 320 wild relatives of crops (51 cereals and millets; 31 grain legumes; 12 oilseeds; 24 fibre plants; 27 spices and condiments; 109 of fruits, 54 of vegetables and 27 of others) in India. The NHCP of ICAR-NBPGR serves as a nodal point for confirming the botanical identity of crop wild relative's taxa. With the identification of diversity-rich spots, availability of location details of intended taxa, India is moving forward in the systematic collecting of CWR from diverse habitats for conservation and sustainable use. Only one third of shortlisted taxa have been assembled by ICAR-NBPGR; among them more than half the taxa with <10 accessions. Analysis of gaps in collection in a scientific manner (keeping in view the conserved material, actual variability/diversity present in habitats, best utilization of GIS tools) through a mission-mode approach is currently being employed the way. In addition, detailed studies on habitat ecology, floral biology and breeding system, crossability (with crop), seed dormancy and storage behaviour of species would enable their meaningful conservation and sustainable utilization. Crossability studies aids in realization of gene-pool concept in crops, and knowing the closer relatives (even from different genera). Ensuring correct taxonomic identity, safe conservation and supply of germplasm to crop-based institutes would strengthen the pre-breeding/base-broadening/gene-pyramiding activities through designing suitable long term multi-parental breeding programmes. All these indicate the need for trained expertise in classical subjects like taxonomy and cytogenetics, with long-term commitment. Also it is imperative to undertake studies on assessing the gene flow between wild (progenitors and naturally crossable relatives) and cultivated taxa in the wake of concerns of biosafety. All taxonomic related species may not have an equal potential as a gene donor to crops (Maxted et al., 2007). Prioritization of CWRs for management preferably on genetic relationship is important for optimization of resources. Economic importance of the crop, crossability relationship, threat and rarity of the taxa and habitat, conservation status in the genebank are the other criteria for prioritization.

Conservation of niche-specific taxa needs attention as they are often rare and endemic. Predicted extinction of species is more likely to affect RET taxa. Various steps involved in the effective management of CWRs such as development of an inventory, prioritization of CWRs taxa and habitats, eco-geographic and genetic analysis of CWRs, threat analysis and genetic erosion assessment of individual CWRs taxa, gap analysis and fixing conservation targets, development of *ex situ*/*in situ* strategies, leading to conservation and finally utilization and sustainable availability for crop improvement (Maxted et al., 2007) are all important in the Indian context also. Constituting specialized group in the country devoted to these aspects of CWRs may be a feasible option.

The above studies would facilitate a national-level mapping of CWR distribution after incorporating additional information from eco-geographic studies, which will help in the identification of CWR hotspots, which can be matched with existing protected area network in the country, thereby areas and taxa demanding conservation can be identified (Maxted et al., 2011). Strong networking among all the stakeholders working on characterization, evaluation and conservation is the need of the hour, as it is difficult for a single institute to collect, conserve and evaluate all the target species due to paucity of land, resources and expertise.

PGR characterization and evaluation

The utilization of PGR in crop improvement programs rests on identification of promising accessions. The collected or introduced germplasm is characterized and evaluated to assess its potential, by recording data on agronomic traits such as yield, quality, and tolerance to biotic and abiotic stresses. The germplasm is also evaluated for new traits using molecular tools to identify the genes to develop new varieties as per requirement of the farmers. Approximately 10,000 accessions are characterized/evaluated every year at ICAR-NBPGR and its regional stations. Till date, more than 2.35 lakhs accessions of different agri-horticultural crops have been characterized and evaluated and passport data is available.

Core sets have been developed to facilitate the enhanced utilization of germplasm. Genetic diversity in large collection has been determined using morphological and DNA fingerprinting markers. Mega programme on characterization and evaluation under the National Initiative for Climate Resilient Agriculture (NICRA) executed in collaboration with SAUs for 21,822 accessions of wheat and 18,775 accessions of chickpea. Generation, validation and utilization of genomic resources is one of the major objective of ICAR-NBPGR. These resources are utilized for value addition to the plant germplasm resources harboured in the genebank and for generating molecular profiles varieties of agri-horticultural crops. The advent of next generation sequencing with improved chemistries and lower input costs have resulted in high throughput data that could be mined for generating SSR and SNP markers.

Application of genomic tools for PGR utilization and pre-breeding

One of the major objectives of ICAR-NBPGR is to supply germplasm, collected indigenously or from exotic sources, to the breeders and other researchers in the country. These germplasm accessions have helped to develop improved varieties in various national programmes. Till date more than 5 lakh samples were supplied for utilization to various stakeholders for use in crop improvement programmes. Ever-increasing significance of conservation and utilization of PGR on one hand and advancements in computer technology for digitization and management of data on the other have catapulted PGR Informatics into limelight.

Genomics has provided various technologies including sequencing and re-sequencing platforms, availability of genome sequences as references, high-throughput genotyping platforms, SNP arrays, genome editing tools, etc. These technologies are shortly described here.

Genome sequencing. The inexpensive sequencing and resequencing technologies are the major driving forces behind increased number of assembled plant genomes of different crops including wild relatives. A single reference genome does not represent the total diversity within a species, hence, resequencing of cultivars, landraces and wild accessions is required to harness the total genetic variation and to identify the superior alleles for the target traits. Genome information availability has generated many next-generation sequencing-based platforms for allele mining and candidate genes identification. Next generation sequencing and whole-genome resequencing is required for discovery, validation, and assessment of diagnostic markers in different crops and it provides genome-wide markers. The draft genome sequences are now available in a number of crops through different genome sequencing consortia for rice International Rice Genome Sequencing Project (IRGSP 2005), pigeonpea (Varshney et al., 2014), chickpea (Varshney et al., 2018), wheat International Wheat Genome Sequencing Consortium (IWGSC 2018), etc.

The genome sequencing using NGS has resulted in large collections of functional markers which enhance gene assisted breeding, reducing the possibility of losing the desirable trait variation due to recombination. Sequencing and resequencing of populations developed in crossing programs or of natural population (germplasm) along with high-throughput phenotyping helps in identification and linking of variations in gene sequences to their phenotypes. Kim et al. (2016) reported the whole-genome resequencing of the 137 rice mini core collection, potentially representing 25,604 rice germplasms in the Korean genebank of the Rural Development Administration (RDA) based on the Nipponbare reference genome, and resequencing data yielded more than 15 million SNPs and 1.3 million INDELs. Further study of this rice mini core with phylogenetic and population analysis using 2,046,529 high-quality SNPs successfully assigned rice accessions to the relevant rice subgroups, suggesting that the SNPs capture evolutionary signatures present in rice subpopulations. Similarly, a population structure analysis of 300 rapeseed accessions (278 representative of Chinese germplasm, plus 22 outgroup accessions of different origins and ecotypes) was carried out based on the 201,817 SNPs obtained from sequencing, divided accessions in nine subpopulations (Zhou et al., 2017). However, hierarchical clustering and principal component analysis showed intermingle of spring type accessions with semi-winter types pointing out towards frequent hybridization between spring and semi-winter ecotypes in China.

