2019 • 23 • 7

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

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

2019 • 23 • 7

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

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

ВАВИЛОВСКИЙ ЖУРНАЛ ГЕНЕТИКИ И СЕЛЕКЦИИ

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Исправление

VAVILOV JOURNAL OF GENETICS AND BREEDING

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Erratum

Пятая международная научная конференция PlantGen2019

Дорогие читатели! Седьмой выпуск журнала тематический, он посвящен Пятой международной научной конференции «Генетика, геномика, биоинформатика и биотехнология растений» (PlantGen2019), которая состоялась 24–29 июня 2019 г. в новосибирском Академгородке. Организаторами конференции выступили Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирский государственный университет, Министерство науки и высшего образования Российской Федерации, межрегиональная общественная организация «Вавиловское общество генетиков и селекционеров», Новосибирская областная общественная организация «Вавиловское общество генетиков и селекционеров», EUCARPIA (European Association for Research on Plant Breeding – Европейская ассоциация по исследованию селекции растений). В работе конференции приняли участие около 300 ученых из 19 стран.

Большинство докладов первой конференции PlantGen (Новосибирск, 2010 г.) было посвящено пшенице – исключительно важной для человечества зерновой культуре. Это обусловливалось результатами, полученными сотрудниками Института цитологии и генетики по данной теме, и вхождением института в крупный международный консорциум по секвенированию генома пшеницы (IWGSC).

Сейчас, спустя десятилетие программа конференции существенно расширилась. В пленарных и ключевых докладах на тематических секциях были рассмотрены новейшие достижения в области изучения структурно-функциональной организации генома растений и применения полученных данных для решения практических задач растениеводства. Прозвучали сообщения, посвященные самым разным сельскохозяйственным культурам и их использованию в селекции и функциональном питании, оценке устойчивости растений к биотическим и абиотическим стрессам, развитию биотехнологии растений в постгеномную эру, системной биологии растений и цифровым технологиям. Для детального обсуждения отдельных методов исследований, в том числе эффективных подходов, направленных на сопровождение селекционного процесса, были организованы дополнительные секции по селекции растений в XXI в., цитогенетике растений в геномную и постгеномную эру, ассоциативному картированию.

На секции «Генетические ресурсы растений для селекции и функциональных продуктов питания» обсуждалось современное состояние исследований по хранению и изучению коллекций растений в различных генбанках. Основной акцент был сделан на актуальные направления изучения коллекций для решения генетических задач, а также их использование в селекции и при создании функциональных продуктов питания.

В центре внимания секции «Устойчивость растений к фитопатогенам и другим биотическим стрессам» оказались работы по поиску новых генов устойчивости к биотическим факторам внешней среды. Механизмы формирования устойчивости растений к засухе, морозам и пониженным температурам, засолению и устойчивости растений к токсическим металлам были представлены в докладах секции «Генетические и эпигенетические механизмы устойчивости растений к абиотическим стрессам». Актуальной задачей признан поиск генетических и эпигенетических факторов, определяющих устойчивость растений к абиотическому стрессу.

В биотехнологии растений, направленной на создание новых форм растений с улучшенными признаками, можно выделить несколько подходов. Предметом рассмотрения на секции «Биотехнология растений в постгеномную эру» были современные подходы, связанные с использованием методов генетической, хромосомной и клеточной инженерии, а также геномного редактирования. Большой интерес вызвали исследования, нацеленные на ускорение создания растений с заранее заданным генотипом с помощью комбинированных биотехнологических подходов.

Секция «Системная биология растений и цифровые технологии» была посвящена геномным и посгеномным методам анализа структурно-функциональной организации генома и интеграции полученных знаний в системную биологии растений. В рамках секции обсуждались вопросы по разработке и использованию генетических моделей для решения задач системной биологии. Особый акцент сделан на методах биоинформатического анализа и сборки *de novo* секвенированных геномов растений. В последние годы существенно расширился круг растительных объектов, для которых получены данные полногеномного секвенирования, и встает вопрос, как успешно интегрировать эти данные в системную биологию, для того чтобы вплотную подойти к пониманию закономерностей организации и функционирования генома растений, реализу-

емого в процессе развития и взаимодействия организма с окружающей средой. Для решения поставленного вопроса было рассмотрено использование методов системной биологии у растительных организмов, различающихся по размерам и плоидности геномов.

В настоящее время генетические технологии активно внедряются в селекционные процессы. На параллельной секции «Селекция растений в XXI веке» обсуждались насущные задачи селекции растений и возможности их решения с помощью методов маркер-ориентированной и геномной селекции, а также современных подходов в геномном редактировании.

На секции «Ассоциативное картирование для диссекции признаков и выявление генов-кандидатов» была показана актуальность данного методического подхода для изучения генетических основ хозяйственно ценных признаков. Технология идентификации генов, контролирующих хозяйственно ценные признаки растений, разнообразна. Докладчики представили наиболее эффективные методы ассоциативного картирования для генома растений и подходы, повышающие уровень достоверности полученных результатов.

В связи с массовым широкомасштабным секвенированием геномов растений существенно изменились направления применения методов цитогенетики для изучения генома растений. Эти актуальные вопросы были поставлены на секции «Цитогенетика растений в геномную и постгеномную эру» с учетом современных технологий изучения генетического материала и новых методик анализа цифровых изображений.

Помимо докладов, важной частью работы на конференции является неформальное общение ученых, которое нередко выливается в совместные исследовательские проекты. Не стала исключением и конференция PlantGen2019, по итогам которой достигнут ряд важных договоренностей между ее участниками.

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Вавиловский журнал генетики и селекции. 2019;23(7):803-808
DOI 10.18699/VJ19.37-o

The Fifth International Scientific Conference PlantGen2019

Haplotypes-based genetic analysis: benefits and challenges

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The increasing availability of Single Nucleotide Polymorphisms (SNPs) discovered by Next Generation Sequencing will enable a range of new genetic analyses in crops, which was not possible before. Concomitantly, researchers will face the challenge of handling large data sets at the whole-genome level. By grouping thousands of SNPs into a few hundred haplotype blocks, complexity of the data can be reduced with fewer statistical tests and a lower probability of spurious associations. Owing to the strong genome structure present in breeding lines of most crops, the deployment of haplotypes could be a powerful complement to improve efficiency of marker-assisted and genomic selection. This review describes in brief the commonly used approaches to construct haplotype blocks and some examples in animals and crops are cited where haplotype-based dissection of traits were proven beneficial. Some important considerations and facts while working with haplotypes in crops are reviewed at the end.
Key words: haplotype; GWAS; genomic selection; SNP.

For citation: Sehgal D., Dreisigacker S. Haplotypes-based genetic analysis: benefits and challenges. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):803-808. DOI 10.18699/VJ19.37-o

Генетический анализ на основе гаплотипов: проблемы и перспективы

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Возросшая доступность однонуклеотидных полиморфизмов (SNP), разработанных с помощью технологий секвенирования нового поколения (NGS), позволяет проводить генетические исследования культурных растений, которые ранее были невозможны. Одновременно с этим ученые сталкиваются с необходимостью обработки больших массивов данных, полученных на основе полногеномного скрининга. Сложность обработки и интерпретации экспериментальных данных может быть уменьшена за счет сокращения числа статистических тестов и снижения вероятности ложных ассоциаций путем группировки тысяч SNP в несколько сотен блоков гаплотипов. Благодаря устойчивой структуре генома в селекционных линиях большинства культур, построение гаплотипов может стать мощным дополнением для повышения эффективности маркер-ориентированной и геномной селекции. В настоящем обзоре кратко перечислены подходы, традиционно применяемые для конструирования гаплотипных блоков, а также приведены успешные примеры исследований, проведенных на культурных растениях и животных, по диссекции хозяйственно важных признаков на основе гаплотипного анализа. Представлены выводы и важные заключения, сделанные по результатам изучения генома культурных растений с использованием подхода, основанного на анализе гаплотипов.
Key words: гаплотип; GWAS; геномная селекция; SNP.

Introduction

Advances in Next Generation Sequencing (NGS) technologies by whole genome (Berkman et al., 2012; Chia et al., 2012), transcriptome (Cavanagh et al., 2013), reduced-representation (Elshire et al., 2011; Poland et al., 2012) and/or exome sequencing (Winfield et al., 2016) have led to new levels of Single Nucleotide Polymorphisms (SNPs) discovery. Hence, a paradigm shift from marker-based to sequencing-based genotyping of breeding populations and diversified germplasm panels has been observed in the post-genome sequencing era. These developments have facilitated development of high-density maps, identification of Quantitative Trait Loci (QTL) and discovery of new genes in several crops, thus assisting the breeding process (Sehgal et al., 2016, 2017; Singh et al., 2016; Pandey et al., 2017; Su et al., 2017). Especially polyploid crops such as wheat have benefited from these advances, as marker number and density were major gaps in conducting in depth genetic analyses. Dense sets of SNPs now available from different marker platforms [90K Illumina iselect, Genotyping-by-Sequencing (GBS), Diversity Array Technology Sequencing (DArTseq), high-density Affymetrix Axiom® genotyping array] have significantly upgraded the genetic toolkit available in wheat. Therefore, rapidly growing numbers of breeding lines are being genotyped at low cost (Poland, 2015). In addition, whole genome sequence (>15 Gb) of wheat is now available, by combining next generation (short Illumina reads)

and third generation sequencing data (long Pacific Biosciences reads), which will make cloning of genes feasible (Shi, Ling, 2018).

With upsurge in dense marker data sets coming from different genotyping platforms leading to more markers than observations, scientists will face the challenge of handling large data sets at the whole-genome level for both reliable gene discovery and genomic predictions. Therefore, new approaches will be required to deal with cumbersome data and to make analysis easier. Constructing haplotypes from SNPs is one of the options to deal with bulky datasets. Being multiallelic, haplotypes are more informative than SNPs and allow more powerful and less exhaustive genome-wide scan. In this review, we have first defined what haplotypes are and what approaches are available to make haplotypes. Many examples are cited in animals and crops where haplotypes-based analysis have yielded better results than using SNPs in Genome Wide Association studies (GWAS), Genomic Prediction (GP) and in candidate gene identification.

What are haplotype blocks?

A haplotype block defines a region in the genome that comprises a set of neighboring SNPs, whereby their phased alleles are likely inherited together with little chance of contemporary recombination (Fig. 1). Mainly, three approaches are used to construct haplotype blocks: (1) user-defined length, (2) sliding-window, and (3) linkage disequilibrium (LD). Any of these three methods can be used depending on the skills of the user and/or on the objective of the research. The user-defined fixed length of haplotype blocks (2 to 15 bp) is the easiest approach; however, generated haplotypes do not reflect any biological phenomenon such as LD (Gabriel et al., 2002) or shared evolutionary history (Templeton et al., 2005). The sliding-window approach is the most widely used, and has been used intensively for building haplotypes in GWAS for quantitative or qualitative traits. In this method, a genomic region under study is divided into windows, either of uniform-size or variable-size (Tang et al., 2009), and a multiple-marker association test is performed for each window. This approach is easy to use and handle, however, when adjacent SNPs are in strong LD, it provides redundant information thus making the sliding-window approach no more informative than a SNP. Similarly, when LD pattern vary over large genomic regions, it is difficult to determine window-size for a genome-wide scan. The LD-based approaches are the most advantageous because they focus directly on the detection of historical recombination in a given population and LD coefficients are easy to visualize.

Today most genomic analyses such as GWAS or GP use bi-allelic SNP markers. However, SNPs can be combined into short, multi-allelic haplotypes to overcome bi-allelic problem and to perform a powerful and less exhaustive genome scan. By using haplotype blocks, information on multiple markers jointly can be used and hence local epistatic interactions can be naturally modelled, and the reduced number of parameters enables a range of genomic analyses including GWAS, GP, and/or detection of selection signatures. Further, haplotype blocks can be coded in a simple numeric (binary) form to be used in different R codes or Java-based programs. Figure 2 shows how a haplotype block composed of two adjoining SNPs and having four alleles (AC, GT, AT and GC) can be converted to a simple binary 1-0 format.

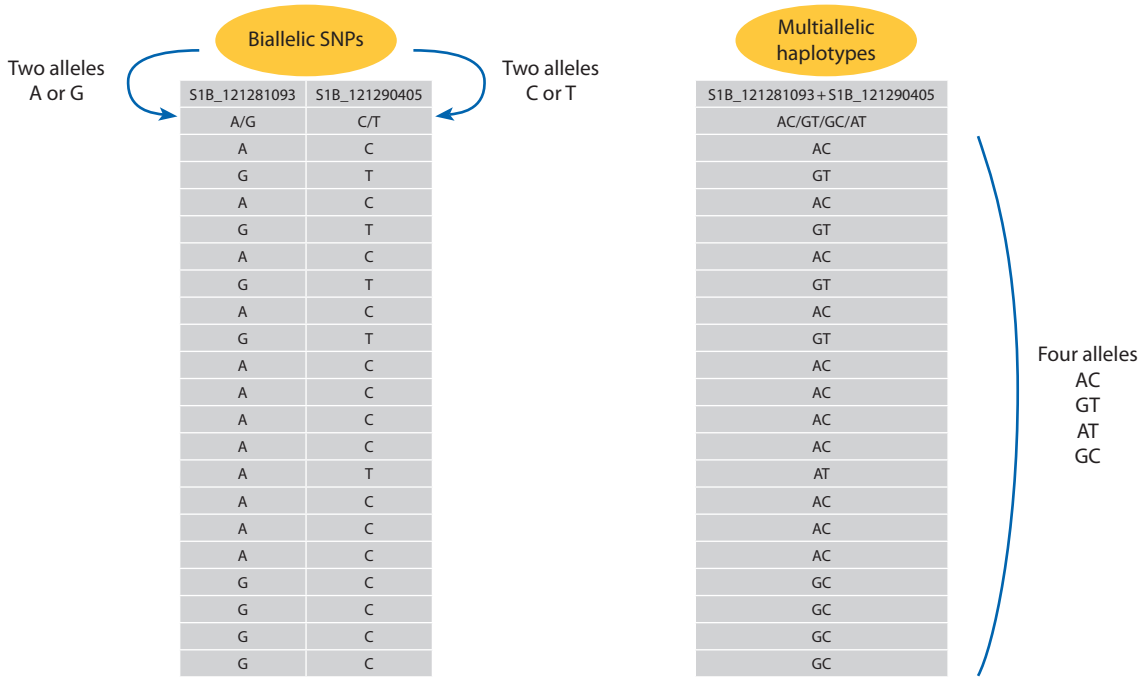
Case studies in animals and humans

GWAS studies based on haplotypes are common in animals and humans (Grapes et al., 2004; Hayes et al., 2007; Calus et al., 2009; Shim et al., 2009; Khankhanian et al., 2015; Jónás et al., 2016; Sato et al., 2016). Studies have generated plethora of evidences to establish that multi-allelic haplotypes significantly improve the power and robustness of association as compared with individual SNPs. A common observation in SNP-based GWAS is the large gap between the variance explained by the identified SNP-associations and the total variance, termed as the 'missing heritability'. J. Yang et al. (2010) showed that a part of the 'missing heritability' could be attributed to a lack of LD between SNP markers and causative variants. Combining neighboring SNPs into haplotype blocks is a simple way to generate a more complete LD. It has been shown that the use of haplotype-based methods have reduced the heritability gap in many cases compared with SNP-based methods when both were applied to the same dataset. P. Khankhanian et al. (2015) investigated the genetic basis of Multiple Sclerosis (MS), a complex genetic disorder in humans controlled by a major histocompatibility complex (MHC) on the short arm of chromosome 6. Haplotypes of various lengths (from 1 up to 15 contiguous SNPs) were constructed at each of the 110 previously identified, MS-associated, genomic regions. The results based on haplotypes outperformed the results using individual SNPs by at least three orders of magnitude. Moreover, when 932 MS-associated haplotypes (identified from 102 genomic regions) were included as independent variables into a logistic linear model; the amount of MS heritability was 38 %, while with individual SNPs it was 29 %.

Simulations based on the LD and population history of livestock have shown that haplotypes can provide greater QTL detection power and mapping accuracy than single markers (Hayes et al., 2007; Calus et al., 2009). Use of haplotypes have also led to the discovery of new genetic regions of interest, which have not been identified by a SNP-based GWAS (Lu et al., 2003; Hagenblad et al., 2004; Shim et al., 2009). W. Barendse (2011) showed that haplotype analysis improved evidence for candidate genes for intramuscular fat (IMF) percentage in cattle as they explained around 80 % more of the phenotypic variance for the five genes that showed some evidence of association to IMF compared to individual SNP analyses. Further studies in animal breeding have also accumulated evidences that integration of haplotypes or haplotype-tagged QTL in genomic selection models can improve GP accuracies for complex traits (Boichard et al., 2012; Cuyabano et al., 2014, 2015a, b; Jónás et al., 2016; Hess et al., 2017; Jiang et al., 2018).