Sequence-based markers associated with rare elite alleles facilitate positional cloning and prebreeding. In case of PGR including landraces and wild relatives, screening of collection to be used for genomic analysis can be done based on passport data (collection site, specific traits, etc.) in combination with evaluation data. Sequencing based approaches provide opportunity to identify novel variations for a large number of genes through genotype-phenotype associations. Resequencing of large number of genotypes helps in determining process of origin, domestication, population structure and identifies lines with deleterious mutations in the genomes that can be eliminated to minimize the genetic load in the crop species as observed in case of maize (Bevan et al., 2017). NGS technologies together with precise phenotyping have been used for identification of marker trait associations in several crops, for example, rare wheat haplotypes effective against abiotic or biotic stresses were developed through introgression of useful and novel stress and quality traits' alleles to lines derived from crosses of exotics with CIMMYT's best elite germplasm under CIMMYT's Seeds of Discovery (SeeD) initiative (Vikram et al., 2016). Singh et al. (2018) used next-generation sequencing, together with multi-environment phenotyping to study the contribution of exotic genomes to 984 three-way-crossderived (exotic/elite1//elite2) pre-breeding lines (PBLs) for accelerating grain yield gains using exotic wheat genetic resources.

Molecular markers and genetic maps. Recent developments in genome sequencing and or resequencing has resulted in development of large number of molecular markers in different crops. Availability of molecular markers linked to specific traits enhances pre-breeding efficiency and effectiveness through marker assisted selection (MAS). Molecular markers that are linked to the genes of a desired trait known as diagnostic markers can be indirectly used for selection of target traits (Xu, Crouch, 2008). A major earlier success for crop breeding using genomic markers was the marker-assisted introgression of the ethylene response factor, known as Submergence 1A (Sub1A) gene, for submergence tolerance into high-yielding commercial rice varieties which acts by limiting shoot elongation during the inundation period (Bailey-Serres

et al., 2010). Riar et al. (2012) used polymorphic D-genome-specific SSR markers for analysing the cosegregation of the 5DS anchored markers (*Xcfd18*, *Xcfd78*, *Xfd81* and *Xcfd189*) with the rust resistance in an F_2 population, and mapped the leaf rust resistance gene (*LrAC*, a novel homoeoallele of an orthologue *Lr57*) on the short arm of wheat chromosome 5D. Vikal et al. (2014) used SSR markers for pyramiding of candidate genes for *xa8*, the resistance gene against Bacterial blight disease in elite rice varieties. Ellur et al. (2016) incorporated a novel Bacterial blight resistance gene *Xa38* in variety PB1121 from donor parent PR114-*Xa38* using a modified marker-assisted backcross breeding (MABB) scheme.

Genomics has provided powerful approaches to understand interaction between many genes and complex signalling pathways in case of polygenic traits like resistance to abiotic and biotic stresses. In rice breeding, high-density genome maps are being effectively used in background selection integrated with foreground selection of bacterial blight resistance (*xa13* and *Xa21* genes), amylose content (waxy gene) and fertility restorer gene in order to identify superior lines with maximum recovery of Basmati rice genome along with the quality traits and minimum non-targeted genomic introgressions of the donor chromosomes (Gopalakrishnan et al., 2008). Quantitative trait loci (QTL) analysis of the genome linked to quantitative phenotypic traits, has yielded climate goverened QTL in diverse crop species (Scheben et al., 2016). Rodrigues et al. (2017) determined protein content and genetic divergence of twenty-nine soybean genotypes using 39 microsatellite markers from QTL regions of the trait grain protein content for plant breeding purposes. The pairs of genotypes with greater genetic distances and protein contents were selected to produce populations with higher means and genetic variances and greater gains with selection.

Genome wide association studies (GWAS) could overcome several constraints of conventional linkage mapping and provide a powerful complementary strategy for dissecting complex traits. GWAS make use of past recombinations in diverse association panels to identify genes linked to phenotypic traits at higher resolution than QTL analysis. GWAS has become a powerful tool for QTL mapping in plants because a broad range of genetic resources may be accessed for marker trait association without any limitation on marker availability. Different approaches used for GWAS include:

(a) SNP marker arrays or SNP chips approach. Discovery and tagging of new genes using GWAS or QTL analysis have now become much easier. The availability of high-density SNP marker arrays has opened a way for cost effective GWAS using natural populations. Wang et al. (2017) developed a high-throughput NJAU 355K SoySNP array and conducted GWAS in 367 soybean accessions (including 105 wild and 262 cultivated) across multiple environments and reported a strong linkage disequilibrium region on chromosome 20 significantly correlated with seed weight. Zhao et al. (2019) carried out meta-analysis GWAS using 775 tomato accessions (including wild accessions) and 2,316,117 SNPs from three GWAS panels and discovered 305 significant associations for the contents of sugars, acids, amino acids, and flavor related volatiles.

(b) Genotyping by sequencing (GBS) approach. As the cost of sequencing is continuously declining, GBS also known as next generation genotyping method, is becoming more common for discovering novel plant SNPs and used them for GWAS studies (Arruda et al., 2016). Kim et al. (2016) reported the whole-genome resequencing of 137 rice mini core collection and conducted genome wide association studies on four agriculturally important traits including 'grain pericarp colour', 'amylose content', 'protein content', and 'panicle number' and identify some novel alleles. Similarly, Arora et al. (2017) genetically characterized 177 *A. tauschii* accessions using GBS to study the variation for grain size using genome-wide association study.

Genomic selection. Genomics assisted breeding approach known as genomic selection (GS) is a better approach which simultaneously uses large genotypic data (genome wide) (exceeding phenotypic data), phenotypic data and modelling using statistical tools to predict the genomic estimated breeding values (GEBVs) for each individual (Meuwissen et al., 2001; Crossa et al., 2017). In genomic selection, a statistical model is generated using a representative population of the breeding population known as training population. This model is subsequently used to calculate the allelic effects of all marker loci, i. e. genomic assisted breeding values without having phenotypic data and these values can be used for preselection of trait-specific genotypes (Heffner et al., 2011). Xu et al. (2012) and Spindel et al. (2016) highlighted that coupling of genome wide data with genomic selection offered great specificity and predictability which can be used to accelerate prebreeding. Using GS, complex traits can be improved rapidly through generation of reliable phenotypes by shortening the selection cycle. GS application in pasture grass *Lolium perenne* resulted in four-year reduction in the breeding cycle (Lin et al., 2016). In genomic selections, genomic estimated and true breeding values were found to be closely correlated, even for polygenic traits with low heritability (Jia, Jannink, 2012). GS can facilitate selection of complex traits, e. g., grain yield (Saint Pierre et al., 2016) and tolerance to abiotic and biotic stress.

In genomic selection, genetic diversity specific to the population or family (species) of interest is captured through markers developed through GBS which minimized the ascertainment bias. GS is superior in respect of fixing all the genetic variation and to select individuals with higher Genomic Breeding Value (GEBV) without any phenotyping. In case of polyploid species, polysomic inheritance and possibility of double reduction requires specific consideration while using for genomic selection.