Case studies in crops

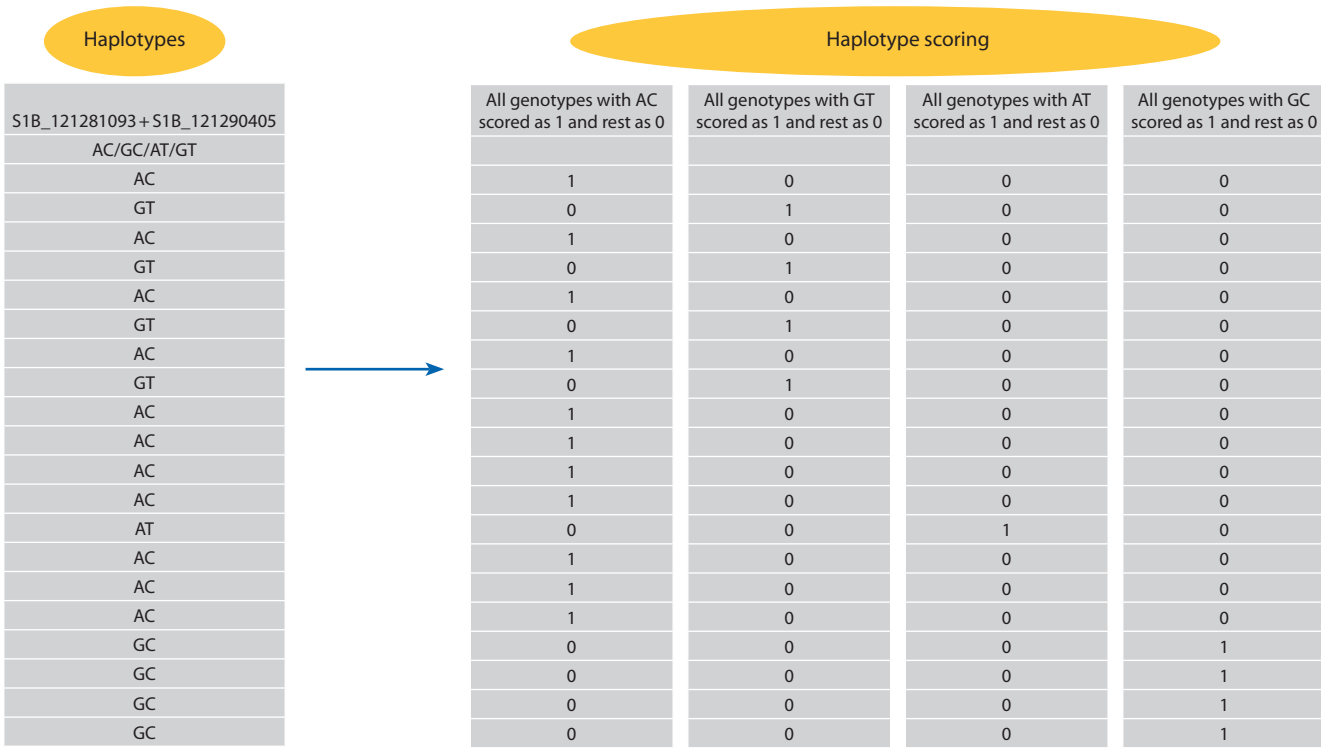
Although only a few case studies have been reported in crops, results have been encouraging towards haplotype-based analyses. A.J. Lorenz et al. (2010) used a sliding window approach and explored the utility of haplotype blocks over individual SNPs for GWAS in barley. They used heading date collected on a large set of barley germplasm from the Barley Coordi-



S1B_121281093	S1B_121290405
A/G	C/T
A	C
G	T
A	C
G	T
A	C
G	T
A	C
G	T
A	C
A	C
A	C
A	C
A	T
A	C
A	C
A	C
G	C
G	C
G	C
G	C

S1B_121281093 + S1B_121290405
AC/GT/GC/AT
AC
GT
AC
GT
AC
GT
AC
GT
AC
AC
AC
AC
AT
AC
AC
AC
GC
GC
GC
GC

Fig. 1. Two close SNPs shown on the left side, each with two alleles. Haplotypes formed by combining these two SNPs are shown on the right side resulting in four alleles.



S1B_121281093 + S1B_121290405	All genotypes with AC scored as 1 and rest as 0	All genotypes with GT scored as 1 and rest as 0	All genotypes with AT scored as 1 and rest as 0	All genotypes with GC scored as 1 and rest as 0
AC/GC/AT/GT				
AC	1	0	0	0
GT	0	1	0	0
AC	1	0	0	0
GT	0	1	0	0
AC	1	0	0	0
GT	0	1	0	0
AC	1	0	0	0
GT	0	1	0	0
AC	1	0	0	0
AC	1	0	0	0
AC	1	0	0	0
AC	1	0	0	0
AT	0	0	1	0
AC	1	0	0	0
AC	1	0	0	0
AC	1	0	0	0
GC	0	0	0	1
GC	0	0	0	1
GC	0	0	0	1
GC	0	0	0	1

Fig. 2. Strategy to convert haplotypes to binary format to be used in well known R scripts and Java-based platforms for genetic analysis.

nated Agricultural Project. Three associations were found for heading date, two of which were detected by haplotype analyses only. Further, the authors determined the effect of three sets of QTL simulations. The power of individual SNP-based analysis was superior to that of haplotypes when the causal SNP was present in genotyping data. In the absence of causal SNP, haplotypes-based GWAS was more powerful to detect QTL than SNPs. In the latter case, however, the type of method used to construct haplotype blocks affected power of the GWAS. Y. Ma et al. (2016) studied the effect of marker preselection on the prediction accuracy in soybean on plant height and yield per plant. The three strategies tested were

(a) a random SNP sampling method (RSM), (b) a haplotype block analysis-based sampling (HBA), and (c) even SNP sampling method (ESM). They found that for grain yield, prediction accuracy increased by approximately 4 % based on HBA-based approach compared with RSM and ESM.

Y. Lu et al. (2012) conducted comparative LD mapping using SNPs and haplotype blocks to identify QTL for plant height and biomass under drought stress in maize. They used a 10 kb sliding-window approach accounting for the average length of LD to construct haplotype blocks. Using haplotype-based LD mapping, three and 12 significant haplotypes were identified for plant height and biomass, respectively, of which six haplotypes contained at least one SNP that was also significantly associated with the specific trait revealed by SNP-based LD mapping. The haplotype-based analysis explained higher phenotypic variation (on average 2.9 %) than SNPs for both traits.

A few genetic studies have attempted to model the effect of interactions between haplotypes (epistasis) on quantitative traits in crops. Some examples include the vernalization response in barley (Cockram et al., 2007) and chlorophyll content in rapeseed (Qian et al., 2016).

Studies in wheat (published and ongoing)

In wheat, studies are so far very few where haplotypes-based genetic analysis have been conducted. K. Voss-Fels et al. (2017) explored molecular interactions connecting root and shoot development and growth in European elite wheat germplasm to investigate plant's demand for water and nutrients along with its ability to access them. They mapped two highly significant haplotypes for root biomass in close proximity to a major locus known to affect spike development. It was concluded that possibly, strong selection for a haplotype variant controlling heading date, has eliminated a specific combination of two flanking, highly conserved haplotype variants whose interaction confers increased root biomass. Breeders could reverse this consequence of selection to recover root diversity that may be useful under stress environments.

N'Diaye et al. (2017) conducted a SNP- and haplotype-based GWAS of semolina and pasta color in elite durum wheat lines. They combined SNPs within a window size of 5.3 cM (based on average LD decay) on the same chromosome to form haplotype blocks. Haplotype-based GWAS resulted in an increase of the phenotypic variance explained (50.4 % on average) and the allelic effect (33.7 % on average) compared to SNP-based GWAS.

In the past decade, various high-throughput genotyping platforms have been adopted by CIMMYT including the 20K and 90K Illumina iselect SNP arrays, the Breeders' 35K Axiom® array (Affymetrix), DArTseq GBS. As a result, large data sets have been generated on different sets of germplasm. Several SNP-based GWAS studies have been performed (reviewed in Dreisigacker et al., 2019) and haplotype-based GWAS has been initially tested. A latest example include haplotype-based quantification of exotic (landrace, synthetics, etc.) genome imprints in pre-breeding germplasm (Singh et al., 2018). A set of 984 pre-breeding lines (PBLs) generated by a three-way cross (exotic/elite1//elite2) were genotyped with DArTseq and phenotyped for a range of agronomic traits under stress environments. Haplotype blocks, generated using the LD approach, identified 361 and 367 blocks in PBLs and exotics, respectively. Haplotype block-by-block comparison on each chromosome revealed that 58 (16 %) blocks identified in PBLs were exotic-specific. Further, a rare and favorable haplotype (GT) was identified on chromosome 6D that minimized grain yield (GY) loss under heat stress without penalty under irrigated conditions.

A large GWAS using haplotypes and individual SNPs was performed for GY and superiority index *Pi* (measure of GY stability) using a large set of advanced bread wheat lines (4,302), which were genotyped with GBS markers and phenotyped under contrasting (irrigated and stress) environments (unpublished work). The average R^2 explained by haplotypes and SNPs showed a 6.1 to 9.9 % higher variation with the haplotype-based GWAS as compared to the individual SNP-based GWAS for GY and *Pi* (Sehgal et al. personal communication). We further explored whether integrating haplotype-tagged QTL for GY as fixed variables in prediction models improved prediction accuracy. It was observed that the model accounting for the haplotype-based GWAS results as fixed effects led to up to 9 to 10 % increase in prediction accuracy, whereas it was only 4 to 5 % with SNP-tagged QTL. Similarly, haplotype-based GWAS conducted for thousand-grain weight identified four major loci in CIMMYT germplasm; all the four loci showed higher *p* values than the associated individual SNPs on chromosomes 4A and 6A.

Considerations and challenges

Due to the growing availability of SNP datasets in crops, haplotype-based approaches for genomic analyses is likely to increase markedly. However, the power of analyses using haplotypes vs. SNPs must be evaluated on a case-by-case basis, as risk factors are common for both approaches. For example, under certain disease models (simple Mandelian or complex multi-gene additive or epistatic inheritance) and certain LD patterns one method outperforms the other, so different architectures of QTL and LD patterns interact with marker characteristics to influence power in GWAS. Similarly, bottlenecks are known to increase LD and shift allele frequency spectra toward higher minor allele frequencies. Hence, after a bottleneck, SNPs are more likely to be in LD with QTL and haplotypes might provide little advantage. Marker ascertainment is another important criterion and is a characteristic of SNP chips. In the standard method of developing a SNP chip or an array, a small SNP discovery panel is used, which means that low frequency mutations often go undetected and SNPs occurring at intermediate to high frequencies dominate in such chips or arrays. This over-sampling of mutations at intermediate frequencies results in lower levels of LD than if SNPs were selected randomly. For GP, haplotype-based prediction approaches are favored only if alleles at QTL are more closely linked to the haplotype than to individual SNPs. Finally, map order errors can play a significant role in determining the safe and best approach for analysis. For example, SNP analysis is unlikely to be affected by ordering errors and hence is the best approach when map order is doubtful.

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

Received June 3, 2019. Revised September 4, 2019. Accepted September 4, 2019. Published online October 8, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):809-816
DOI 10.18699/VJ19.554

The Fifth International Scientific Conference PlantGen2019

Detection of CRISPR cassettes and *cas* genes in the *Arabidopsis thaliana* genome

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The state of the art in the evolution of plant viruses allows the genetic foundations of antiviral immunity in higher (including the most important crops) plants to be categorized as one of the most pressing issues of genetics and selection. According to the endosymbiotic theory, mitochondria descended from alphaproteobacteria that had been absorbed but not degraded by the host cell. The discovery of CRISPR-Cas systems (clustered regularly interspaced short palindromic repeats (CRISPR)-associated proteins), which implement the adaptive immunity function in prokaryotes, raises the question whether such a mechanism of antiviral protection could be caught up by evolution and used by representatives of eukaryotes (in particular, plants). The purpose of this work was to analyze the complete sequences of nuclear, mitochondrial, and chloroplast genomes of *Arabidopsis thaliana* in order to search for genetic elements similar to those in CRISPR-Cas systems of bacteria and archaea. As a result, *in silico* methods helped us to detect a locus of regularly intermittent short direct repeats in the mitochondrial genome of *A. thaliana* ecotypes. The structure of this locus corresponds to the CRISPR locus of the prokaryotic adaptive antiviral immune system. The probable connection between the locus found in the mitochondrial genome of the higher plant and the function of adaptive immunity is indicated by a similarity between the spacer sequences in the CRISPR cassette found and the genome of *Cauliflower mosaic virus* affecting Arabidopsis plants. Sequences of repeats and spacers of CRISPR cassettes in Arabidopsis C24 and Ler lines are perfectly identical. However, the locations of the CRISPR locus in the mitochondrial genomes of these lines differ significantly. The CRISPR cassette in the Col-0 line was found to be completely broken as a result of four deletions and one insertion. Although *cas* genes were not detected in the mitochondrial genome of the studied Arabidopsis ecotypes, their presence was detected in the nuclear genome. Both *cas* genes and numerous CRISPR cassettes were found on all the five chromosomes in the nuclear genome of the Col-0 ecotype. The results suggest the existence of a system of adaptive immunity in plants, which is similar to the CRISPR immunity of bacteria and archaea.
Key words: *Arabidopsis thaliana*; ecotypes; mitochondrial genome; nuclear genome; CRISPR cassette; *cas* genes; homology of CRISPR spacers; plant virus genome; adaptive immunity; RNA interference.

For citation: Konstantinov Yu.M., Petrushin I.S. Detection of CRISPR cassettes and *cas* genes in the *Arabidopsis thaliana* genome. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7): 809-816. DOI 10.18699/VJ19.554

Обнаружение CRISPR-кассет и генов *cas* в геноме *Arabidopsis thaliana*

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Современный уровень знаний в области эволюции растительных вирусов позволяет отнести проблему генетических основ противовирусного иммунитета высших растений (в том числе важнейших сельскохозяйственных культур) к разряду наиболее актуальных проблем генетики и селекции. В соответствии с эндосимбиотической теорией принято считать, что митохондрии произошли от альфа-протеобактерий, которые были поглощены, но не подвергнуты деструкции клеткой-хозяином. В связи с открытием у прокариот CRISPR-Cas (clustered regularly interspaced short palindromic repeats – CRISPR-associated proteins) систем, выполняющих функцию адаптивного иммунитета, возникает вопрос, мог ли подобный механизм противовирусной защиты быть подхвачен эволюцией и использован представителями эукариот, например растениями. Задачей настоящей работы был анализ полных последовательностей ядерного, митохондриального и хлоропластного геномов *Arabidopsis thaliana* с целью поиска генетических элементов, сходных с таковыми в CRISPR-Cas системах у бактерий и архей. В результате методами *in silico* в митохондриальном геноме экотипов *A. thaliana* обнаружен локус регулярно перемежающихся коротких прямых повторов, соответствующий по своей организации CRISPR-локусу адаптивного CRISPR-Cas иммунитета прокариот. На вероятную связь обнаруженного в митохондриальном геноме высшего растения локуса с функцией адаптивного иммунитета

указывает наличие у спейсерных последовательностей в составе найденной CRISPR-кассеты гомологии с геномом вируса мозаики цветной капусты, поражающего растения арабидопсиса. У линий арабидопсиса C24 и Ler последовательности повторов и спейсеров CRISPR-кассеты полностью идентичны. В то же время локализация самого CRISPR-локуса в митохондриальном геноме этих линий существенно различается. Установлено, что у линии Col-0 в результате четырех делеций и одной инсерции CRISPR-кассета полностью нарушена. Хотя гены *cas* в митохондриальном геноме исследуемых экотипов арабидопсиса не были найдены, установлено их наличие в ядерном геноме. В ядерном геноме экотипа Col-0 на всех пяти хромосомах обнаружены гены *cas* и многочисленные CRISPR-кассеты. Полученные результаты позволяют предположить существование у растений системы адаптивного иммунитета, аналогичного CRISPR-иммунитету бактерий и архей.

Ключевые слова: *Arabidopsis thaliana*; экотипы; митохондриальный геном; ядерный геном; CRISPR-кассета; гены *cas*; гомология CRISPR-спейсеров; геном растительного вируса; адаптивный иммунитет; РНК-интерференция.

Introduction

The acquisition of alphaproteobacteria (which subsequently gave rise to mitochondria) as endosymbionts by the archaeal host is now unquestionably accepted to be one of the most important events in the nascence of the eukaryotic cell (Archibald, 2015). In recent years, methods of phylogenomics provided fundamentally new data demonstrating the possibility of several evolutionary scenarios for the genesis of the eukaryotic cell, including "late" or "early" acquisition of mitochondria by the host cell (Poole, Gribaldos, 2014; Pittis, Gabaldon, 2016). The discovery of the CRISPR-Cas adaptive immunity system based on the phenomenon of RNA interference in a significant percentage of bacterial and archaeal species (Jansen et al., 2002; Mojica et al., 2005; Makarova et al., 2006; Barrangou et al., 2007; Lander, 2016) poses the question whether such a protective system may exist in eukaryotic mitochondria, organelles that have an obvious evolutionary relationship with their bacterial ancestors. In this regard, mitochondria of higher plants, which have an extremely large genome compared to the genomes of animals and yeast, are of particular interest.

The mitochondrial genome of plants is also characterized by unusual dynamism, which manifests itself as a high recombination rate caused by repetitive sequences (Gualberto, Newton, 2017). The recombination activity results in the formation of a set of subgenomic forms and high genomic variability even within the same species. Such changes in the genomic structure lead to the rapid evolution of the plant mitochondrial genome. Moreover, the mitochondrial genome of higher plants is tightly involved in horizontal gene transfer processes, where it can act as both a donor and an acceptor of the gene (Kleine et al., 2009; Zhao et al., 2018).

Another important feature of the mitochondrial genome of higher plants is the presence of species-specific sets of linear and circular plasmids in these organelles of many plant species studied in this regard. The composition of the sets within the species can vary significantly (for example, in fertile and sterile forms) (Esser et al., 1986; Thomas, 1986). The origin of mitochondrial plasmids is still unknown. Double-stranded plasmids are believed to be introduced into the cells of higher plants by a symbiotic or pathogenic pathway (Douce, Neuburger, 1989). This hypothesis is supported by the fact that mitochondrial linear plasmids are associated with a protein at their 5′-ends that resembles the structure of some viral nucleic acids (Douce, Neuburger, 1989). Moreover, the detection of genes in linear plasmids S1 and S2 of maize mitochondria encoding viral-type nucleic exchange proteins speaks for their probable viral origin (Kuzmin, Levchenko, 1987; Kuzmin et al., 1988). In recent years, a significant progress has been made in the study of mitoviruses, viruses with the simplest RNA genome that specifically infect fungal mitochondria (Shahi et al., 2019). However, there is also evidence for the existence of plant mitoviruses, which are believed to have arisen as a result of horizontal transfer events of the corresponding genes from plant-infecting fungi (Marienfeld et al., 1997; Bruenn et al., 2015; Nibert et al., 2018). Thus, if to compare bacteria and plant mitochondria, it can be said that the latter, like prokaryotes, also badly needed the protection against infectious nucleic acids of viral and/or plasmid origin during evolution.

Nevertheless, data on the existence of a similar mechanism of protection against pathogenic DNA among representatives of eukaryotes have not been obtained until recently, with the exception of the single detection of a typical CRISPR locus on the mitochondrial plasmid of the higher plant *Vicia faba* in (Mojica et al., 2000). However, that study has gone nowhere in the search for *cas* genes in the mitochondrial, chloroplast, and nuclear genomes of this plant species. In addition, no data on the existence of genetic elements of the CRISPR-Cas immunity in the nuclear plant genome has been obtained thus far.

Considering the evolutionary origin of mitochondria and the plant mitochondrial genome structure, search for genetic elements similar to those of CRISPR-Cas systems of bacteria and archaea in the mitochondrial, chloroplast, and nuclear genomes of the model plant *Arabidopsis thaliana* has been attempted by *in silico* methods. Taking into account the high dynamism of the plant mitochondrial genome, the genome-wide analysis of the mitochondrial genomes of three *A. thaliana* ecotypes (C24, Ler, and Col-0) was carried out with the purpose of searching for elements presumably associated with adaptive CRISPR-Cas immunity.

Materials and methods

The complete sequences of the nuclear (Col-0 ecotype), mitochondrial (C24, Ler, Col-0 ecotypes), and chloroplast (Ler, Col-0 ecotypes) genomes of the model plant *Arabidopsis thaliana* (L.) Heynh. were examined. DNA sequences were taken from the GenBank database (accession numbers of the nuclear genome: NC_003070, NC_003071, NC_003074,

NC_003075, NC_003076; of the mitochondrial genome: JF729200, JF729202, NC_037304; of the chloroplast genome: KX551970, NC_000932.) The sequences were first analyzed with the UGENE program (Okonechnikov et al., 2012). The same program was used to build illustrations (together with the Inkscape vector graphics package).

To seek elements of CRISPR-Cas systems in genomes, the CRISPROne online service was used (Zhang, Ye, 2017). To determine the origin of the detected CRISPR spacers, a search through the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the default parameters for viral taxa was carried out. Cases of coincidence with the numbers of mismatches fewer than 3 nucleotides were subsequently selected.

Sequence alignment of CRISPR loci in the mitochondrial genomes of *A. thaliana* ecotypes was carried out with the programs Matcher (paired) (Rice et al., 2000) and MUSCLE (multiple) (Edgar, 2004). The analysis of CRISPR spacer similarity to the genomes of species-specific viruses was carried out as in (Mihara et al., 2016) (according to Virus-Host DB https://www.genome.jp/virushostdb/3702).

Results and discussion

To date, the CRISPR locus, upstream leader sequence, and *cas* genes have been convincingly shown to be the critical components of CRISPR-Cas systems in bacteria and archaea as a general matter (Jansen et al., 2002; Richter et al., 2012). Resting on the known evolutionary relationship between mitochondria and bacteria, we searched for elements of the CRISPR-Cas system in the mitochondrial genome of three ecotypes of *A. thaliana* using approaches and methods of bioinformatics that are widely used in studying CRISPR-Cas systems of prokaryotes nowadays (Jansen et al., 2002; Makarova et al., 2006, 2015; Grissa et al., 2007; Zhang, Ye, 2017; Couvin et al., 2018).

The context analysis of the complete mitochondrial genome sequence of *A. thaliana* (C24 and Ler ecotypes) revealed a site whose structure is fully consistent with the organization of CRISPR cassettes of prokaryotic origin. The features of the nucleotide organization of the CRISPR-like locus in the mitochondrial genome of these ecotypes are shown in Fig. 1, *a*. As seen from the data presented, the CRISPR cassette found in the plant mitochondrial genome is formed by three 20-bp perfect direct repeats separated by two spacer sequences of 42 bp and 33 bp, respectively. By contrast, the genome-wide analysis of the Col-0 ecotype mitochondrial DNA showed that the CRISPR cassette structure is completely broken there as a result of four deletions and one insertion in the repeat unit (see Fig. 1, *b*).

In our opinion, a noteworthy result of the analysis of the ecotype-specific features of the mitochondrial CRISPR cassette is the fact that the localization of the CRISPR cassette (and its damaged variant) in the mitochondrial genomes of Arabidopsis C24, Ler, and Col-0 lines varies significantly with the complete match of the succession of repeats and spacers (Fig. 2). Such changes in the localization of the CRISPR cassette in the mitochondrial DNA of the studied Arabidopsis ecotypes are most likely to result from intense rearrangements in the mitochondrial genome due to high recombination activity, which is characteristic of the mitochondrial genomes of higher plants (Gualberto, Newton, 2017).

A special search revealed the presence of numerous CRISPR cassettes in the nuclear genome of *A. thaliana* (Fig. 3). Their sizes and arrangement on chromosomes are presented in Supplementary Material[1]. The total number of spacers present in 110 nuclear CRISPR cassettes is 330. We have not performed a detailed analysis of the similarity of the spacers of nuclear CRISPR cassettes to the genomes of plant viruses.

The results of the analysis of spacer sequences in a CRISPR cassette localized in Arabidopsis mitochondrial DNA with reference to the database of plant viruses are summarized in Table 1. The detected spacers were found to contain sections of nonrandom homology to the genomes of three strains of cauliflower mosaic virus able to infect *A. thaliana* plants. Moreover, regions of homology to mismatching genome units of different strains of this virus were identified in individual spacers (data not shown).

Search of the mitochondrial genome of the *A. thaliana* C24, Ler, and Col-0 ecotypes detected *cas* genes neither in the sequences immediately adjacent to the CRISPR locus nor in the rest of the genome. By contrast, sequences of individual *cas* genes were found in the nuclear genome (Table 2).

The *in silico* search of three Arabidopsis chromosomes (chromosomes 1, 2, and 3, respectively) made it possible to map the *cas5* gene, which is part of the effector module of type I CRISPR-Cas systems according to the existing classification of CRISPR-Cas systems (Makarova et al., 2015; Koonin et al., 2017). The *csm6* gene is located on the same chromosome 3 as the *cas5* gene. This gene encodes RNAse III-A, associated with the CRISPR-Cas system and involved (in prokaryotes) in the implementation of immunity against phages through degradation of phage transcripts (Jiang et al., 2016). The *csa5* gene, whose protein product is a universal component of type I-A CRISPR-Cas systems, was detected on chromosome 4 (Daume et al., 2014). This protein is believed to participate in the R-loop stabilization during the interference stage (Daume et al., 2014). Finally, chromosome 5 contains three regions of different lengths corresponding to the gene previously annotated in the Arabidopsis nuclear genome as *DEDDh*, which is a representative of the 3′→5′ exonuclease gene family involved in the metabolism of small noncoding RNAs (Chen et al., 2018). The attribution of this gene to the *cas* family may mean that its protein product can perform several functions *in vivo*, including plant protection from the nucleic acids of viruses and plasmids.

The reverse transcriptase (RT) genes associated with type I and III CRISPR-Cas systems were found to be represented on all the five Arabidopsis chromosomes by a significant number of copies (43 in total) (see Table 2). Presently, these enzymes are assigned a particularly important role in the functioning of type III CRISPR-Cas systems, which is to incorporate new spacers into the existing CRISPR cassette, both with the direct RT participation and with the participation of the RT Cas1 fusion protein (Silas et al., 2016; Toro et al., 2017).

Thus, for the first time ever, our search for elements of CRISPR-Cas systems in the mitochondrial and nuclear genomes of *A. thaliana* made it possible to detect the main genetic elements of prokaryotic adaptive immunity, including CRISPR loci and *cas* genes, in the genome of this plant. With

[1] Supplementary Material is available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx18.pdf

a

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C24              86224  AACTCGACTGAAAGGAGAGGTTGTGAACACAAACTCGACTGAAAGGAGAG  86273
                        ||||||||||||||||||||||||||||||||||||||||||||||||||
Ler             163198  AACTCGACTGAAAGGAGAGGTTGTGAACACAAACTCGACTGAAAGGAGAG 163247

C24              86274  GTTGTGAACACAAACTCGACTGAAAGGAGAGGTCCAAGGTAATTTATTAC  86323
                        ||||||||||||||||||||||||||||||||||||||||||||||||||
Ler             163248  GTTGTGAACACAAACTCGACTGAAAGGAGAGGTCCAAGGTAATTTATTAC 163297

C24              86324  TCTTATAAAAGAGGGAACTCGACTGAAAGGAGAGG   86358
                        ||||||||||||||||||||||||||||||||||
Ler             163298  TCTTATAAAAGAGGGAACTCGACTGAAAGGAGAGG  163332
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в

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C24      AACTCGACTGAAAGGAGAGGTTGTGAACACAAACTCGACTGAAAGGAGAGGTTGTGAACA
Ler      AACTCGACTGAAAGGAGAGGTTGTGAACACAAACTCGACTGAAAGGAGAGGTTGTGAACA
Col-0    -ACTC----------AGAGATCAGGA----AAACTTGATAGAAAG-------CCCTAGCT
          ****          **** *   **    ***** **  *****          * *
C24      CAAACTCGACTGAA-----------------AGGAGAGGTCCAAGGTAATTTATTACTC
Ler      CAAACTCGACTGAA-----------------AGGAGAGGTCCAAGGTAATTTATTACTC
Col-0    CTAACTTGATAGAATGCCCCAGCAATGGCCATCGGAGAGGTCCAACGTAATTTATTACTC
         * **** **  ***                  ************ **************
C24      TTATAAAAGAGGGAACTCGACTGAAAGGAGAGG
Ler      TTATAAAAGAGGGAACTCGACTGAAAGGAGAGG
Col-0    TTATAAAAGAGGGAACTCGACTGAAAGGAGAGG
         *********************************
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Fig. 1. (*a*) Pair alignment of the CRISPR loci in the mitochondrial genomes of the C24 and Ler ecotypes. Repeats are shown in boldface. (*b*) Multiple alignment of the CRISPR loci in the mitochondrial genomes of *A. thaliana* ecotypes.

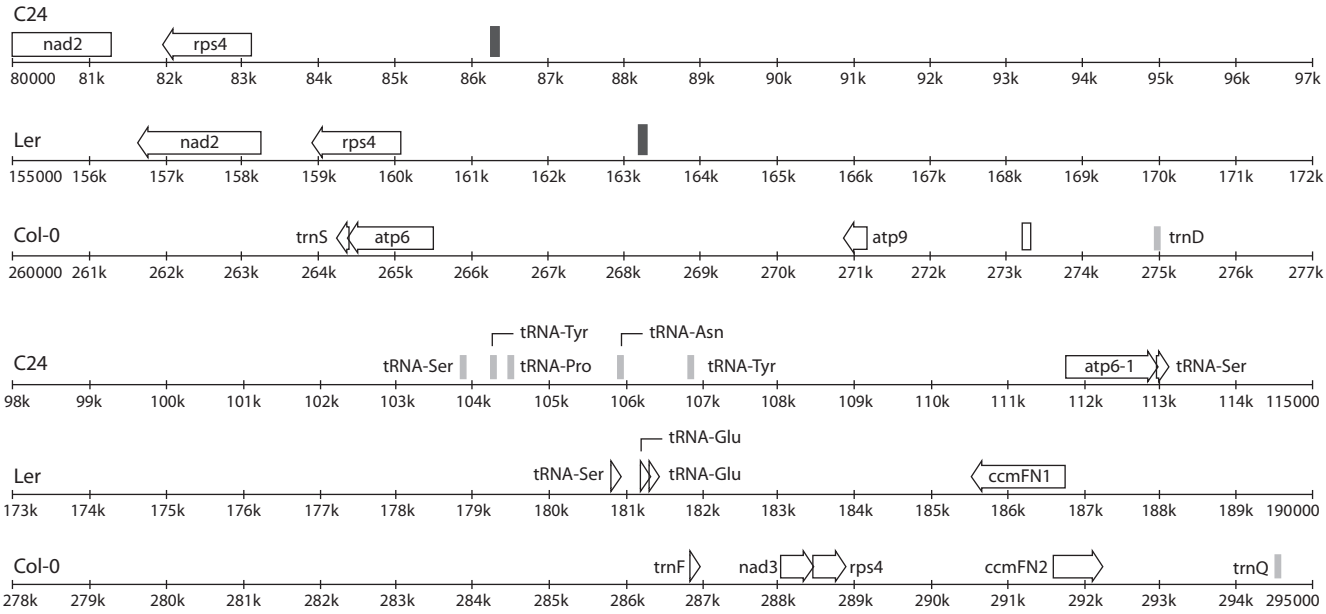

Fig. 2. Localization of the CRISPR cassette in the mitochondrial genomes of *A. thaliana* ecotypes.

The positions of the CRISPR cassette are indicated by black boxes above the C24 and Ler lines. The position of the damaged CRISPR cassette in the mitochondrial genome of the Col-0 line is indicated by open box.

the exception of the CRISPR cassette found in the mitochondrial genome, the structural elements of the system are localized in the nuclear genome. In general, in accordance with the classification proposed in (Makarova et al., 2015; Koonin et al., 2017), the still incomplete list of genes (*cas5*, *csm6*, *csa5*, cd06127, and RT) associated with CRISPR-Cas immunity of prokaryotes found in the *A. thaliana* genome allows the system found in this plant to be assigned to class 1 systems, which have a multi-subunit effector module.

However, during our study, we failed to find any structure corresponding to the characteristics of leader sequences of the prokaryotic type in the mitochondrial or nuclear genomes

of *A. thaliana* (Alkhnbashi et al., 2016). Our attempt to seek elements of CRISPR-Cas systems in the chloroplast genomes of *A. thaliana* Ler and Col-0 ecotypes was unsuccessful as well.

It is the first finding of such canonical elements of CRISPR-Cas systems of prokaryotic origin as CRISPR loci and *cas* genes in a higher eukaryote, namely, the model plant *A. thaliana*. Only a single CRISPR cassette, whose spacer sequences exhibit nonrandom homology with the cauliflower mosaic virus genome, was found in the mitochondrial genome of Arabidopsis (see Table 1). The ability of this virus to infect plants of the species under investigation is of special importance. The Arabidopsis lines studied are characterized by a significant difference between the C24 and Ler ecotypes in the genomic localization of the CRISPR locus. In the Col-0 ecotype, the CRISPR cassette structure is completely broken as a result of four deletions and one insertion in the region of direct repeats (see Fig. 1, *b*). This fact points to intense mitochondrial genome reorganization in higher plants, which manifest itself as a rapid occurrence of interline differences at the level of mitochondrial DNA.

From the evolutionary point of view, the possible existence of CRISPR-Cas immunity in plants seems quite justified since DNA-containing plant organelles – mitochondria and chloroplasts – are obviously attractive targets for viruses and plasmids of alien origin. Plant mitochondria are particularly vulnerable in this regard due to the existence of mitoviruses that attack this type of organelles (Marienfeld et al., 1997; Bruenn et al., 2015; Nibert et al., 2018) and the natural ability of plant mitochondria for DNA uptake (Koulintchenko et al., 2003). In general, with regard to the currently available data, it seems premature to form any hypotheses on the evolutionary origin of CRISPR-Cas system elements in the Arabidopsis genome. However, it should be noted that a lot of experimental data for reconstructing the scenarios of the origin and evolution of CRISPR-Cas systems in prokaryotes have already been reported (Koonin, Makarova, 2019). When trying to consider the issue of the origin of CRISPR-Cas systems in plants in the context of eukaryogenesis, the data may be useful (Koonin, 2015; Lopez-Garia et al., 2017). In this case, it should be taken into account that similar protection systems against pathogenic nucleic acids might have been present in both the alpha-proteobacterial symbiont that gave rise to mitochondria and the archaean host of the protomitochondrial endosymbiont. Notionally, in evolutionary terms the presence of such a protective system cannot be ruled out for the cyanobacterial ancestor of modern chloroplasts either.