Genome editing. Recent advancements in genomics have also made feasible the editing of genomes and their use in crop improvement programs. Pre-breeding involves genetic transformation through recombination and genome editing (GE) tools provide an alternative. To replace conventional genetic engineering, a number of genome editing technologies have been developed during last two decades including antisense, RNA interference (RNAi), virus-induced gene silencing (VIGS), oligonucleotide directed mutagenesis (ODM), zinc finger nuclease (ZFN), transcription activator-like effects nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) (Sauer et al., 2015). These genome editing technologies can accelerate pre-breeding programs through beneficial knockout mutations,

e. g. identification of genes for disease resistance or suppressing of unwanted traits linked with desired traits in wild species, as products of these technologies are not considered a genetically modified organism (GMO) (Huang et al., 2016). Most of these genome editing tools except RNAi, act by inserting, removing or replacing specific regions of genome with the help of specific nucleases known as "molecular scissors" (Esvelt, Wang, 2014).

GE approaches can be used to modify genes with defined quantitative trait nucleotides (QTNs) that cause a sizeable phenotypic effect. Further, GE tools can be used for broadening the allele pool through generating targeted variations useful for genomic selection (Scheben, Edwards, 2017; Scheben et al., 2017). Using GE tools, desired traits can be physically linked to ensure their co-segregation known as "trait stacking" (Urnov et al., 2010). For example, ZFN-assisted gene targeting helped insertion of heritably insert herbicide-resistant genes (SuRA/SuRB and PAT) in the *Z. mays* genome (Shukla et al., 2009). Zhang et al. (2016) recently used CRISPR/Cas9 system for production of homozygous transgene free wheat mutants. Jenko et al. (2015) showed that GE and GS, both can be combined and referred as the promotion of alleles by genome editing (PAGE) which also has a great potential for pre-breeding. Recently, Gupta (2019) reviewed the latest modification of CRISPR/Cas9 system, a base editing technology applicable to DNA as well as RNA, has revolutionized GE and demonstrated in several crops including rice, maize, wheat, etc. will be highly useful for base broadening to be used for genomic selection and relating phenotypes to genes through mutant development, particularly in primitive landraces and wild species and will provide a new direction to pre-breeding programmes.

PGR Informatics

PGR Informatics is the management (creation, storage, retrieval and presentation) and analyses (discovery, exploration and extraction) of diverse information (facts, figures, statistics, knowledge and news). PGR Informatics has assumed significance because of the following factors: (i) increased awareness about PGRFA, (ii) various international agreements (CBD, GPA, ITPGRFA) coming into force, (iii) availability of information in text, images, maps, videos, etc., (iv) technologies to record, link and archive such diverse types of information, (v) growing power (and falling costs) of computers and internet to facilitate access and retrieval. Fundamental merit of an organized digital information system is that it provides fair and just opportunity for all to access. On-line portals, as a consequence of PGR Informatics, enable non-exclusive access to PGR information to a large number of users involved in overlapping research areas on PGR management. Typically information is collected on details of multitude of passport data including taxonomy, biogeography, and ethnobotany of the germplasm acquisitions (domestic collections and exotic introductions), their seed health, multiplication for supply and conservation, regeneration, experimental data on characterization and evaluation leading to utilization. In addition to field data, it also includes biochemical and genomic data as well as publications. Once the information is digitized and stored, computer technologies allow management and analysis irrespective of the scale and types of data leading to better visualization and predictions.

The need for countries to develop, maintain and exchange information "from all publicly available sources, relevant to conservation and sustainable use of biological diversity" including "results of technical, scientific and socio-economic research" has been recognized in the Convention on Biological Diversity (CBD, Articles 7d, 17), and the Global Plan of Action (GPA, priority activities 17 and 18). Information of this nature is imperative for planning and implementing activities; sustainable use and sharing of benefits accrued from its use. Global assessment indicates that many of the world's PGR are insufficiently and poorly documented. The passport information and characterization and evaluation data on genebank accessions conserved in genebanks are either lacking or poorly recorded or scattered at different places, such as passport data sheets, reports of collection and exploration missions, crop catalogues, published articles, etc. In addition, there exist informal or non-coded knowledge held by traditional farmers and indigenous people. To use this information efficiently and effectively, valuable information needs to be collected, collated, maintained and exchanged with the help of PGR Informatics.

Important PGR Informatics applications developed and maintained at NBPGR are:

- PGR Portal pgrportal.nbpgr.ernet.in
- Import Permit and EC Data Search exchange.nbpgr.ernet.in
- Genebank Dashboard genebank.nbpgr.ernet.in
- PGR Map pgrinformatics.nbpgr.ernet.in/pgrmap 5
- National Herbarium of Crop Plants pgrinformatics.nbpgr.ernet.in/nhcp
- Biosystematics Portal pgrinformatics.nbpgr.ernet.in/cwr
- PGR Climate pgrinformatics.nbpgr.ernet.in/pgrclim
- PGR and IPRs http://pgrinformatics.nbpgr.ernet.in/ip-pgr/

Recent advances in PGR Informatics in India

NBPGR has been striving to establish PGR information set up since 2002 (Archak, Agrawal, 2012). Development of mobile apps in PGR Informatics facilitates enhanced access to PGR information which in turn could lead to enhanced utilization. NBPGR has developed two mobile apps "Genebank" and "PGR Map". Both the apps are first of their kind for any genebank in the world. The apps have been developed for both Android and iOS. No other ICAR app is available for iPhone. Licenses were purchased and the apps have been hosted on Google Play and App Store.

Genebank app provides a dashboard view of indigenous collections (state-wise), exotic collections (country-wise), addition of accessions to genebank, etc. The app also helps generate routine genebank reports. The app uses databases live on the backend and hence always gives updated information.

PGR Map app offers three benefits: "What's around me" helps user to obtain quickly the accessions that have been collected and conserved in the genebank from a particular location in India where the user is located at the moment; "Search the map" helps user to list the accessions that have been collected and conserved in the genebank from any selected location in India; "Search for species" helps user to map the collection sites of a crop species.

Perspectives

All countries are interdependent for their PGR requirements and cannot acquire and conserve resources to satisfy all their needs. There is a need to collaborate at local, regional and international levels for the acquisition and conservation of the germplasm. It is also imperative to obey the quarantine and biosafety rules for the safe movement of germplasm. Priority collection trips are required to identify areas which are not sufficiently covered so far and a repeated visits are required to be made in areas that showed diversity in the past. New tools of geographical information system (GIS) and remote sensing need to be deployed to supplement the existing ground data in PGR programmes to exploit agro-biodiversity particularly in difficult/inaccessible areas.

There is a need to adopt complementary conservation strategies involving both *in situ* and *ex situ* approaches. For *in situ* conservation due attention is required to be given to genetically rich hotspots including tribal belts and to strengthen and expand the network of germplasm conservation by including all the stakeholders, including the communities. Characterization and evaluation are essential to promote the utilization of materials. These tasks require substantial inputs and a decentralized evaluation network. There is a need to modify the descriptors for evaluation accordingly, and make the search for the desired characteristics in the database as quick and efficient as possible. The core collection concept is more structured and efficient approach to identify limited sets of diverse germplasm and utilise the same more effectively. Pre-breeding is needed to incorporate new kinds of pest resistance, to bring in new levels of productivity and stability of performance, and to provide quality traits for food and feed products. Awareness generation of the people at various levels (policy makers, scientific, administration, farmers, etc.) about the value of PGR wealth, its protection and conservation is essential. In addition the interface among different stakeholders is likely to bring out new useful PGR management alternatives.

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Conflict of interest. The authors declare no conflict of interest.
Received April 3, 2019. Revised April 3, 2020. Accepted April 3, 2020.

Вавиловский журнал генетики и селекции. 2020;24(3):315-324
DOI 10.18699/VJ20.623

In cherished memory of Lyudmila Pozdnyakova, who made indispensable contribution to the creation and population of the collection

The Collection of Rhizosphere Microorganisms: its importance for the study of associative plant-bacterium interactions

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Abstract. Microbial culture collections are very important components of biological science. They provide researchers with material for studies and preserve biological resources. One such collection is the Collection of Rhizosphere Microorganisms, kept at the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences, Saratov (IBPPM). Its activity is primarily directed toward the isolation and preservation of microorganisms from the plant root zone. The international research interest in microorganisms from this ecological niche is not waning, because they are very important for plant growth and development and, consequently, for plant breeding. The group of bacteria with properties of significance for plants has been given the name "plant-growth-promoting rhizobacteria" (PGPR). This group includes nitrogen-fixing soil alpha-proteobacteria of the genus *Azospirillum*, which form the core of the IBPPM collection. First discovered by Brazilian scientists in the 1970s, azospirilla are now a universally recognized model object for studying the molecular mechanisms underlying plant-bacterium interactions. The broad range of useful properties found in these microorganisms, including the fixation of atmospheric nitrogen, production of phytohormones, solubilization of phosphates, control of pathogens, and formation of induced systemic resistance in the colonized plants, make these bacteria an all-purpose tool that has been used for several decades in basic and applied research. This article reviews the current state of *Azospirillum* research, with emphasis on the results obtained at the IBPPM. Scientific expeditions across the Saratov region undertaken by IBPPM microbiologists in the early 1980s formed the basis for the unique collection of members of this bacterial taxon. Currently, the collection has more than 160 *Azospirillum* strains and is one of the largest collections in Europe. The research conducted at the IBPPM is centered mostly on the *Azospirillum* structures involved in associative symbiosis with plants, primarily extracellular polysaccharide-containing complexes and lectins. The development of immunochemical methods contributed much to our understanding of the overall organization of the surface of rhizosphere bacteria. The extensive studies of the *Azospirillum* genome largely deepened our understanding of the role of the aforesaid bacterial structures, motility, and biofilms in the colonization of host plant roots. Of interest are also applied studies focusing on agricultural and environmental technologies and on the "green" synthesis of Au, Ag, and Se nanoparticles. The Collection of Rhizosphere Microorganisms continues to grow, being continually supplemented with newly isolated strains. The data presented in this article show the great importance of specialized microbial culture repositories, such as the IBPPM collection, for the development and maintenance of the microbial research base and for the effective solution of basic and applied tasks in microbiology.

Key words: microbial culture collection; *Azospirillum*; rhizosphere; plant-growth-promoting rhizobacteria; associative symbiosis.

For citation: Turkovskaya O.V., Golubev S.N. The Collection of Rhizosphere Microorganisms: its importance for the study of associative plant-bacterium interactions. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(3):315-324. DOI 10.18699/VJ20.623

Коллекция ризосферных микроорганизмов: значение для исследования растительно-бактериальной ассоциативности

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Аннотация. Коллекции микроорганизмов – один из важнейших компонентов биологической науки, который обеспечивает исследователей необходимым материалом и сохраняет биологические ресурсы. Таковой является Коллекция ризосферных микроорганизмов Института биохимии и физиологии растений и

микроорганизмов Российской академии наук (ИБФРМ РАН), деятельность которой сосредоточена в первую очередь на выделении и сохранении микроорганизмов, выделенных из корневой зоны растений. Интерес мировой науки к микроорганизмам этой экологической ниши не ослабевает по причине их большой значимости для роста и развития растений и, следовательно, для растениеводства. Группа бактерий, обладающих полезными для растений свойствами, получила название PGPR (plant growth promoting rhizobacteria – стимулирующие рост растений ризобактерии). К ним относятся и почвенные азотфиксирующие альфа-протеобактерии рода *Azospirillum*, составляющие ядро вышеназванной коллекции. Азоспириллы, открытые в 70-х гг. прошлого века бразильскими учеными, в настоящее время являются признанными во всем мире модельными объектами для изучения молекулярных механизмов растительно-микробных взаимодействий. Фиксация атмосферного азота, продукция фитогормонов, солюбилизация фосфатов, контроль патогенов, формирование у растений индуцированной системной устойчивости – целый комплекс полезных свойств делает их универсальным инструментом для фундаментальных исследований и практического применения. В обзоре обсуждается современное состояние исследований по бактериям рода *Azospirillum* с акцентом на результатах, полученных коллективом ИБФРМ РАН (г. Саратов). Экспедиции по Саратовской области, проведенные микробиологами института в начале 1980-х гг., заложили основу уникального собрания представителей этого бактериального таксона, которое сегодня включает более 160 штаммов и считается одним из самых крупных в Европе. Исследования сотрудников ИБФРМ РАН преимущественно сосредоточены на структурах азоспирилл, вовлеченных в образование ассоциативного симбиоза с растениями. Прежде всего это внеклеточные полисахаридсодержащие комплексы и лектины. Развитие методов иммунохимии во многом позволило выяснить, как в целом организована поверхность бактерий. Благодаря изучению генома азоспирилл существенно углубилось понимание роли вышеупомянутых структур, а также подвижности бактерий и формирования ими биопленок при заселении корней. В прикладном аспекте заслуживают внимания исследования азоспирилл, направленные на развитие агро- и экотехнологий, а также технологии «зеленого» синтеза наночастиц золота, серебра и селена. Коллекция продолжает развиваться, пополняясь новыми штаммами, показывая большое значение специализированных собраний микроорганизмов для создания и поддержания исследовательской базы и эффективного решения фундаментальных и прикладных задач микробиологии.
Ключевые слова: коллекция микроорганизмов; *Azospirillum*; ризосфера; стимулирующие рост растений ризобактерии; ассоциативный симбиоз.