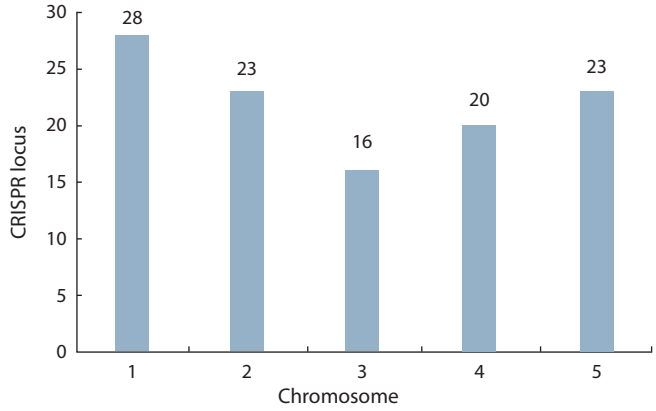

Fig. 3. Quantitative distribution of CRISPR cassettes over nuclear chromosomes of *A. thaliana.*

At the current stage of Arabidopsis CRISPR-Cas systems research, we were unable to identify a set of prokaryotic *cas* genes whose products would form the adaptation and effector modules of a class 1 CRISPR-Cas system and thus support the stages of adaptation, expression, and interference (Koonin et al., 2017). With regard to the known data on the wide variety of CRISPR-Cas systems found in bacteria and archaea (Westra et al., 2016; Koonin et al., 2017; Koonin, Makarova, 2019), it is natural to expect that the plant adaptive immunity mechanism can differ significantly from that in prokaryotes. Therefore,

Table 1. Alignment of the CRISPR spacers found in the *A. thaliana* mitochondrial genome with the cauliflower mosaic virus genome

Alignment*	Isolate	NCBI GenBank identifier
Spacer 1 TCCAAGGTAATTTATTACTCTTATAAAAGAGGG ||||.||.|||....||||||| KJ716236 7512 AAGGGAAATTAGGGTTCTTATA 7533	Cabb B-JI	KJ716236
Spacer 1 TCCAAGGTAATTTATTACTCTTATAAAAGAGGG |.||||..||..|||.|..||||||| M10376 3301 TACAAGAAAAAATATAAGGCTTATAAA 3327	D/H	M10376
Spacer 2 TTGTGAACACAAACTCGACTGAAAGGAGAGGTTGTGAACACA |.||||||| |.|||.|| AB863182 1782 ATAAACTCGA-TCAAAGAAG 1800	TUR239	AB863182

* Numerals indicate the localization of the regions of homology in the virus genome.

Table 2. Description of *cas* genes detected *in silico* in the nuclear genome of *A. thaliana*

Chromosome	Gene	Type of CRISPR-Cas system	Start–End of ORF*	Cas gene identification according to the classification (Makarova et al., 2015; Koonin et al., 2017)
1	*cas5*	I	11918718–11919893	COG1688
2	*cas5*	I	2540882–2541148	cd09693
3	*cas5*	I-B	12023929–12024258	mkCas0191
	csm6	III-A, III-D	10339967–10340668	cd09742
4	*csa5*	I-A	5072131–5072607	mkCas0163
5	*DEDDh*	I	9481429–9481962 9482093–9482377 9482399–9482647	cd06127
1	RT	I, III	11916164–11916466 11916554–11917162	pfam00078
2			10826–12517 17624–20293 23971–25653 30408–31532 3223463–3224872 3224999–3225685 3228729–3230522 5235099–5235800 5241327–5241869 5241873–5242298 5600424–5602496 5602506–5603024 5619463–5619846 5619956–5620156	
3			9435745–9436644 9436766–9437260 9444515–9444907 9445029–9445523 10341391–10343106 10343111–10343806 12021817–12022311 13167127–13168218 13174969–13175118 13175226–13175606 13467703–13469727 13469756–13470868 13476637–13477317 15489669–15490154 15490132–15490506 15493988–15496318	
4			3296005–3296457 3303483–3304247 3304260–3305717 3744233–3746887 3749914–3752901 3754635–3756176 5071003–5071551 5081577–5082176	
5			14576286–14579228 14582070–14582471 14582509–14584656	

* ORF, open reading frame.

it is obvious that only the use of a comprehensive approach, including transcriptomics and proteomics techniques along with genomics ones, will allow getting a more complete idea of the genes and their protein products that form the plant adaptive immunity system.

Conclusions

For the first time ever, such elements of the CRISPR-Cas system of prokaryotes as CRISPR cassettes and *cas* genes were detected *in silico* in the genome of the higher plant *A. thaliana*. This finding can provide a staging ground for

further detailed genomic, transcriptomic and proteomic studies of a wider set of plant species (including the most important crops) in addition to Arabidopsis in order to determine groups of genes whose expression may be associated with the activity of the natural adaptive plant immunity mechanism. The applied relevance of the expected scientific results on the molecular nature of adaptive plant immunity can hardly be overestimated.

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Acknowledgements. The authors are grateful to Prof. Ian Max Møller and Dr. R. Shyama Prasad Rao for their interest in the research, critical remarks, and useful discussion. The authors acknowledge Dr. Alexey Fadeev's valuable advice and useful comments on the paper structure. The authors sincerely express their deep gratitude to the anonymous reviewer, whose favorable comments and analysis of the original paper version were helpful in its substantial processing, which improved the presentation of the results obtained.

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

Received June 5, 2019. Revised August 20, 2019. Accepted August 21, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):817-826
DOI 10.18699/VJ19.555

The Fifth International Scientific Conference PlantGen2019

Genome constitution and differentiation of subgenomes in Siberian and Far Eastern endemic species of the genus *Elymus* (Poaceae) according to the sequencing of the nuclear gene *waxy*

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Fifty-three species of perennial grasses in the genus *Elymus* L. (Poaceae), which are widespread in Russia, are generally assumed to have three haplome combinations: StH, StY and StHY. The StH-genome species, endemic to Russia, remain the least studied. R. Mason-Gamer and co-authors have previously shown in a series of studies that a molecular phylogenetic analysis of the low-copy gene *waxy* (*GBSS1*) sequences significantly complements cytogenetic data on the genomic constitution and evolutionary relationships among both North American and Asian species of the genus *Elymus*. To determine the species' genomic constitution and to evaluate the level of phylogenetic differentiation, we examined the *GBSS1* gene in 18 species of *Elymus* from Siberia and the Russian Far East, including the following 14 endemics: *E. charkeviczii*, *E. jacutensis*, *E. kamczadalorum*, *E. komarovii*, *E. kronokensis*, *E. lenensis*, *E. macrourus*, *E. margaritae*, *E. subfibrosus*, *E. sajanensis*, *E. transbaicalensis*, *E. peschkovae*, *E. uralensis*, and *E. viridiglumis*. PCR amplification products of *GBSS1* gene fragments (including exons 9–14) were cloned and 6–8 clones per accession were sequenced. It appears that all the species studied have St and H subgenomic gene variations. The most significant differences between the subgenomic variants St and H were found in intron 13. The H subgenome contains a 21-bp-long deletion in intron 13 in all *Elymus* genotypes, probably derived from a common ancestor of the H and P genomes. Instead of this deletion, all St subgenomes have a relatively conservative sequence similar to that of the genus *Pseudoroegneria*, whose ancestor is considered to be the donor of the modern St subgenome for all *Elymus* species. Cluster phylogenetic analysis revealed differentiation in St and H subgenome sequences into two evolutionary variants: St_1 vs. St_2 and H_1 vs. H_2, respectively. Variants of the St and H subgenomes were found homologous to various modern species of the ancestral genera *Pseudoroegneria* and *Hordeum*: St_1 to *P. strigosa*, St_2 to *P. spicata*, H_1 to *H. jubatum*, and H_2 to *H. californicum*. The details of the relationships between Russian and North American species of the genus, as well as a number of microevolutionary interconnections in the group of boreal endemic species of Siberia and the Russian Far East were revealed. The new results obtained here are essential for the development of a phylogenetically oriented taxonomic system for the genus *Elymus*.
Key words: *Elymus*; phylogeny; allopolyploids; genome constitution; *GBSS1*.

For citation: Agafonov A.V., Asbaganov S.V., Shabanova (Kobozeva) E.V., Morozov I.V., Bondar A.A. Genome constitution and differentiation of subgenomes in Siberian and Far Eastern endemic species of the genus *Elymus* (Poaceae) according to the sequencing of the nuclear gene *waxy*. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):817-826. DOI 10.18699/VJ19.555

Геномная конституция и дифференциация субгеномов эндемичных сибирских и дальневосточных видов рода *Elymus* (Poaceae) по данным секвенирования ядерного гена *waxy*

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В России распространены 53 вида многолетних трав рода *Elymus* L. (Poaceae) предположительно трех гаплоомных комбинаций: StH, StY и StHY. Наименее изученными остаются бореальные StH-геномные виды – эндемики РФ. Ранее R. Mason-Gamer с соавторами в серии исследований было показано, что молекулярно-филогенетический анализ последовательностей низкокопийного гена *waxy* (*GBSS1*) существенно дополняет цитогенетические данные по геномной конституции и эволюционным взаимоотношениям как среди североамериканских, так и среди азиатских видов рода *Elymus*. Мы исследовали ген *GBSS1* у 18 видов *Elymus*

из Сибири и Дальнего Востока России (включая 14 эндемичных), чтобы определить их геномную конституцию и оценить уровни филогенетической дифференциации: *E. charkeviczii*, *E. jacutensis*, *E. kamczadalorum*, *E. komarovii*, *E. kronokensis*, *E. lenensis*, *E. macrourus*, *E. margaritae*, *E. subfibrosus*, *E. sajanensis*, *E. transbaicalensis*, *E. peschkovae*, *E. uralensis*, *E. viridiglumis*. Продукты ПЦР-амплификации фрагментов гена *GBSS1* (область экзонов 9–14) были клонированы и секвенированы (по 6–8 клонов на образец). Все изученные виды включали субгеномные вариации St и H. Наиболее существенные различия между субгеномными фрагментами St и H обнаружены в интроне 13. Этот интрон в субгеноме H содержит делецию в 21 п.н. во всех генотипах *Elymus*, вероятно, унаследованную от общего предка геномов H и P. Вместо этой делеции все субгеномы St имеют относительно консервативную последовательность, близкую по нуклеотидному составу к таковой у рода *Pseudoroegneria*, предок которого является донором современного субгенома St всех видов *Elymus*. Кластерный филогенетический анализ выявил дифференциацию последовательностей каждого из субгеномов St и H на два эволюционных варианта – условно St_1 и St_2, H_1 и H_2. Установлено, что варианты субгеномов St и H гомогенны с различными современными видами предковых родов *Pseudoroegneria* и *Hordeum*: St_1 – *P. strigosa*, St_2 – *P. spicata*, H_1 – *H. jubatum*, H_2 – *H. californicum*. Выявлены особенности взаимоотношений между российскими и североамериканскими видами рода, а также ряд микроэволюционных связей в группе эндемичных бореальных видов Сибири и Дальнего Востока. Полученные новые данные необходимы для построения филогенетически ориентированной таксономической системы рода *Elymus*.
Ключевые слова: *Elymus*; филогения; аллополиплоиды; геномная конституция; *GBSS1*.

Introduction

The genus *Elymus* L. (wildrye) is the largest genus of the tribe *Triticeae* Dumort in the family Poaceae Barn. It contains exclusively amphiploid self-pollinating perennial grasses (Dewey, 1984; Löve, 1984). Species of the genus are widespread on all continents from the Holarctic to the subtropics, with more than half populations growing in Central Asia (Lu, 1994). The genomic constitution of all species formed by haplomes from the ancestors of several modern genera: *Pseudoroegneria* (Nevski) Á. Löve (St haplome), *Hordeum* L. (H haplome), *Agropyron* Gaertn. (P haplome) and *Australopyrum* (Tzvelev) Á. Löve (W haplome), as well as the Y haplome from an unknown ancestor. The St haplome is common for all species of the genus. After institution and recognition of the genomic classification system for the tribe *Triticeae* (Dewey, 1984), a taxonomic system began to spread, in which the genus *Elymus* in the broadest sense is divided into several separate genera on the basis of the genomic composition of the species (Baum et al., 2011): *Elymus* L. (StStHH genome), *Roegneria* C. Koch (StStYY genome), *Campeiostachys* Drobov (StStHHYY genome), and *Kengylia* C. Yen & J.L. Yang (StStYYPP genome), *Douglasdeweya* C. Yen, J.L. Yang et B.R. Baum (StStPP genome). According to the current concepts, the genus *Elymus* within Russia is divided into four sections: *Turczaninovia* (Nevski) Tzvelev (includes 4 species), *Goulardia* (Husn.) Tzvelev (includes 42 species), *Elymus* (includes 6 species), and *Clinelymopsis* (Nevski) Tzvelev (includes 1 species) (Tsvelev, Probatova, 2010). This system was built according to the traditional criteria (comparative-morphological and ecological-geographical) and ensures the integrity and consistency of the genus, but the same sections include species with different genomic constitutions.

Nowadays it becomes evident that a balanced integrated approach is required to construct a phylogenetically oriented system of taxa of the genus *Elymus*. The main difficulty here is in combining two entirely different methodologies in botany: traditional taxonomy with the priority of morphological criteria and molecular genomics based on the modern molecular technologies. Significant results on the use of molecular markers were obtained by R. Mason-Gamer with collaborators (Helfgott, Mason-Gamer, 2004; Mason-Gamer, 2001, 2004, 2008, 2013; Mason-Gamer et al., 1998; 2010a, b). In particular, their studies have shown that comparative data on nucleotide sequences of the low-copy gene *waxy* (granule-bound starch synthase 1, *GBSS1*) are consistent with cytogenetic data in regard to the genomic constitution and evolutionary origin of North American (Mason-Gamer, 2001) and Asian (Mason-Gamer et al., 2010a) species of the genus *Elymus*.

We have analyzed the applicability of the nuclear low-copy genes *bmy2* and *waxy*, as well as ITS rRNA clusters as genetic markers for the assessment of phylogenetic relationships between species of the genus growing in Siberia and in the Russian Far East (Shmakov et al., 2015). We confirmed that comparative analysis of selected locus sequences in combination with other molecular markers allow phylogenetic relationships between taxa to be reconstructed. Moreover, our studies proved that data on the genomic constitution of species and their microevolutionary relationships are to be taken as a fundamental basis to construct phylogenetically-oriented genus systematics for the species grown in Russia. The availability of numerous *GBSS1* gene sequences in the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) enables a more detailed assessment of the relationships between a large number of genotypes of each species in comparative studies.

Here we present a comparative analysis of nucleotides sequences of an ~1300-bp-long fragment of the *GBSS1* gene including exons 9 to 14 in 18 *Elymus* species (including 14 endemics) growing in Siberia and in the Russian Far East in order to establish or confirm their genomic constitution and to assess the evolutionary differentiation levels of subgenomes in different species. This information is essential for the construction of a phylogenetically oriented taxonomic system of the genus species growing in Russia.

Materials and methods

The analyzed accessions included genus *Elymus* species widespread in the Asian part of Russia, mainly with unknown or unconfirmed genomic constitutions (Supplementary

Material 1)[1]. Known *GBSS1* gene sequences of the St, H and Y genomes deposited in the NCBI database were used as references for comparative analysis (Supplementary Material 2). Genomic DNA was extracted from fresh or dried leaves as previously described by Khanuja et al. (1999) with modifications, or by using Nucleospin Plant II kits (Macherey-Nagel, Germany) according to the manufacturer's recommendations.

The previously described (Mason-Gamer et al., 1998) primers F-for (TGCGAGCTCGACAACATCATGCG) and M-bac (GGCGAGCGGCGCGATCCCTCGC) were used for PCR-amplification of an ~1300-bp-long *GBSS1* fragment overlapping gene exons from 9 to 14. The PCR reaction mixture of a total volume of 15 µl contained Taq buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 µM of each primer, 20 ng genomic DNA, and 1.0 unit of HS Taq DNA polymerase (Eurogen, RF). The following temperature profile was used (a C-1000 thermal cycler, Bio-Rad, USA): primary denaturation for 4 min at 94 °C, then 38 three-step cycles with denaturation for 25 sec at 94 °C, annealing for 30 sec at 65 °C and elongation for 1 min at 72 °C, followed by final elongation for 20 min at 72 °C to enhance the terminal non-matrix addition of deoxyadenosine at the 3′-end of the PCR product (Mason-Gamer et al., 1998). Amplification products were analyzed by 1.7 % agarose gel electrophoresis in TAE buffer at an electric field strength of 4 V/cm.

Since allopolyploid *Elymus* genomes contain at least two subgenomic variations of the *GBSS1* gene, amplification was performed in three replicates to minimize the "PCR drift" effect due to stochastic fluctuations at the initial stages of PCR (Wagner et al., 1994). The combined PCR product was ligated into vector pAL2-T (Eurogen, RF) and then cloned in chemically competent XL1-Blue *E. coli* cells. Positive colonies containing recombinant plasmids with a *GBSS1* insert were selected by blue/white coloring of *E. coli* grown on Amp(+) LB-Agar containing X-gal/IPTG. Twenty white colonies for each accession were tested for an insert of the expected length by PCR-amplification with universal M13 primers (Eurogen, RF) followed by electrophoresis analysis. At least 6 colonies containing pALT2 with an insert of the expected size (~1300 bp) per each accession have been grown in 4 ml LB liquid medium for 16 hours at 37 °C and 220 rpm. Plasmid DNA was isolated with a Plasmid Miniprep Kit (Eurogen, RF) according to the manufacturer's instructions.