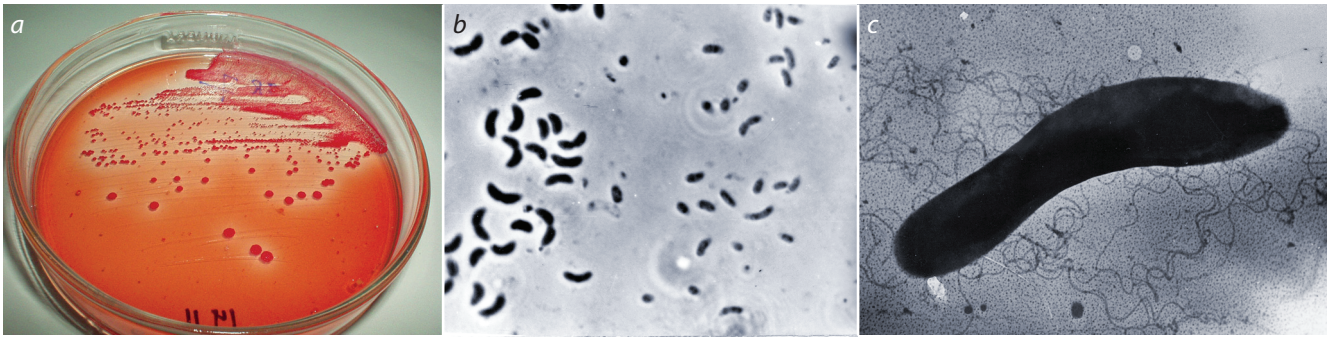
Introduction

The conservation and development of biological collections in the Russian Federation is part of the interdepartmental and interdisciplinary priority problem of preserving biological resources and biodiversity, as well as the national biological and food security building. The solution of this problem is the basis for the sustainable development of Russian science as a whole, modern science-intensive industries, and training of qualified personnel (Recommendations of the "round table"…, 2011). The currently existing collections (according to the World Federation for Culture Collections (WFCC), there are 715 of them (http://www.wfcc.info/ccinfo)) are tentatively divided into three categories (Kalakutskii et al., 1996; Ivshina, 2012): nonspecialized collections (service, integrated, and public), specialized collections (for the study and preservation of microorganisms of specific groups for specific purposes), and research collections (private, highly specialized). Until recently, a very large number of Russian collections were on the verge of ceasing their activities owing to the lack of funding. In recent years, with the approval of the Plan for the Development of Biotechnologies and Genetic Engineering (Decree of the Government of the Russian Federation, 2013), an attempt was made to provide targeted organizational and financial support to intensely working collections.

The above Plan includes the foundation of large bioresource centers (BRCs), which will be the most important element for the development of biotechnology in Russia (Kalakutsky, Ozerskaya, 2011), and integration into the European and global information networks of biological resources. It should be noted that there are already many BRCs in the world, which are represented by both departmental and private non-profit organizations. One such BRC was established in 2014 on the basis of a leading Russian collection (the Russian Collection of Industrial Microorganisms, VKPM), designed to become the basis of the infrastructure in the field of microbial genetic resources for biotechnological purposes necessary for supporting research in living systems (http://www.genetika.ru/vkpm). Several large Russian collections may claim the BRC status. Most of the collections existing as specialized units at scientific and educational organizations cannot meet the criteria of such large structures, but they deserve attention as well. As a rule, they possess both a significant panel of cultures and the most complete information about them, which is their significant advantage. One such specialized unit is the IBPPM Collection of Rhizospheric Microorganisms (CRM IBPPM).

The IBPPM Collection of Rhizospheric Microorganisms

The Institute of Biochemistry and Physiology of Plants and Microorganisms, Academy of Sciences of the USSR (IBPPM), founded in Saratov in 1980, was focused on the tasks of increasing crop yields in the Volga region. One of them was the justification of the possibility of using microbial fertilizers and, in this regard, the clarification of the role of associative nitrogen-fixing microorganisms in the nitrogen nutrition of crops. To solve this problem, the diazotrophic bacterium *Azospirillum* was chosen as a model research object, publications about which had appeared shortly before (Tarrand et al., 1978). The Institute's microbiologists mastered the methods



Visualization of *Azospilillum* bacteria:

a, growth of azospirilla on potato agar; *b*, azospirilla in a liquid medium, 18 h, phase contrast; *c*, azospirilla under the electron microscope, 18 h, ×25,000, contrasted with uranyl acetate (Pozdnyakova et al., 1988).

of isolation and identification of these microorganisms and worked out methods for their conservation. In the course of expeditions in the Saratov region, undertaken to obtain a representative collection of *Azospirillum* strains associated with wild and cultivated cereals (wheat, rye, oats, millet, maize, sorghum, etc.) (Fedorova et al., 1985; Pozdnyakova et al., 1988), the investigators laid the foundation for a unique set of members of the studied bacterial taxon (see the Figure), becoming part of the IBPPM Collection of Non-Pathogenic Microorganisms.

The key characteristics of azospirilla in the first stages of isolation were: colony growth on a medium with Congo red (Caceres, 1982) and on potato agar (Tarrand et al., 1978), as well as microscopy and Gram staining. Because the main features of the isolated bacteria were the fixation of atmospheric nitrogen and the promotion of plant growth, the isolates were screened for nitrogenase and IAA-producing activities.

Taxonomic studies were carried out with original strains of azospirilla. In addition to interspecific differentiation by methods based on DNA–DNA hybridization, intraspecific differences were evaluated with two variants of genomic fingerprinting: RFLP and AFLP (Pozdnyakova et al., 1988; Nikiforov et al., 1994a). The plasmid composition of the strains was studied (Matveev et al., 1988). The obtained isolates were screened for restriction endonuclease activity (Nikiforov et al., 1994b). Storage methods were being optimized for four years. Long-term observations showed that *Azospirillum* strains frozen in liquid nitrogen retained high viability and basic differential properties at –70 °C.

In 2014, the Collection of Non-Pathogenic Microorganisms was transformed into the CRM IBPPM (www.collection.ibppm.ru; http://ckp-rf.ru). It is a specialized scientific collection focused on the gathering and maintenance of non-pathogenic bacteria isolated mainly from the root zone of plants. It is a member of the WFCC with the number 975 and is registered at the World Data Center for Microorganisms (WDCM), No. 1021.

Currently, the collection catalog (www.collection.ibppm.ru) includes about 500 cultures of rhizospheric bacteria belonging to 28 genera: *Acidovorax*, *Aeromonas*, *Alcaligenes*, *Aquaspirillum*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Brevundimonas*, *Comamonas*, *Ensifer* (*Sinorhizobium*), *Enterobacter*, *Herbaspirillum*, *Kocuria*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Nitrospirillum*, *Niveispirillum*, *Nocardioides*, *Ochrobactrum*, *Paenibacillus*, *Pectobacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Stenotrophomonas*, and *Xanthomonas*. Among them are bacteria isolated from the rhizosphere and rhizoplane of various wild and cultivated plants, endophyte strains, strains associated with aquatic plants (macrophytes), etc.

Members of the genus *Azospirillum* constitute approximately half of the collection. In addition to the original strains isolated in the Saratov region, the collection includes azospirilla of various geographical origins (Brazil, India, Senegal, the USA, Ecuador, etc.) provided by researchers or other culture collections. In addition, almost all the type strains of known *Azospirillum* species available in public depositories are maintained. Among the collection strains, there may be members of new species of this genus (Golubev et al., 2018). Thus, the *Azospirillum* set in the CRM IBPPM is highly representative. For comparison, in DSMZ (Deutcsche Sammlung von Mikroorganismen und Zellkulturen, www.dsmz.de), the total bacterial stock of the genus *Azospirillum* is represented by 27 strains, of which 6 are type strains; at BCCM/LMG (Belgian Co-ordinated Collections of Microorganisms/Laboratory of Microbiology, Faculty of Sciences of Ghent University, bccm.belspo.be), 37 and 10; at JCM (Japan Collection of Microorganisms, jcm.brc.riken .jp), 13 and 5; in ATCC (American Type Culture Collection, www.lgcstandards-atcc.org), 14 and 3, respectively.