The Sanger sequencing reaction in a total volume of 40 µl contained 0.7 µg of plasmid DNA with a total length of ~4300 bp, 20 pmol of primer M13F or M13R, 1.8 µl of reagent BigDye v. 3.1 (ABI, USA), 7.2 µl of 5X sequencing buffer (ABI, USA) and water up to the final volume. The temperature profile for the Sanger reaction included primary denaturation for 2 min at 95 °C, then 50 three-step cycles with denaturation for 30 sec at 95 °C, then annealing for 10 sec at 55 °C and elongation for 4 min at 60 °C. Sanger reaction products were purified from excess of BigDye components by gel filtration through micro columns containing 600–700 µl of prepared Sephadex G-50 (GE Healthcare) with liquid removal from the dead volume by centrifugation for 2 min at 900 g and subsequently analyzed on an ABI 3130XL automatic gene analyzer (ABI, USA) at the Genomics Core Facility SB RAS. DNA sequences obtained were assembled into contigs overlapping *GBSS1* from exon 9 to 14, including 5 introns, by using Unipro UGENE v1.31.0 (Okonechnikov et al., 2012). Finally, at least 6 clones of *GBSS1* per each of 22 *Elymus* accessions have been sequenced. Additionally, 42 nucleotide sequences from the NCBI GenBank were used for comparative analysis.

Multiple sequence alignment was performed using the T-Coffee program (www.tcoffee.org) and refined manually. The alignments of the *GBSS1* fragment were used to generate phylogenetic trees using the maximum likelihood (ML) method on the IQ-TREE web server (Trifinopoulos et al., 2016). For each exon and intron, the best models of nucleotide substitutions were determined in PartitionFinder version 2.1.1 (Lanfear et al., 2016) with the following parameters: the AICc selection model, "greedy" search algorithm and related (linked) lengths of the branches (Lanfear et al., 2012). The previously proposed (Mason-Gamer, 2004) sequence of *Bromus tectorum* AY362757.1 from the NCBI GenBank was used to root the dendrograms. The statistical support of topology in IQ-TREE analysis was evaluated using 1000 replications produced by SH-aLRT (Guindon et al., 2010) and UFBoot (Minh et al., 2013) methods.

Results and discussion

The results obtained using reference accessions carriers of the genus *Elymus* ancestor genomes, St (genus *Pseudoroegneria* species) and H (genus *Hordeum* species), clearly indicated the presence of only the St and H genomes in all the studied species from Siberia and the Russian Far East, thus confirming that these species belong to the tetraploid StH genome group. Obviously, the center of species diversity for the StH genome group is shifted to the north from the center of origin of most StY genome species located in China (Lu, Salomon, 1992). Interestingly, the allotetraploid group of *Elymus* species of North America is also represented mainly by StH genome species (Mason-Gamer, 2001). Only rare individuals of several alien Asian StHY and StY genome species were reported there (Barkworth et al., 2007).

The most notable differences between the St and H subgenomic fragments are located in intron 13 (Fig. 1). The H subgenome sequences of this intron in all *Elymus* genotypes analyzed contained a 21-bp-long deletion, most likely coming from a common ancestor of the H and P subgenomes, since it is also present in modern representatives of related monogenomic species from the genera *Hordeum* and *Agropyron*. However, all St and Y subgenomes had at the very site of this deletion a relatively conservative sequence, which largely matches a sequence in the genus *Pseudoroegneria*, whose ancestor is believed to be the donor of the modern St genome. Small deletions are also common for other regions of this intron, but are less frequent in the other *GBSS1* fragment regions analyzed.

In addition, our analysis of the accessions did not confirm the previously published data on the existence of conservative sites that are absolutely specific to the H and St haplomes (Shmakov et al., 2015). This was true only partially of some sequences belonging to different haplomes.

Cluster analysis of the whole *GBSS1* region from 9 to 14 exon sequences, as well as separate sequences of introns or

[1] Supplementary Materials 1 and 2 are available in the online version of the paper: https://vavilov.elpub.ru/jour/manager/files/SupplAgafonov_engl.pdf

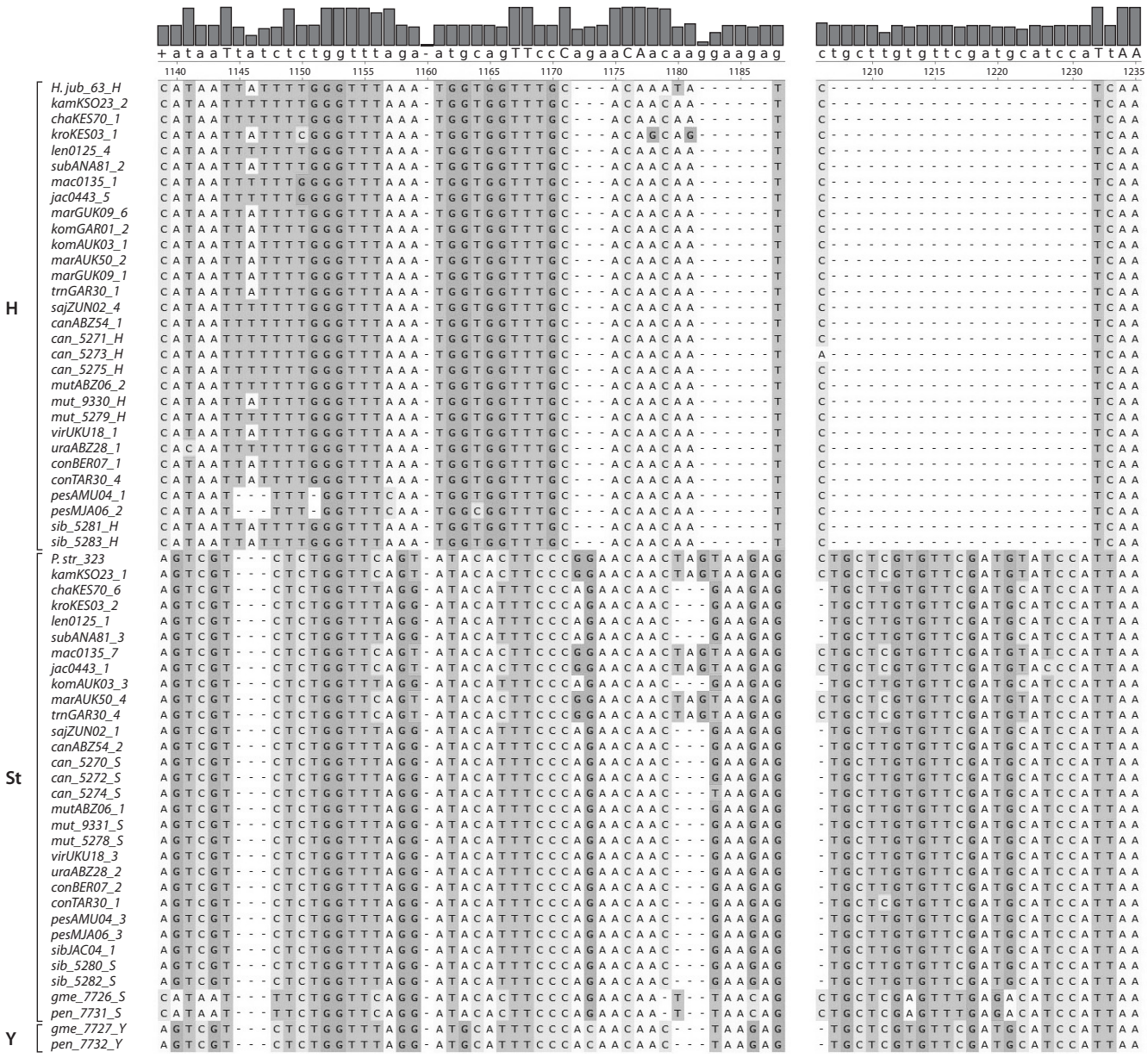

Fig. 1. H and St subgenome differences in intron 13 of the *GBSS1* gene nucleotide sequences among *Elymus* species from the Asian part of Russia in comparison with the sequences of the reference species of Eurasia.

exons, showed common patterns with certain nuances of phyletic connections both within and between related groups of the *Elymus* taxa analyzed. The analysis of the most conservative sites (exons 9–14) showed uniformity within the same subgenomes and at the same time distinction among different subgenomes (Fig. 2).

In the species studied the two subgenomes were found clearly differentiated. For instance, the sequences of the St subgenome were divided into two groups: St_1 and St_2. The sequences of the St_1 subgenome for Siberian species are probably older since they were found not only in the northern biotypes *E. macrourus*, *E. jacutensis*, *E. kamczadalorum* and more southern StY genome species *E. gmelinii* and *E. pendulinus*, but also in *P. strigosa* accession PI 499637 from the northeastern part of China.

The subgenomic group St_2 was formed by a larger part of the species, including both strictly local (*E. komarovii*, *E. uralensis*, *E. sajanensis*, *E. margaritae*) and widely vicarious (*E. caninus*, *E. sibiricus*) species. This fact is clearly illustrated by nucleotide sequence peculiarities in different regions of the gene, as shown in Fig. 3. Remarkably, accession AUK-0650 of the Altai species *E. margaritae* contained both variants of the St subgenome. At the same time, in the set of 8 sequences for each *E. komarovii* accession GAR-0501 and *E. margaritae* accession GUK-1709 the sequences belonging to the St subgenome were not detected.

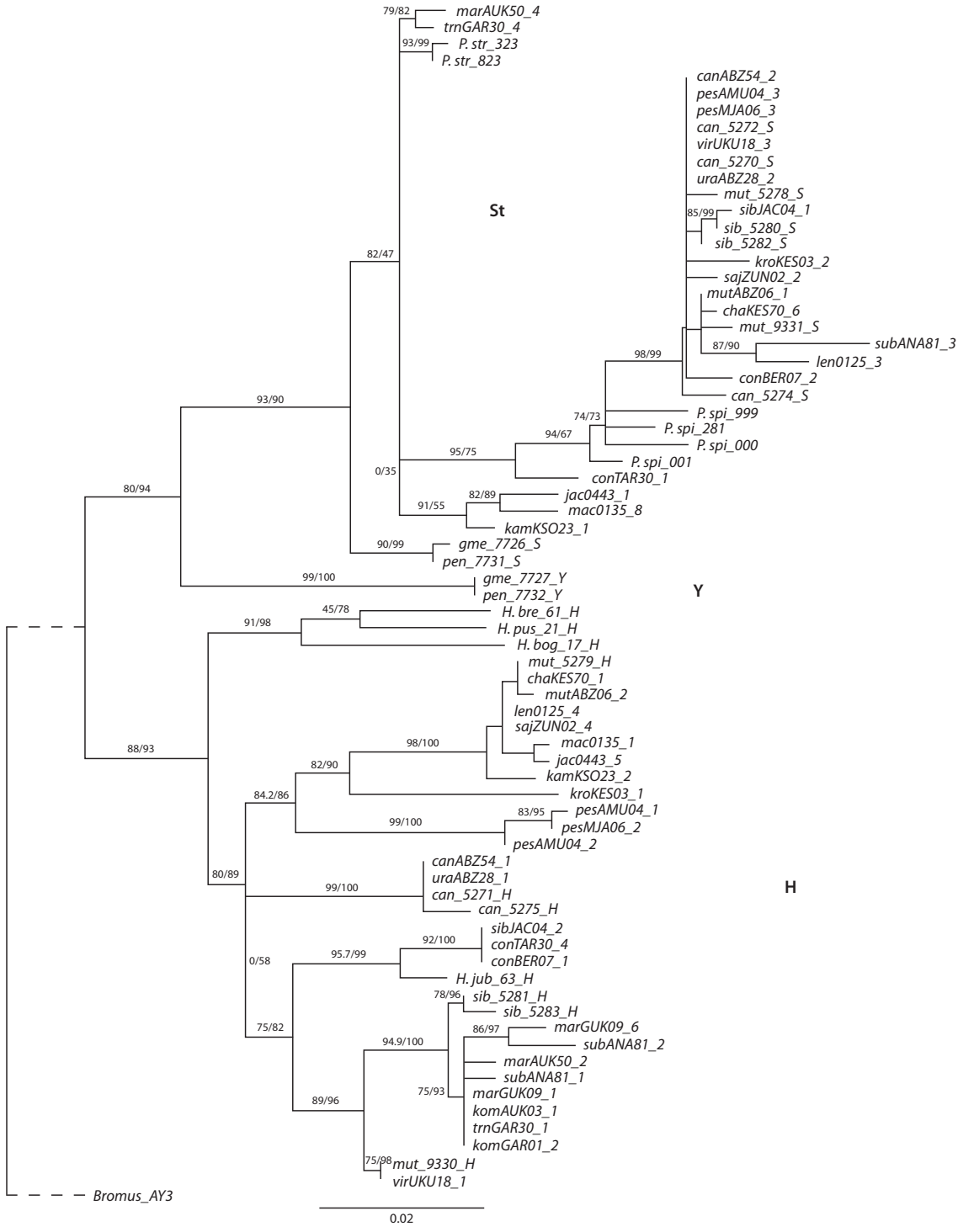

Fig. 2. The maximum likelihood tree constructed from multiple alignment of all exon (9–14) sequences of the *GBSS1* gene of the St and H subgenomes in *Elymus* species from the Asian part of Russia in comparison with the sequences of the reference species of Eurasia (St, H and Y subgenomes).
SH-aLRT (%)/UFboot (%) support values are shown.

Sequences of a greater number of the *Elymus* species from North American natural accessions initially were subdivided according to the same principle (Mason-Gamer, 2001), therefore we have built a dendrogram that included the endemic species of Asian Russia in comparison with some sequences of North American species. The sequences of the St subgenome including exons 9 to 14 with introns were used. The results are shown in Fig. 4.

In this version of the dendrogram, Asian species were also distributed among two clades with the same composition as in

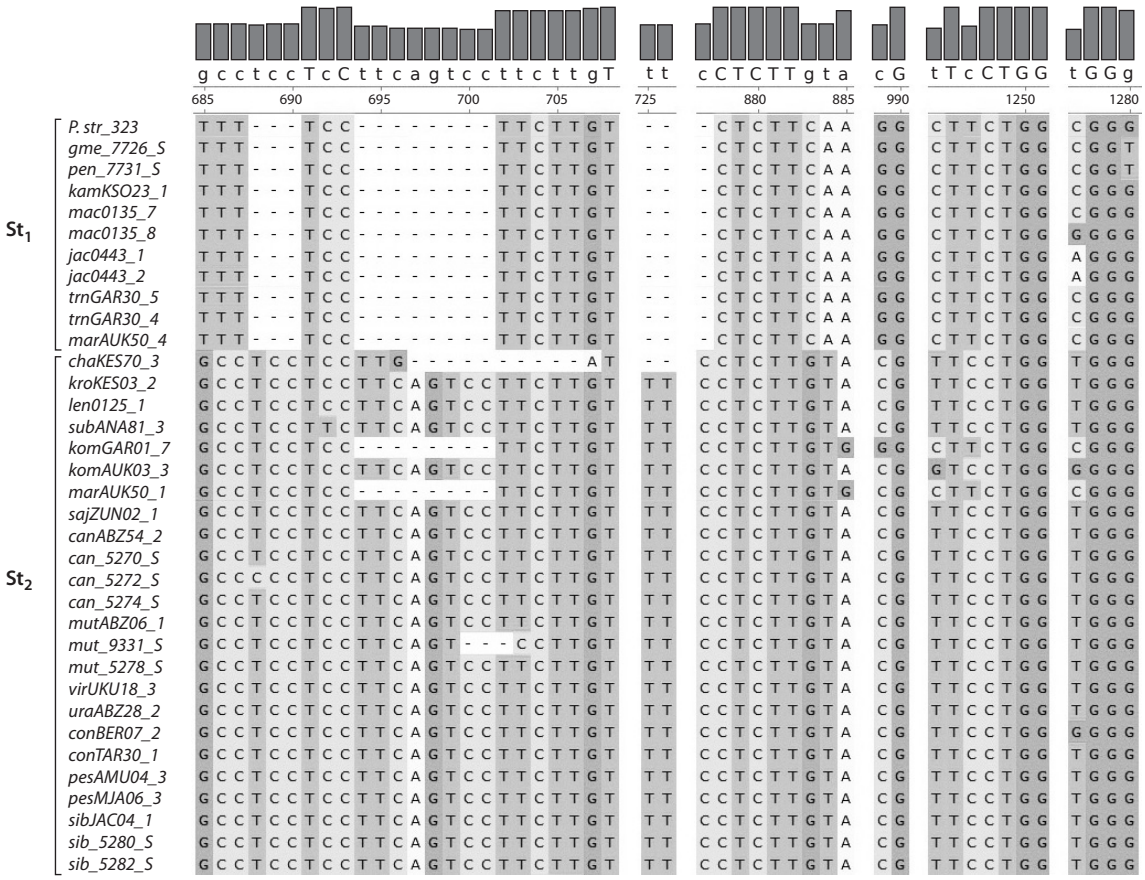

Fig. 3. Differentiation of St subgenomes on the basis of differences in nucleotide sequences in different parts of the *GBSS1* gene in *Elymus* species from the Asian part of Russia in comparison with sequences in reference accessions of the Eurasian species.

the dendrogram constructed using exons alone. Some of the North American species (marked by dots on the dendrogram) together with the Asian species *P. strigosa* formed a joint clade with the group of the St_1 subgenome, while the others met in the St_2 group along with all accessions of the North American species *P. spicata*. *GBSS1* sequences of the Y subgenome in *E. gmelinii* and *E. pendulinus* showed a closer relationship with the St_2 group, which does not contradict the data on the evolutionary origin of this subgenome (Mason-Gamer et al., 2010a).

The H subgenome showed a similar pattern of differentiation. Figure 5 shows a dendrogram constructed from complete sequences of the H genome introns and exons from Russian and North American species (the latter are marked with dots in the figure). Two perennial *Hordeum* species (marked with asterisks) were taken as references. Gene copies from the H genome appeared to be divided into two main clades (designated as H_1 and H_2). Clade H_1 included exclusively Russian species, while clade H_2 was formed by Russian northeastern and all North American species. Each of these clades has its own ancestral taxon from the contemporary genus *Hordeum*: widespread in Eurasia *H. jubatum* for the Russian H_1 group and North American *H. californicum* Covas & Stebbins for the H_2 group.