Characterization of the genus *Azospirillum*

This microorganism was first isolated by Beijerinck in 1923 and described as a “nitrogen-fixing Spirillum”, but the researcher was unable to confirm its ability to fix nitrogen in pure culture and called this microorganism *Spirillum lipoferum* (Beijerinck, 1925). The genus *Azospirillum* was rediscovered and described by Tarrand et al. (1978) but became widely known thanks to the scientific enthusiasm and competence of Johanna Döbereiner, who made a significant contribution to the basic and applied research on azospirilla (Döbereiner, Day, 1976; Hartmann, Baldani, 2006).

The taxonomy of the genus *Azospirillum*, belonging to the family *Rhodospirillaceae* of the order *Rhodospirillales* in the

class α-Proteobacteria, is developing fast. From the moment the first two species, *A. lipoferum* and *A. brasilense*, were described (Tarrand et al., 1978) and until the end of the second millennium, only four more species were discovered: *A. amazonense* (Magalhaes et al., 1983), *A. halopraeferens* (Reinhold et al., 1987), *A. irakense* (Khammas et al., 1989) and *A. largimobile* (Dekhil et al., 1997). However, since 2000, 16 new species have been described with valid published names: *A. doebereinerae* (Eckert et al., 2001), *A. oryzae* (Xie, Yokota, 2005), *A. melinis* (Peng et al., 2006), *A. canadense* (Mehnaz et al., 2007a), *A. zeae* (Mehnaz et al., 2007b), *A. rugosum* (Young et al., 2008), *A. picis* (Lin et al., 2009), *A. thiophilum* (Lavrinenko et al., 2010), *A. formosense* (Lin et al., 2012), *A. humicireducens* (Zhou et al., 2013), *A. fermentarium* (Lin et al., 2013), *A. soli* (Lin et al., 2015), *A. agricola* (Lin et al., 2016), *A. ramasamyi* (Anandham et al., 2019), *A. griseum* (Yang et al., 2019), and *A. palustre* (Tikhonova et al., 2019). In 2014, *Azospirillum irakense* was reclassified as *Niveispirillum irakense* comb. nov. and *Azospirillum amazonense*, as *Nitrospirillum amazonense* gen. nov., sp. nov. (Lin et al., 2014). Thus, at the time of this writing, the genus *Azospirillum* includes 20 species with valid published names. It is important to note that two more species are recognized as members of the genus: *A. palatum* (Zhou et al., 2009) and *A. himalayense* (Tyagi, Singh, 2014), whose names were not validly published in conformity with the rules of the International Code of Nomenclature of Bacteria.

The onset of the era of genomic sequencing highlighted the development of reliable criteria for the comparative assessment of the genomes of bacteria and archaea for taxonomy and systematics. Recently, the first results of applying a set of phylogenetic tests in the context of the development of microbe genomic taxonomy were obtained for the group of bacteria of the genus *Azospirillum* whose genomes were present in the GenBank database (Shchyogolev, 2018). The author revealed the dependence of the assessment of the taxonomic position of strains on the type of full-genome data used related to the core or core and variable components of the pangenome. It was noted that there was no unified system of assigning isolates to one or another species yet. Until the genomic databases are filled to the necessary extent with high-quality material, the so-called "polyphase" approach, based on a combination of phenotypic, chemotaxonomic, and genotypic characteristics, remains the most correct in the taxonomy and systematics of prokaryotes.

Azospirilla, chosen as an object of research in the infancy of IBPPM, turned out to be an excellent model for studying associative plant-microbe interactions. Currently, *Azospirillum* is one of the universally recognized and widely studied plant-growth-promoting rhizobacteria (PGPR) (Fukami et al., 2018). Such bacteria play an important role in helping the plant adapt to external influences. In this case, a plant-microbial association (associative symbiosis) with new properties determined by positive interactions between partners is often formed.

The extreme diversity of colonizable plant species is characteristic of azospirilla, which indicates the multitude of ecological strategies implemented by these bacteria, as well as their wide adaptive capabilities (Bashan et al., 2004; Baldani et al., 2014; Pereg et al., 2016). Bacteria adapt to their environments through such abilities as fixation of atmospheric nitrogen; solubilization of phosphates; and production of exopolysaccharides, lectins, phytohormones, siderophores, poly-β-hydroxybutyrate, etc. There is evidence (Fukami et al., 2018) that *Azospirillum* bacteria are involved in the formation of so-called induced systemic resistance (ISR) in partner plants exposed to biotic stress, as well as induced systemic tolerance (IST) to abiotic stress.

Most *Azospirillum* species were isolated from the rhizosphere of land plants. So far, only three species were isolated from aquatic biotopes: *A. largimobile* (Dekhil et al., 1997), *A. thiophilum* (Lavrinenko et al., 2010), and *A. griseum* (Yang et al., 2019). Sequencing of the *Azospirillum* genome showed that this bacterium shifted from aquatic to terrestrial existence at the same time as vascular plants appeared on land: about 400 million years ago (Wisniewski-Dyé et al., 2011). Almost half of the *Azospirillum* genome was acquired as a result of horizontal gene transfer from other terrestrial bacteria. Most horizontally acquired genes encode functions that are critical to environmental adaptation.

The possession of phytostimulation mechanisms makes azospirilla one of the best inoculants that are employed in various countries to manufacture commercial biological products increasing crop yields: Azo-Green™, Zea-Nit™, Graminante™, BioPower®, etc. (Mehnaz, 2015).

The key results obtained at the IBPPM RAS in studies of *Azospirillum* bacteria as model objects

At the IBPPM RAS, the use of *Azospirillum* strains as model objects was focused mainly on the study of the structures involved in the formation of associative symbiosis and/or having important taxonomic significance. First of all, these are extracellular polysaccharide-containing complexes, which play very important and diverse roles in the formation and successful functioning of plant–microbe associations. Azospirilla produce intricate highly aggregated compounds of polysaccharides (PSs), lipids and proteins, as well as free PSs with molecular weights up to 20 kDa. These compounds are stored in the capsular material and released to the environment (Konnova et al., 1994). The capsular PS-containing components of azospirilla are involved in the adsorption of the bacteria to plant roots. Their ability to induce deformations of root hairs of wheat was shown for the first time (Yegorenkova et al., 2001). Novel information was obtained on the primary structures of repetitive PS units in surface lipopolysaccharides (LPSs) and capsular PSs of more than 40 *Azospirillum* strains of various origins (Fedonenko et al., 2013, 2015). In several strains isolated from plant roots on different continents, molecular mimicry of bacterial surface glycopolymers owing to the presence of identical or structurally similar repeating units in an O-specific PS (O-PS) was observed. It may be conjectured that this mimicry is associated with the implementation of certain strategies during the formation of associations with plants, possibly owing to the presence of several interaction

mechanisms, for example, endo- and ectosymbiosis (Konnova et al., 2008; Fedonenko et al., 2015).