Russian species from clade H_1 showed significantly greater differentiation than the species from heterogeneous clade H_2. Clade H_1 appeared to be divided into 3 subclades. Primarily three clones of the northeastern species *E. kronokensis* and *E. peschkovae* went to a separate group. This fact indirectly confirms the significant isolation of the latter from Siberian *E. confuses*, although they are similar in spike morphology. *E. confusus*, in turn, clustered most closely with the reference *H. jubatum*. The most distant cluster was formed by all accessions of *E. caninus* with an addition of the clone of South Ural endemic *E. uralensis*. The largest cluster was formed by the Siberian mountain species *E. komarovii*, *E. transbaicalensis* and *E. margaritae*, which an addition of a pair of reference clones of *E. sibiricus* and, as the most unexpected fact, a clone of *E. subfibrosus* accession from Chukotka. Remarkably, the different reference accessions of *E. mutabilis* fell into different H subgenome clades.

Mixed clade H_2 included not only all North American clones of *Elymus* species together with *H. californicum*, but also clones from different regions of Russia: *E. kamczadalorum* and *E. charkeviczii* (species from the Kamchatka Peninsula), *E. macrourus*, *E. jacutensis*, *E. lenensis* (northern accessions from the Taymyr Peninsula), *E. sajanensis* (a Siberian mountain species) and two of three *E. mutabilis* (reference Chinese

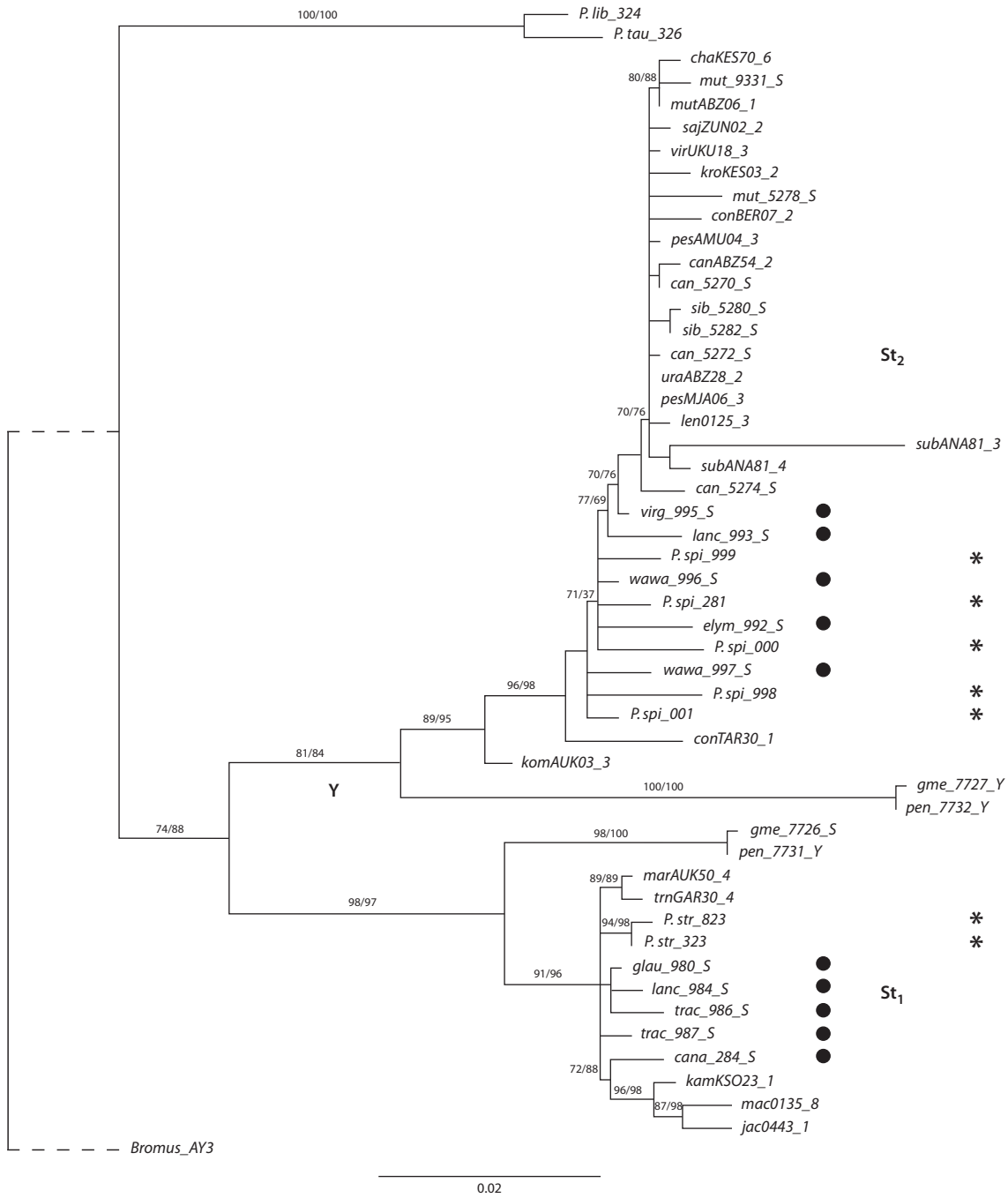

Fig. 4. The maximum likelihood tree constructed from multiple alignment of St subgenome *GBSS1* intron and exon (9–14) sequences in *Elymus* species from the Asian part of Russia in comparison with sequences in Eurasian and North American (marked by dots) reference species (St and Y subgenomes). Asterisks indicate *Pseudoroegneria* species carriers of the St genome.

SH-aLRT (%)/UFboot (%) support values are shown.

mut_5279_H and South Ural ABZ06_2). The third reference, mut_9330_H *E. mutabilis*, from Altai fell into clade H_1. Thereby, only some tendency toward relations between the North American accessions and northern or eastern accessions of Russian species can be derived from the H subgenome sequence analysis. The close relationship between American and Kamchatka species is easier to understand, taken into account the historical connections of these flora with each other, as well as with the species from the wide northern distribution areas of *E. macrourus* and *E. jacutensis*. It is more difficult to explain the close proximity of Chinese and South Ural accessions of *E. mutabilis* to this group.

Nevertheless, *GBSS1* gene variability provides a tool to trace evolutionary relations of species and local geographical

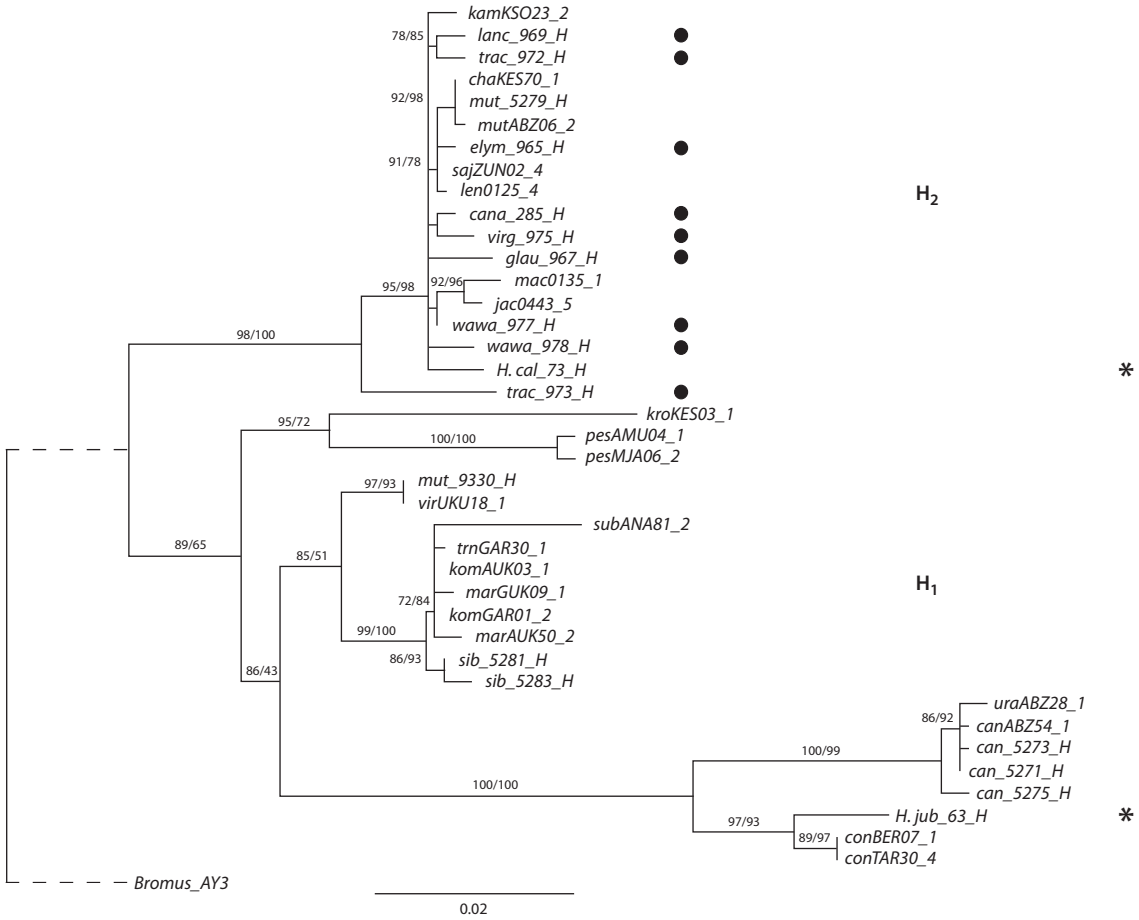

Fig. 5. The maximum likelihood tree constructed from multiple alignment of H subgenome *GBSS1* gene intron and exon (9–14) sequences in *Elymus* species from the Asian part of Russia in comparison with sequences in Eurasian and North American (marked by dots) reference species. Asterisks indicate the carriers of the H genome: the North American species *Hordeum californicum* and the widely distributed species of Asian origin *H. jubatum*.

SH-aLRT (%)/UFboot (%) support values are shown.

races from Siberia and the Russian Far East. If we consider the relative position of the accessions inside the clades of the subgenomes, we will see that the clusters combined the species accessions according to their perceived relationship. *E. jacutensis* and *E. macrourus* species, for instance, united into the common clusters in both H and St clades (see Fig. 2), as well as on separate dendrograms of these subgenomes (see Fig. 4, 5), thereby confirming the earlier assumptions about *E. jacutensis* being an aristate subspecies of *E. macrourus* (Tsvelyov, 1964). This fact is consistent with data on comparative morphological and peptide electrophoretic analyses and hybridization of these species' particular biotypes (Agafonov, 2008).

A comparative sequence analysis confirmed the isolation of *E. kamczadalorum* from the Kamchatka species *E. charkeviczii*, which was previously established using data on comparative morphology, electrophoresis of seed endosperm storage proteins, sexual hybridization (Agafonov, Gerus, 2008) and molecular ISSR analysis (Kobozeva et al., 2017). The species *E. komarovii* and *E. transbaicalensis* formed indistinguishable branches inside clade H_1 together with the Altai species *E. margaritae*, while *E. transbaicalensis* and *E. margaritae* clones were grouped inside clade St_1. The phylogenetic proximity of the first two species has been repeatedly experimentally confirmed previously (Agafonov et al., 2019), while the degree of *E. margaritae* isolation is currently being studied using biosystematic methods. The most unexpected data were obtained regarding the relationships in the group of South Ural biotypes of *E. uralensis*, *E. viridiglumis*, *E. caninus*, and *E. mutabilis*. These data are currently being verified in the field and laboratory experiments.

Conclusion

Therefore, despite a complicated reticulate evolution in parallel with various related allopolyploid genera and constantly ongoing active microevolutionary transformations, basic genomes seem to have retained unique ancestral features. This makes it relatively easy to identify the genomic composition and to classify modern species within the framework of a phylogenetically oriented taxonomic model of the genus. In our opinion, the integrity of the genus ought to be preserved, because some species in the independent genus *Roegneria* with the genomic formula StY (Baum et al., 1991) are similar

in morphology to species in the newly proposed genus *Campeiostachys* with the genomic formula StHY (Baum et al., 2011), the species of which are significantly different from each other in morphology. The St genome originating from the ancestors of the genus *Pseudoroegneria* seems to be an anchoring constant for a genetic unification of all members of the genus.

We suppose that differentiation of the genus should be based on a model of microevolutionary complexes representing an aggregate of taxa evolving through hybridization and introgression. The degree of taxa relationship within the complex should be confirmed using biosystematic methods with the obligatory determination of the ability to cross, i. e. taking into account the position in the system of recombination (RGP) and inrogressive (IHP) gene pools (Agafonov, Salomon, 2002). In fact, the microevolutionary complex is a projection of the RGP collection onto the taxonomic model of the genus, considering the genomic constitution of the species. Each microevolutionary complex should be thought of as a branched system of different ranks of taxa (species and subspecies), remaining therefore a phylogenetically confirmed structure.

In the future, it is necessary to determine the taxonomic rank of microevolutionary complexes, which can be sections or aggregates of the same species in a broad sense, as shown by the example of a revision of *Pendulini* (Nevski) Tzvelev sub-section of the *Goulardia* (Husn.) Tzvelev section (Kobozeva, Agafonov, 2015).

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Acknowledgements. This work was carried out as part of the state grant for the Central Siberian Botanical Garden SB RAS "Estimation of the morphogenetic potential of the North Asian plant population by experimental methods" (state registration number: AAAA-A17-117012610051-5) with partial support from the Russian Fund for Basic Research (project No. 18-04-01030). The materials of the bio-resource scientific collection of the CSBG SB RAS "Collections of living plants in open and closed ground", USU No. 440534 were used. DNA sequencing was performed at SB RAS Genomics Core Facility (ICBFM, Novosibirsk). The authors would like to acknowledge engineer M.V. Yemtseva (CSBG SB RAS) for important assistance in the *GBSS1* gene sequencing result analysis.

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

Received June 6, 2019. Revised July 30, 2019. Accepted July 31, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):827-835
DOI 10.18699/VJ19.556

The Fifth International Scientific Conference PlantGen2019

Создание и изучение интрогрессивных линий мягкой пшеницы, полученных на основе синтетической формы RS7

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Синтетическая форма RS7 (BBAAUS), у которой первые два генома, A и B, происходят от мягкой пшеницы, а третий состоит из хромосом *Aegilops speltoides* (S) и *A. umbellulata* (U), получена от скрещивания синтетических форм Авродес (BBAASS) и Авролата (BBAAUU). От беккроссов с восприимчивыми к листовой ржавчине, желтой ржавчине и мучнистой росе сортами мягкой пшеницы Краснодарская 99, Фишт и Ростислав были созданы устойчивые к этим болезням интрогрессивные линии. ПЦР-анализ показал наличие амплификации фрагментов с маркером SCS421, специфичным для гена *Lr28*, у линии 4991п17. Цитологическое изучение (C-banding и FISH) 14 линий выявило хромосомные перестройки у 12 из них. В большинстве случаев линии несут транслокации от *Ae. speltoides*, установленные в хромосомах 1D, 2D, 3D, 2B, 4B, 5B и 7B. Были идентифицированы также линии с замещенными хромосомами 1B (1S), 4D (4S), 5D (5S) и 7D (7S). Отобраны линии, несущие одновременно генетический материал от *Ae. speltoides* и *Ae. umbellulata*. У линии 3379п14 идентифицированы транслокации: T7US-7DS.7DL – в коротком плече хромосомы 7D от *Ae. umbellulata* и в хромосомах 5BL, 1DL, 2DL от *Ae. speltoides*. В линии 4626п16 определены транслокации: 2DS.2DL-2UL от *Ae. umbellulata* и T7SS.7SL-7DL от *Ae. speltoides*. Транслокации T1DS.1DL-1SL, T3DS.3DL-3SL от *Ae. speltoides* и T2DS.2DL-2UL, T7DL.7DS-7US от *Ae. umbellulata* выявлены впервые. Сделано предположение, что линии могут нести неидентифицированные ранее гены устойчивости к грибным болезням, в частности к листовой ржавчине, от видов *Ae. speltoides* и *Ae. umbellulata*.
Ключевые слова: мягкая пшеница; синтетические формы; устойчивость к болезням; цитологический анализ; замещенные хромосомы; транслокации; C-banding; FISH.