Immunochemical methods are a good tool for the study of bacterial surface structures. Scientists can examine structural details of the main surface antigens of azospirilla to find out how the surface of rhizosphere bacteria is generally organized. LPS is a major antigen on the surface of azospirilla; therefore, the O-PS structure determines the immunochemical specificity of these microorganisms (Matora et al., 2005). With account taken of the immunochemical characteristics of the carbohydrate antigens of *Azospirillum* bacteria, a biotest system for their serological identification has been developed (Bogatyrev et al., 1992).

With the example of azospirilla, a fundamentally new type of microbial R-S dissociation was described, owing to the redistribution of the contributions of two different (full-fledged) O-PSs to the architecture of the bacterial cell surface, depending on the culture age (Matora et al., 2005). Carbohydrate fragments of the glycosylated flagellin of the polar flagellum from *A. brasilense* type strain Sp7 were isolated and studied, and their chemical structures were determined (Belyakov et al., 2012). These fragments were immunochemically identical to one of the two O-PS somatic antigens of strain Sp7. With account taken of the results obtained, which indicate the identity of antigenic determinants in capsular PSs, exopolysaccharides, and LPSs of azospirilla, it is reasonable to suggest a common pathway (or several intersecting pathways) for the biosynthesis of carbohydrate surface structures in *Azospirillum* bacteria.

For the first time, a variant of enzyme-linked immunosorbent assay (ELISA) of microsediments of soil suspensions was proposed that uses antibodies against *Azospirillum* LPSs. The assay allows the detection of the somatic bacterial antigen in soil. With the optimized ELISA variant, the dynamics of *in situ* detection of the somatic antigen of *A. brasilense* associative bacteria introduced into soil was studied (Shirokov et al., 2015).

A prominent direction in the progress of the immunochemical methodology at the IBPPM RAS is the study of unique physicochemical and biochemical properties of gold nanoparticles and their bioconjugates. By using colloid gold as a carrier and an adjuvant, as well as the phage display method, procedures have been developed to prepare antibodies to various antigens and haptens (Matora et al., 2005; Dykman et al., 2010). Gold and gold-silver nanoparticles were conjugated with antibodies to flagellin, LPS, and genus-specific surface-protein determinants of *A. brasilense* type strain Sp7. Electron microscopic analysis of the *A. brasilense* Sp245 cell surface involving antibodies labeled with metal nanoparticles revealed flagellin determinants of the polar flagellum, originally shielded from their environment by an LPS sheath in these bacteria (Shirokov et al., 2017).

Significant progress has been made in the genetics of motility, plasmid biology, and the genome organization and dynamics of *Azospirillum* bacteria (Katsy, 2011, 2014). A new type of social motility was revealed: spreading in a semiliquid medium with the formation of microcolonies. It is such spreading that is of decisive importance when wheat roots are colonized by azospirilla, while swarming is the dominant mode of social motility under laboratory conditions (Shelud'ko et al., 2010). It was shown that external factors (the presence of certain plant lectins, plant exudates, etc.) and spontaneous and induced changes in the genome, in particular, in the structure of megaplasmids, have a great influence on the social behavior of the bacteria. The genome changes are accompanied by phenotypic variations in the social motility of azospirilla (swarming → accelerated swarming; swarming → spreading with the formation of microcolonies; spreading with the formation of microcolonies → accelerated swarming), and they can lead to changes in the formation of biofilms and in the early stages of plant-root colonization (Katsy, Prilipov, 2009; Schelud'ko et al., 2009).

Insertion elements responsible for the plasticity of *A. brasilense* megaplasmids were described for the first time. New knowledge was obtained on the primary structures and functions of several such plasmids (Katsy, Prilipov, 2009). The insertion elements IS*Azba1* and IS*Azba3*, which mediate the fusion of the resident plasmid from *A. brasilense* Sp245 with foreign DNA, contribute to the enrichment of the *Azospirillum* genome with genetic material. The genome dynamics of *A. brasilense* has a significant effect on the structure of the bacterial LPSs and their antigenic properties, as well as on the resistance of these bacteria to heavy metals and nitrites (Katsy, Petrova, 2015). The *Azospirillum* genes that regulate motility, denitrification, and the production of LPSs and flagella have been identified. A collection of *A. brasilense* mutants, recombinant plasmids, and *Escherichia coli* strains containing cloned *Azospirillum* genes was established (Kovtunov et al., 2013).

Another component of the *Azospirillum* cell surface is carbohydrate-binding proteins, lectins. These are important structures in the system of "recognition" and the establishment of partnerships at the initial stages of associative bacterium–plant relationships. Studies of *Azospirillum* lectins at the IBPPM RAS began in the second half of the 1980s (Nikitina et al., 2005). It was for the first time that lectins with interstrain differences in carbohydrate specificity were found on the surface of azospirilla isolated from various sources (30 strains). It was revealed that lectins are distributed evenly on the outer membrane of the azospirilla and do not belong to any morphological structures such as pili or flagella (Karpunina et al., 1995). The dependence of lectin activity in bacteria on culturing conditions was found. Conditions unfavorable for culture growth stimulated lectin activity and vice versa. For the first time, the role of *Azospirillum* lectins associated with the outer membrane in the adhesion of the bacteria to wheat seedling roots was revealed. Bacterial lectins were found to interact with exocomponents, components of the membrane fraction, and root lectins of plant seedlings (Nikitina et al., 1996). Confocal laser scanning microscopy showed that the location of tritium-labeled *Azospirillum* lectins was confined to the plasma membrane of wheat seedling root cells. At the initial stages of interaction with the roots, lectins can elicit a broad range of biochemical responses that are part of plant signaling systems (Alen'kina et al., 2014).

A dose-dependent effect (inhibiting or promoting) of bacterial lectins on the germination of seeds of higher plants was shown. The regulatory effect of *Azospirillum* lectins on a number of their own and plant hydrolytic enzymes was revealed (Alen'kina et al., 2006). From the detected interaction of the polysaccharide-containing complexes of azospirilla with intrinsic lectins, as well as with surface-localized agglutinating proteins of other soil microorganisms (bacilli and rhizobia), participation of these extracellular glycopolymers in *Azospirillum* aggregation and in interbacterial contacts during the formation of soil communities may be inferred.

For the first time, the ability of azospirilla to reduce gold (III) ($AuCl_4^-$) and selenium (IV) (SeO_3^{2-}) to the elementary state (Kupryashina et al., 2013; Tugarova et al., 2014a, b) with the formation of nanoparticles was described. A simple scheme of bacterial synthesis of selenium nanoparticles with extracellular localization was proposed (Tugarova et al., 2018).