Для цитирования: Давоян Р.О., Бебякина И.В., Давоян Э.Р., Миков Д.С., Зубанова Ю.С., Болдаков Д.М., Бадаева Е.Д., Адонина И.Г., Салина Е.А., Зинченко А.Н. Создание и изучение интрогрессивных линий мягкой пшеницы, полученных на основе синтетической формы RS7. Вавиловский журнал генетики и селекции. 2019;23(7):827-835. DOI 10.18699/VJ19.556

The development and study of common wheat introgression lines derived from the synthetic form RS7

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Synthetic recombination form RS7 (BBAAUS), in which the first two genomes, A and B, originate from common wheat, and the third recombinant genome consists of *Aegilops speltoides* (S) and *Ae. umbellulata* (U) chromosomes, was obtained from crossing synthetic forms Avrodes (BBAASS) and Avrolata (BBAAUU). Resistant to leaf rust, yellow rust and powdery mildew, introgression lines have been obtained from backcrosses with the susceptible varieties of common wheat Krasnodarskaya 99, Fisht and Rostislav. PCR analysis showed the presence of amplification fragments with marker SCS421 specific for the *Lr28* gene in the line 4991n17. The cytological study (C-banding and FISH) of 14 lines has revealed chromosomal modifications in 12 of them. In most cases, the lines carry translocations from *Ae. speltoides*, which were identified in chromosomes 1D, 2D, 3D, 2B, 4B, 5B and 7B. Also, lines with the substituted chromosomes 1S (1B), 4D (4S), 5D (5S) and 7D (7S) were identified. Lines that have genetic material from *Ae. speltoides* and *Ae umbellulata* at once were revealed. In the line 3379n14, translocations in the short arm of chromosome 7D from *Ae. umbellulata* and chromosomes 5BL, 1DL, 2DL from *Ae. speltoides* were revealed. The line 4626p16 presumably has a translocation on the long arm of chromosome 2D from *Ae. umbellulata* and the T7SS.7SL-7DL translocation from *Ae. speltoides*. The T1DS.1DL-1SL and T3DS.3DL-3SL translocations from *Ae. speltoides*, and T2DS.2DL-2UL and T7DL.7DS-7US from

Ae. umbellulata have been obtained for the first time. These lines may carry previously unidentified disease resistance genes and, in particular, leaf rust resistance genes from *Ae. speltoides* and *Ae. umbellulata*.
Key words: common wheat; synthetic forms; resistance to diseases; cytological analysis; substituted chromosomes; translocations; C-banding; FISH.

For citation: Davoyan R.O., Bebyakina I.V., Davoyan E.R., Mikov D.S., Zubanova Yu.S., Boldakov D.M., Badaeva E.D., Adonina I.G., Salina E.A., Zinchenko A.N. The development and study of common wheat introgression lines derived from the synthetic form RS7. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7): 827-835. DOI 10.18699/VJ19.556 (in Russian)

Введение

Мягкая пшеница (*Triticum aestivum* L.) – важнейшая продовольственная культура. Генетический потенциал ее продуктивности в настоящее время практически исчерпан, и урожайность во многом зависит от устойчивости возделываемых сортов к неблагоприятным абиотическим и биотическим факторам среды. Генетических ресурсов самой мягкой пшеницы недостаточно для решения этих проблем. В особенности это касается генов устойчивости к болезням, ограниченное разнообразие которых является одним из основных лимитирующих факторов селекции.

Значительный резерв генов, контролирующих хозяйственно ценные признаки, находится в генофонде многочисленных родственных мягкой пшенице видов и родов. Многие из них были с успехом использованы для решения актуальных задач селекции мягкой пшеницы (Knott, 1987; Friebe et al., 1996). В настоящее время значительная часть эффективных генов устойчивости к болезням происходит из этого генофонда (McIntosh et al., 2013). Один из эффективных методов передачи ценного генетического материала от диких сородичей – создание и использование синтетических геномно-замещенных и геномно-добавленных форм в качестве «мостиков» для передачи генетического материала в культурную пшеницу от диких сородичей.

В Национальном центре зерна (НЦЗ) им. П.П. Лукьяненко разработан оригинальный подход, позволивший создать синтетические геномно-замещенные формы Авродес (BBAASS), Аврозис (BBAA$S^{sh}S^{sh}$), Авролата (BBAAUU), Авротата (BBAA$N^{n}N^{n}$), Авроале (BBAARR), у которых геном D мягкой пшеницы был замещен, соответственно, на геномы *Ae. speltoides*, *Ae. sharonensis*, *Ae. umbellulata*, *Ae. uniaristata* и *S. cereale* (Жиров и др., 1984). Эти формы были использованы для передачи хозяйственно ценных генов в мягкую пшеницу, а также для получения «вторичных» синтетических форм (Давоян и др., 2012). Основной формой для создания рекомбинантных синтетиков была синтетическая форма Авродес. Она обладает полученной от *Ae. speltoides* способностью стимулировать гомеологичную конъюгацию хромосом (Tsatsenco et al., 1993). Кроме этого, синтетическая форма Авродес проявляет высокую устойчивость к листовой ржавчине (*Puccinia triticina* Eriks.), желтой ржавчине (*Puccinia stiifomis* West.), мучнистой росе (*Blumeria graminis* f. sp. *tritici*), а также отличается повышенным содержанием белка. Предполагалось, что общие для всех форм геномы BA мягкой пшеницы могут стать основой для возможного рекомбинационного процесса между хромосомами двух различных геномов диких видов. Такие формы можно использовать для получения множественных интрогрессий, новых транслокаций, они способствуют в отдельных случаях передаче генетического материала одновременно от двух дикорастущих видов. От скрещивания формы Авродес с другими геномно-замещенными формами были получены синтетические формы (RS-формы), у которых первые два генома, A и B, происходят от мягкой пшеницы сорта Аврора, а третий геном сочетает хромосомы генома S от *Ae. speltoides* с хромосомами других диких видов: *Ae. umbellulata* (U), *Ae. sharonensis* (S^{l}), *Secale cereale* (R) (Давоян и др., 2012).

В настоящей работе приведены результаты создания, молекулярно-цитологического анализа и оценки по устойчивости к грибным болезням интрогрессивных линий мягкой пшеницы, полученных с использованием синтетической формы RS7 (BBAASU) (Авролата × Авродес).

Материалы и методы

Исходным материалом были интрогрессивные линии мягкой пшеницы (BC_2F_6–BC_3F_5), полученные от последовательных скрещиваний синтетической формы RS7 с восприимчивыми к листовой ржавчине, желтой ржавчине и мучнистой росе сортами мягкой пшеницы селекции НЦЗ им. П.П. Лукьяненко: Краснодарская 99, Фишт и Ростислав. Сорт Ростислав несет в себе ржаную транслокацию 1RS.1BL. Сорт Фишт имеет транслокацию 5BS.5BL-5GL и замещенные хромосомы 1D($1D^{t}$) и 6D($6D^{t}$), полученные от видов *T. militinae*, *Ae. tauschii* через синтетическую форму *T. miguschovae*.

Конъюгацию хромосом в метафазе I мейоза изучали на давленых препаратах, окрашенных уксуснокислым гематоксилином. Для приготовления маточного раствора гематоксилина брали 4 г порошка гематоксилина, 1 г железо-аммиачных квасцов, доводили до 100 мл 45 % уксусной кислотой. Реактив созревал в течение 160 ч в темноте в термостате при подогреве до 40 °С. Полученный реактив черного цвета фильтровали с помощью складчатого фильтра и оставляли в темной бутылке на хранение в холодильнике. Для окрашивания колосков пшеницы, взятых в стадии прохождения мейоза, реактив разбавляли 45 % уксусной кислотой в 1.5–2 раза. Предварительно зафиксированные в фиксаторе Карнуа колосья (Паушева, 1974) окрашивали разбавленным раствором гематоксилина в термостате при 25 °С в течение 1–2 суток. В мейозе подсчитывали число би-, уни- и мультивалентов.

Заражение и оценку по устойчивости к болезням растений во взрослой стадии проводили в полевых условиях. Популяцию линий заражали смесью уредоспор желтой и листовой ржавчины, собранных с разных сортов пшеницы в фазах выхода в трубку и трубка–колошение соответственно. Устойчивость к желтой ржавчине определяли по шкале Гасснера и Штрайба в фазу молочной спелости зерна (Gasner, Straib, 1934). Учет поражения листовой

Table 1. PCR conditions and primers used for identification of genes for leaf rust resistance

Gene	Primers	Annealing temperature, °C	Amplicon size, bp	Reference
Lr28	CS421570-R	60	570	Cherukuri et al., 2005
	CS421570-L			
Lr35	BCD260F1	59	931	Seyfarth et al., 1999
	35R2			
Lr47	PS10R	61	224	Dubcovsky et al., 2000
	PS10L			
Lr51	S30-13L	52	422	Helguera et al., 2005
	AGA7-759R			
Lr9	FJ13/1	63	1100	Schachermayer et al., 1994
	FJ13/2			

ржавчиной проводили в фазу молочно-восковой спелости зерна по международной шкале Майнса и Джексона (Mains, Jakson, 1926). К устойчивым относили растения с типом реакции 0 (иммунные), 1 (высокоустойчивые) и 2 (умеренно устойчивые). Растения с промежуточным типом реакции от 0 до 1 (единичные очень мелкие пустулы с некрозом) обозначали баллом 01. К восприимчивым относили растения с типом реакции 3–4. Оценку устойчивости к мучнистой росе проводили на естественном инфекционном фоне в фазу колошения по шкале Гешеле (Пересыпкин, 1979). Растения со степенью поражения мучнистой росой 0–20 % считались устойчивыми.

ДНК выделяли из 5–7-дневных этиолированных проростков пшеницы по методу Плашке с коллегами (Plaschke et al., 1995). Идентификацию генов *Lr* осуществляли с помощью метода полимеразной цепной реакции (ПЦР) с праймерами, маркирующими гены *Lr9*, *Lr28*, *Lr35*, *Lr47*, *Lr51* и *Lr66*. Праймеры подбирали на основании литературных данных, их названия и условия амплификации приведены в табл. 1.

Реакционная смесь объемом 25 мкл содержала 1× буфер для Taq-ДНК-полимеразы (50 мМ KCl, 20 мМ трис-HCl, pH 8.4, 2–5 мМ $MgCl_2$, 0.01 % твин-20), 2 мМ $MgCl_2$, по 0.2 мМ каждого dNTP, 12.5 мМ каждого праймера, 50 нг ДНК и 1 ед. Taq-полимеразы. Амплификацию вели согласно условиям, приведенным в табл. 1, с незначительными модификациями. Продукты ПЦР разделяли с использованием электрофореза в 1.8 % агарозном геле с 0.5× буфером TBE. Гели окрашивали бромистым этидием и фотографировали в ультрафиолетовом свете с помощью фотобокса Infiniti 1000.

В качестве положительных контролей для определения генов были использованы почти изогенные линии сорта Thatcher с генами устойчивости к листовой ржавчине *Lr9* (TcLr9) и *Lr35* (TcLr35), в качестве отрицательного контроля был восприимчивый к листовой ржавчине сорт Аврора.

Дифференциальное окрашивание хромосом (C-banding) осуществляли в Институте общей генетики им. Н.И. Вавилова по методике, разработанной в лаборатории функциональной морфологии хромосом Института молекулярной биологии им. В.А. Энгельгардта РАН (Badaeva et al., 1994).

Флуоресцентную *in situ* гибридизацию (FISH) проводили в Институте цитологии и генетики СО РАН по ранее опубликованной методике (Salina et al., 2006) с применением зондов pSc119.2 (Bedbrook et al., 1980) и pAs1 (Rayburn, Gill, 1986) для идентификации хромосом (Schneider et al., 2003), Spelt1 (Salina et al., 2004) – для выявления генетического материала *Ae. speltoides* в исследуемых линиях.

Результаты

Синтетическая форма RS7 проявляла высокую устойчивость к листовой и желтой ржавчине и мучнистой росе, но была полностью стерильна. Для передачи устойчивости и восстановления фертильности эту форму последовательно скрещивали с восприимчивыми к этим болезням сортами мягкой пшеницы Краснодарская 99, Фишт и Ростислав. Первое поколение гибридных растений было частично фертильным либо полностью стерильным и также проявляло устойчивость к комплексу болезней пшеницы. В зависимости от уровня фертильности этих растений проводили беккроссирование мягкой пшеницей от 1 до 3 раз, но в большинстве случаев для ее восстановления было достаточно двух беккроссов. Полученные от беккроссов растения имели от 40 до 42 хромосом. Результаты цитологического изучения хромосомных ассоциаций в метафазе I мейоза приведены в табл. 2.

В первых поколениях растений, полученных от беккроссов с мягкой пшеницей, наблюдается большое количество растений с мультивалентами (80 %), которое существенно уменьшается (до 8.2 %) по мере увеличения числа беккроссов и отбора фертильных растений с нужными признаками в следующих поколениях. Примеры конъюгации хромосом в метафазе I мейоза у гибридных растений показаны на рис. 1.

В результате отбора 42-хромосомных растений со стабильным протеканием мейоза из популяции гибридных растений RS7 × *T. aestivum* поколения BC_2F_6–BC_3F_5 получено 130 линий. В настоящей статье представлены результаты изучения 14 линий, наиболее интересных по своим морфо-биологическим признакам.

Поскольку главной задачей являлась передача от RS7 мягкой пшенице устойчивости к болезням, линии оценивались по устойчивости к наиболее распространенным и

Table 2. Presence of multivalents at MI of meiosis in hybrid plants F_1 and BC_1 F_1–BC_3 F_1 of RS7 × *T. aestivum*

Generation	Numbers of plants examined	Numbers (percentages) of plants with multivalents
F_1	25	20 (80 %)*
BC_1 F_1	60	35 (58.3 %)
BC_1F_1–BC_2 F_1	75	25 (33.3 %)
BC_2F_1–BC_3 F_1	134	11 (8.2 %)

* Percent from total number of plants.

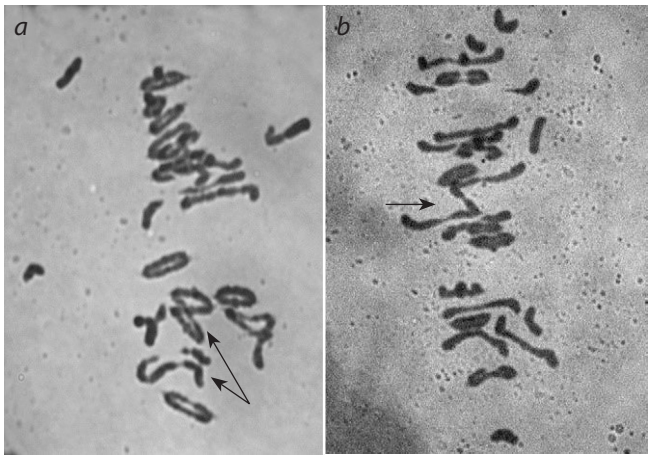

Fig. 1. Chromosome pairing at metaphase I of meiosis in hybrid plants: *a*, F_1 RS7 × Krasnodarskaya 99 ($12^{II}+6^{I}+1^{VIII}+1^{IV}$); *b*, BC_3 (RS7 × Krasnodarskaya 99) × Rostislav (16II + 5I + 1V). Arrows indicate of multivalents.

Table 3. Resistance to diseases in RS7 × *T. aestivum* introgression lines (2017–2018)

Line	Resistance to		
	leaf rust, reaction type, score	yellow rust, reaction type, score	powdery mildew, %
4572p16	2	2	20
3379p14	01	2	15
4581p16	01	01	15
4586p16	2	1	20
4607p16	2	2	15
4623p16	2	01	15
4626p16	2	01	15
4635p16	2	2	20
4662p16	1	1	20
4665p16	1	2	25
4670p16	2	1	25
4671p16	2	2	20
4991p17	1	1	15
5026p17	1	2	20
Krasnodarskaya 99	4	3	25
Fisht	3	3	30
Rostislav	3	3	30

вредоносным болезням – листовой и желтой ржавчине и мучнистой росе. В табл. 3 приведена их характеристика за 2017–2018 гг. Все 14 линий были устойчивы к листовой ржавчине. Высокую резистентность с типом реакции 01 и 1 имели 6 линий: 3379п14, 4581п16, 4662п16, 4665п16, 4991п17 и 5026п17. Остальные линии обладали средней устойчивостью к этой болезни. Резистентность к желтой ржавчине несли также все 14 линий, 3 из которых, 4581п16, 4623п16 и 4626п16, имеют тип реакции на заражение 01. Устойчивость к мучнистой росе проявили 12 линий.

Особую ценность для селекции представляют линии, устойчивые к комплексу болезней. Две линии, 4665п16 и 4670п16, имели групповую устойчивость к двум и 12 линий ко всем трем болезням. Следует отметить линии 3379п14, 4581п16 и 4991п17, обладающие высокой резистентностью ко всем трем болезням.

Разный тип реакции линий по устойчивости к листовой ржавчине может свидетельствовать о различных интрогрессиях чужеродного генетического материала в геном мягкой пшеницы. Для того чтобы выяснить природу переданного материала от синтетической формы RS7, были скрещены 10 изучаемых линий с одним из наиболее мейотически стабильных сортов мягкой пшеницы Краснодарская 99 и изучен мейоз у гибридов F_1 (табл. 4).

Ассоциация хромосом у гибридных растений F_1, $20^{II}+2^{I}$ и $19^{II}+4^{I}$ может свидетельствовать о замещении одной или двух пар пшеничных хромосом на чужеродные. Такие замещения могут быть у 8 линий из 10 анализируемых – 3379п14, 4581п16, 4586п16, 4623п16, 4626п16, 4635п16, 4662п16 и 4671п16. Наличие мультивалентов у гибридов с линиями 3379п14 (0.5 %); 4626п16 (1.7 %); 4635п16 (2.9 %); 4662п16 (2.3 %) и 4671п16 (1.9 %) свидетельствует о том, что эти линии могут нести транслокации от синтетика RS7 и сортов Фишт и Ростислав.