Various aspects of the azospirilla life are extensively studied by modern instrumental methods, including various spectroscopy options: Mössbauer, IR Fourier, and Raman scattering (Kamnev et al., 2001, 2018; Kamnev, Tugarova, 2017). By using glutamine synthetase isolated from *A. brasilense* Sp245 cells as an example, the possibility of applying nuclear gamma resonance spectroscopy (^{57}Co nuclei) to the examination of the structural organization of metal cation binding sites in active centers of enzymes was shown (Kamnev, Tugarova, 2017). For the first time in relation to *Azospirillum* bacteria, the assimilation of iron and the composition and structure of iron-containing cellular components were studied (Kovács et al., 2016), and so were the interaction and metabolic transformations of cobalt ions by *A. brasilense* cultures (Kamnev, Tugarova, 2017).

The synthesis of poly-β-hydroxybutyrate (PHB) by azospirilla is the most pronounced response to negative effects in these bacteria. For the first time, changes in bacterial PHB accumulation under prolonged exposure to stress and differences between *A. brasilense* strains Sp7 (epiphyte) and Sp245 (endophyte) in response to heavy metal stress were shown (Kamnev et al., 2018). A reduced PHB content in 6-day old biofilm formed by the flagella-free mutant *A. brasilense* Sp245.1610, as compared to the wild-type strain Sp245 was shown by Tugarova et al. (2017). The decrease in PHB content may affect the formation and stability of *Azospirillum* biofilms.

It is known that under adverse living conditions plants gain advantage if the protective rhizosphere associations contain microorganisms that perform a wide range of functions, including plant nutrition, resistance to abiotic stresses, biocontrol (protection against pathogens), and the removal of pollutants from soil (Tikhonovich, Provorov, 2009). The last function is performed by pollutant-degrading microorganisms. Azospirilla, possessing almost all the above properties, are typical representatives of protective rhizosphere associations. Screening of *Azospirillum* strains from the CRM IBPPM allowed the first detection of oil-oxidizing activity in some of them (Muratova et al., 2005). Use of the *A. brasilense* SR80-wheat model showed that oil neither interferes with the plant-growth-promoting activity of the micropartner nor affects the synthesis of bacterial IAA. *A. brasilense* SR80 showed chemotaxis not only toward root exudates of wheat but also toward crude oil (Muratova et al., 2005). This strain was also resistant to the toxic effects of glyphosate and showed a consistently high level of IAA production in the presence of the herbicide (Kryuchkova et al., 2005). On the basis of these results, a phytoremediation method was developed for hydrocarbon-contaminated soil in which *A. brasilense* SR80 was used as one of the bacterial cultures for inoculating plants (a mixture of leguminous and cereal seeds) (Patent RU 2403102). *A. brasilense* Sp245 associated with the roots of wheat seedlings of cv. Saratovskaya 29 can transform inorganic forms of arsenic (arsenite to arsenate); owing to this ability, the bacteria reduce the toxicity of the element (Lyubun et al., 2006).

On the basis of *A. zeae* strain from the CRM IBPPM, employees of the Bionovatic group of companies developed and produced a biological product named Organit N, aimed at improving the nitrogen nutrition of plants (bionovatic.ru). According to the manufacturer's recommendations, the preparation is applicable to cereals, legumes, corn, and sugar beet.

The high efficiency of artificial plant-microbial associations established *in vitro* with the participation of azospirilla was shown in the development of microclonal propagation technologies for plants to improve the quality of planting material of crops and preserve rare plant species that are sources of valuable biologically active compounds (Tkachenko et al., 2015).

Conclusions

Despite the rather long period of studying *Azospirillum* bacteria, the interest of the world scientific community in them does not wane; the number of publications dedicated to azospirilla has been steadily growing during the past decade, reaching 250 articles per year (according to www.scholar.google.com). The knowledge gained serves as a basis for expanding research on the variety of bacteria that form associations and symbioses with plants. It is in this direction that the CRM IBPPM develops at present, and in this regard, the role of such specialized collections can hardly be overestimated. As a result of IBPPM participation in the Russian-European project of the 7th Framework Program of the European Union "Banking Rhizosphere Micro-Organisms" (BRIO No. 266106, 2011–2014), some strains of the Collection were included in the pan-European database on rhizospheric microorganisms, designed to support both research on the rhizospheric microbiome and practical biotechnology (Declerk et al., 2015).

It should be noted that the results described above were obtained by modern methods, including those obtained in Russian and international projects: Russian Science Foundation, Russian Foundation for Basic Research, grants of the President of Russia, state contracts within governmental programs, ISTC, INTAS, NATO, FP-7, etc. The obtained data formed the basis for more than 80 candidate's and doctoral dissertations defended by researchers of the IBPPM. More than 600 articles were published in Russian and international scientific journals.

A scientific school was created under the guidance of Professor V.V. Ignatov, D. Sc. (Biol.), Honored Scientist of the Russian Federation. The school was repeatedly supported by grants of the President of the Russian Federation. Several inventions are protected by patents of the Russian Federation. PGPR strains and pollutant-degrading strains have become the subject of intense interest from domestic small businesses.

Thus, the example of the IBPPM Collection shows clearly the significance of such special collections for the fundamental and applied aspects of biological science.

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Acknowledgements. This work was supported in part by FP7 Banking Rhizosphere Micro-Organisms (project BRIO 266106), a European–Russian initiative to set up a network of rhizosphere microbiological resource centers.
The authors are grateful to the staff of the IBPPM RAS for advice on writing this manuscript: E.I. Katzy Dr. Sc. (Biol.), V.E. Nikitina Dr. Sc. (Biol.), L.Yu. Matora Dr. Sc. (Biol.), S.Yu. Shchyogolev Dr. Sc. (Chem.), A.A. Kamnev Dr. Sc. (Chem.), A.Yu. Muratova Dr. Sc. (Biol.), E.V. Dubrovskaya Ph. D. (Biol.), Yu.P. Fedonenko Ph. D. (Biol.), A.V. Tugarova Ph. D. (Biol.), and D.N. Tychinin, translator.

Conflict of interest. The authors declare no conflict of interest.

Received June 19, 2019. Revised August 21, 2019. Accepted September 10, 2019.

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Издатель: Федеральное государственное бюджетное научное учреждение
«Федеральный исследовательский центр Институт цитологии и генетики
Сибирского отделения Российской академии наук»,
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Адрес редакции: проспект Академика Лаврентьева, 10, Новосибирск, 630090.
Секретарь по организационным вопросам С.В. Зубова. Тел.: (383)3634977.
Издание подготовлено информационно-издательским отделом ИЦиГ СО РАН. Тел.: (383)3634963*5218.
Начальник отдела: Т.Ф. Чалкова. Редакторы: В.Д. Ахметова, И.Ю. Ануфриева. Дизайн: А.В. Харкевич.
Компьютерная графика и верстка: Т.Б. Коняхина, О.Н. Савватеева.

Подписано в печать 20.05.2020. Выход в свет 29.05.2020. Формат 60 × 84 $^{1}/_{8}$. Усл. печ. л. 11.39.
Уч.-изд. л. 13.9. Тираж 150 экз. (1-й завод 1–50 экз.). Заказ № 31. Цена свободная.

Отпечатано в типографии ФГУП «Издательство СО РАН», Морской проспект, 2, Новосибирск, 630090.