Для идентификации перестроек хромосом и изменений в геноме у интрогрессивных линий нами были использованы методы дифференциального окрашивания хромосом (C-banding) и флуоресцентной *in situ* гибридизации (FISH). Применение метода дифференциальной окраски хромосом для двух линий, 5026п17 и 4991п17, с генетическим материалом RS7 позволило выявить у них неизвестные ранее перестройки и замещения хромосом (рис. 2). Линия 5026п17, кроме известной транслокации 1RS.1BL, имеет замещение хромосомы 5D на хромосому 5S от *Ae.speltoides*. Линия 4991п17 несет замещения 7D (7S) от *Ae. speltoides* и 6D ($6D^{ae}$) от другого вида *Aegilops*. Предположительно, замещение 6D ($6D^{ae}$) происходит от сорта-реципиента Фишт, который, в свою очередь, получил это замещение от *T. miguschovae* (GGAADD), в которой присутствует геном D от *Ae. tauschii* (Давоян и др., 2012).

Table 4. Analysis of meiosis at MI in F_1 hybrids obtained by crossing cytologically stable RS7 × *T. aestivum* lines to cv. Krasnodarskaya 99

Hybrid	Numbers of cells examined	21^{II}, %	$20^{II}+2^{I}$, %	$19^{II}+4^{I}$, %	Cells with multivalents, %
4572p16×K99*	168	89.5	7.9	2.6	–
3379p14×K99	205	56.3	43.2	–	0.5
4581p16×K99	226	66.7	30.5	2.8	–
4586p16×K99	178	69.3	30.7	–	–
4607p16×K99	128	92.2	7.0	0.8	–
4623p16×K99	256	45.6	42.3	12.1	–
4626p16×K99	305	79.6	14.4	4.3	1.7
4635p16×K99	238	79.8	11.2	6.1	2.9
4662p16×K99	334	67.4	20.1	10.2	2.3
4671p16×K99	312	46.5	39.4	12.2	1.9
Krasnodarskaya 99	112	91.0	6.3	2.7	–

* K99 – common wheat cultivar Krasnodarskaya 99.

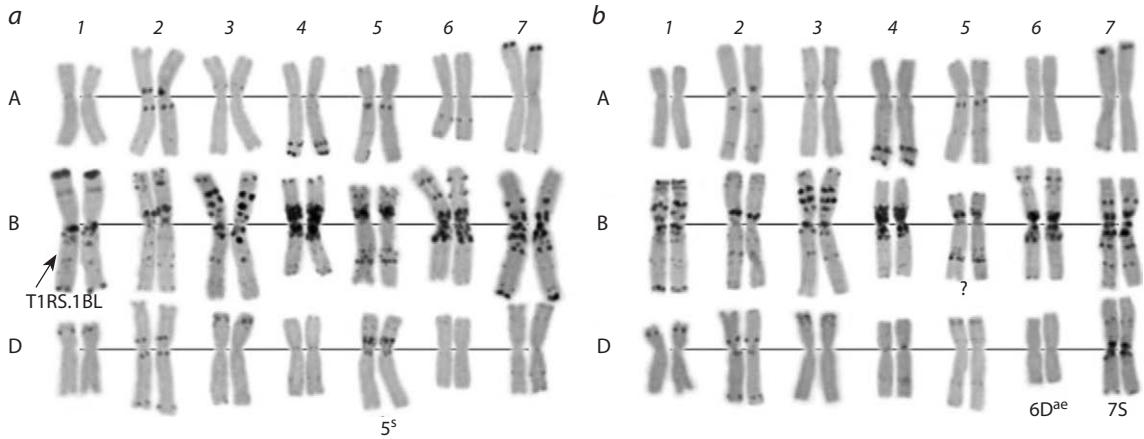

Fig. 2. Karyotype of introgression lines: (*a*) 5026p17 and (*b*) 4991p17 C-banded with genetic material from RS7 synthetic forms.

С использованием метода флуоресцентной *in situ* гибридизации проанализировано 12 линий (табл. 5). Хромосомные перестройки затронули 10 из 12 изученных линий. Участие хромосом генома A в перестройках не установлено. Перестройки хромосом генома B определены у 8 линий и генома D – 11 линий. Наиболее часто перестройки затрагивают хромосомы 5B (6 линий) и 2D (4 линии). В большинстве случаев линии несут транслокации от *Ae. speltoides*. Транслокации от этого вида идентифицированы на хромосомах 1D, 2D, 3D, 5D, 7D, 4B, 5B. Выявлены также линии с замещением хромосом пшеницы на хромосомы *Ae. speltoides*: 1B(1S) и 4D(4S).

Линий, несущих транслокации и замещенные хромосомы только от *Ae. umbellulata*, не обнаружено. В то же время две линии несут одновременно генетический материал от *Ae. speltoides* и *Ae. umbellulata*. В линии 3379п14 (рис. 3, *а*) определены транслокации: T7DL.7DS-7US в коротком плече хромосомы 7D от *Ae. umbellulata* и на плечах хромосом 5BL, 1DL, 2DL от *Ae. speltoides*. Кроме этого, у этой линии обнаружено замещение хромосомы 4D на хромосому 4S *Ae. speltoides*. Линия 4626п16 (см. рис. 3, *б*), предположительно, имеет транслокацию T2DS.2DL-2UL на длинном плече хромосомы 2D от *Ae. umbellulata* и транслокацию в хромосоме 7D от *Ae. speltoides*. В ней могут присутствовать также транслокации от *Ae. speltoides* на хромосомах 5B и 7B. Следует отметить, что транслокации T1DS.1DL-1SL, T2DS.2DL-2UL, T7DL.7DS-7U и хромосомное замещение 4D (4S) получены впервые.

Выявленные интрогрессивные линии представляют особый интерес как возможные доноры новых генов устойчивости к болезням, в частности к листовой ржавчине, переданных от видов *Ae. speltoides* и *Ae.umbellulata*. В настоящее время в каталог генных символов пшеницы внесено шесть генов устойчивости, переданных от *Ae. speltoides*: *Lr 28*, *Lr 35*, *Lr36*, *Lr47*, *Lr51*, *Lr66* и один ген, *Lr9*, от *Ae. umbellulata* (McIntosh et al., 2013).

Для постуляции генов устойчивости к листовой ржавчине в геноме полученных нами линий использовали ДНК-маркеры. Ранее (Давоян и др., 2012) был проведен анализ синтетической формы Авродес на присутствие

Table 5. Results of the analysis of RS7 × *T. aestivum* introgression lines by *in situ* hybridization (FISH)

Line	Source	Revealed translocations and substitution
4572p16	*Ae. speltoides* *Ae. umbellulata*	The full number of chromosomes of wheat. No chromosomal reorganizations are detected
3379p14	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL; T1DS.1DL-1SL; T2DS.2DL-2SL; T5BS.5BL-5SL; 4S(4D); a translocation or deletion in 6DL T7DL.7DS-7US
4581p16	*Ae. speltoides* *Ae. umbellulata*	1B(1S), T5BS.5BL-5SL Not detected
4586p16	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL; T3DS.3DL-3SL Not detected
4607p16	*Ae. speltoides* *Ae. umbellulata*	The full number of chromosomes of wheat. No chromosomal reorganizations are detected
4623p16	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL; T5BS.5BL-5SL Not detected
4626p16	*Ae. speltoides* *Ae. umbellulata*	T7DL-7SL.7SS; T5BS.5BL-5SL T2DS.2DL-2UL
4635p16	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL; T2DS.2DL-2SL; T5BS.5BL-5SL Not detected
4662p16	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL; T5D Not detected
4665p16	*Ae. speltoides* *Ae. umbellulata*	T5BS.5BL-5SL Not detected
4670p16	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL. Putative translocation from *Ae. speltoides* on the long arm of an unidentified A genome chromosome Not detected
4671p16	*Ae. speltoides* *Ae. umbellulata*	T1RS.1BL; T2DS.2DL-2SL; T4BS (origin unknown); T5BL (origin unknown) Not detected

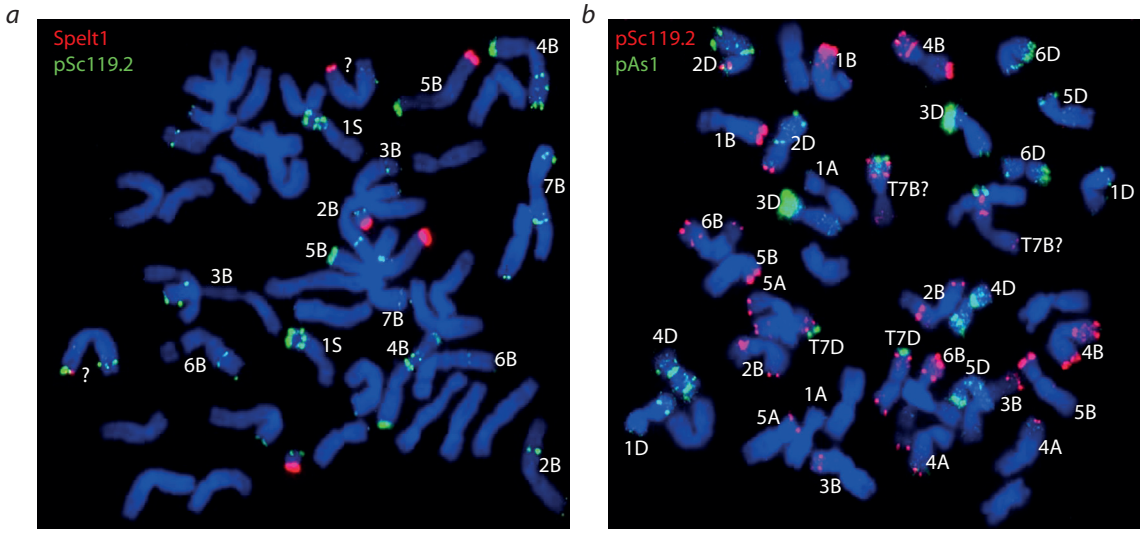

Fig. 3. Hybridization *in situ* (FISH): *a,* with the probes Spelt1 (red) and pSc119.2 (green) on metaphase chromosomes of line 4581p16; *b*, with the probes pAs1 (green) and pSc119.2 (red) on metaphase chromosomes of line 4626p16.

генов устойчивости к листовой ржавчине *Lr28*, *Lr35*, *Lr47*, *Lr51* и формы Авролата – на наличие гена *Lr9*. Ген устойчивости *Lr36* в анализ не был включен в связи с отсутствием эффективного молекулярного маркера к нему. Идентификацию гена *Lr66* на данном этапе не проводили.

Установлено, что синтетическая форма Авродес имеет из перечисленных генов только *Lr28*, *Lr35* и *Lr51*. В синтетической форме Авролата было подтверждено наличие гена *Lr9*. Исходя из этого, полученные интрогрессивные линии анализировали только на наличие у них эффектив-

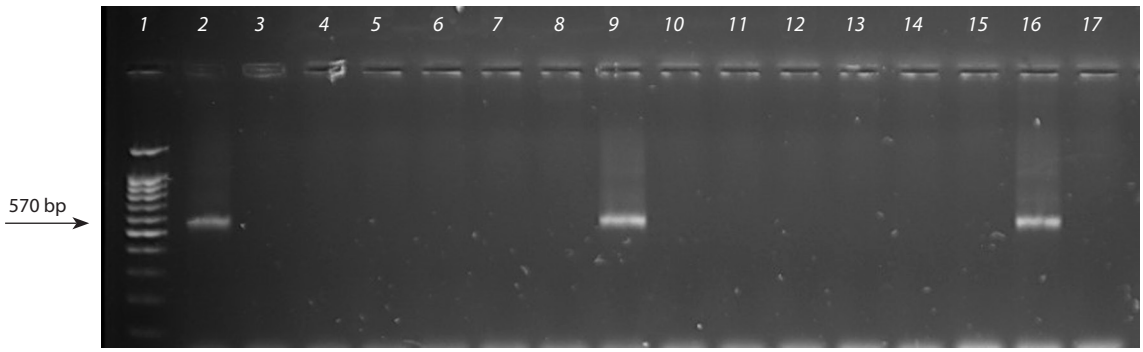

Fig. 4. Amplification products with primers CS421570-R and CS421570-L to the diagnostic marker linked to the leaf rust resistance gene *Lr28*.

1, molecular weight ladder; *2*, TcLr28; *3–14*, introgression lines; *9*, line 4991p17; *15*, Avrolata; *16*, Avrodes; *17*, cultivar Avrora.

ных генов устойчивости к листовой ржавчине *Lr9*, *Lr28*, *Lr35* и *Lr51*.

В анализ было включено 12 линий, кроме двух линий, 4572п16 и 4607п16, в которых, по результатам цитологического анализа, не было выявлено чужеродных интрогрессий. Только у одной линии, 4991п17, установлена амплификация фрагмента для маркера SCS421, специфичного для гена *Lr28* (рис. 4). В остальных линиях искомые гены маркерным анализом не обнаружены.

Обсуждение

Основной практической задачей при создании и использовании синтетической формы RS7 была возможность передачи от *Ae. speltoides* и *Ae. umbellulata* мягкой пшенице новых генов устойчивости к болезням. Такая работа основана на получении интрогрессивных линий с генетическим материалом от этих видов. Анализ конъюгации хромосом в метафазе I мейоза у гибридных растений, полученных от скрещивания RS7 с мягкой пшеницей, выявил большое количество растений с мультивалентами (80 %) в F_1, которое по мере увеличения числа беккроссов и отбора фертильных растений с нужными признаками в следующих поколениях существенно уменьшилось (до 8.2 % в BC_3). При получении рекомбинантных синтетических форм геномно-замещенная форма Авродес использована нами не только как источник ценных генов устойчивости к болезням, но и как промотор гомеологичной конъюгации хромосом. Можно предположить, что наиболее активно гомеологичная конъюгация хромосом осуществлялась в первых поколениях. В популяции гибридных растений происходил естественный отбор, стабилизирующий количество хромосом и их ассоциацию в мейозе в сторону мягкой пшеницы. Таким образом, в поколениях BC_2–BC_3 можно было проводить отбор фертильных растений с полезными признаками.

Отобранные для изучения 14 линий RS7 × *T. aestivum* поколения BC_2F_6–BC_3F_5 различались по устойчивости к листовой и желтой ржавчине и мучнистой росе. Выявлены линии с типами реакции к листовой ржавчине 01, 1 и 2, к желтой ржавчине 01, 1 и 2, со степенью поражения мучнистой росой 15 и 20 %. Линии отличаются также по устойчивости к комплексу перечисленных болезней. Разнообразие линий по устойчивости к болезням может свидетельствовать о различных интрогрессиях генетического материала RS7 в геноме мягкой пшеницы и возможной передаче нового гена(ов) устойчивости.

Цитологический анализ (C-banding и FISH) выявил хромосомные перестройки у 12 из 14 линий. Установлено, что генетический материал от синтетической формы RS7 в изученных линиях представлен как в форме транслокаций, так и в виде замещенных хромосом. Несмотря на небольшое количество изученных линий, было выявлено 12 хромосом с интрогрессиями, при этом в основном перестройками были затронуты хромосомы геномов B (4 хромосомы) и D (7 хромосом) мягкой пшеницы. Полученные результаты вполне ожидаемы. Они объясняются, во-первых, тем, что в синтетических формах Авродес (BBAASS) и Авролата (BBAAUU) геном D мягкой пшеницы замещен на геномы S *Ae. speltoides* и U от *Ae. umbelllata*, во-вторых, *Ae. speltoides* является вероятным донором генома B и, в-третьих – способностью синтетика Авродес стимулировать гомеологичную конъюгацию хромосом (Tsatsenco et al., 1993). Особо следует отметить обнаруженные впервые у мягкой пшеницы транслокации: T1DS.1DL-1SL, T2DS.2DL-2UL, T3DS.3DL-3SL, T7DL.7DS-7U, так как они могут быть вероятными источниками новых генов устойчивости к болезням, в частности к листовой ржавчине.

У некоторых линий результаты цитологического анализа и оценки по устойчивости к болезням не совпадают. Так, например, у линии 4623п16 методом гибридизации *in situ* идентифицировано всего две транслокации: T1RS.1BL и T5BS.5BL-5SL. В то же время анализ мейоза в метафазе I у гибридов F_1, полученных от скрещивания этой линии с сортом Краснодарская 99, свидетельствует о наличии в ней большего количества интрогрессий. В относительно устойчивых линиях 4572п16 и 4607п16 хромосомные перестройки не выявлены. Вероятно, в этих линиях присутствуют перестройки, которые не определяются методом FISH. В частности, это могут быть замещения 1D(1D^t) и 6D(6D^t), полученные от сорта Фишт. В нашей работе замещение 6D(6D^t) у линии 4991п17 было установлено методом дифференциального окрашивания хромосом (C-banding).

От вида *Ae. speltoides* мягкой пшенице переданы гены устойчивости к листовой ржавчине *Lr28*, *Lr35*, *Lr36*, *Lr47*,

Lr51 и *Lr66* (McIntosh et al., 2013). Эти гены были перенесены в хромосомы мягкой пшеницы 4A, 2B, 6B, 7A, 1B и 3A (Friebe et al., 1996; Dubcovsky et al., 2000; Helguera et al., 2005; Marais et al., 2010). Кроме этого, И.Г. Адонина с коллегами (2012) охарактеризовали новую транслокацию T5BS·5BL-5SL от *Ae. speltoides* с эффективным геном, обозначенным как *LrASP5*. Несмотря на довольно большое количество переданных генов, не исключено, что у *Ae. speltoides* могут присутствовать другие гены устойчивости к листовой ржавчине, о чем также свидетельствуют полученные нами ранее результаты (Давоян и др., 2017).

От *Ae. umbellulata* передан единственный ген устойчивости *Lr9*, локализованный в хромосоме 6B (Sears, 1956). Из перечисленных генов в селекции в основном используются гены устойчивости *Lr9*, *Lr28*, *Lr47*, *Lr ASP5* (Friebe et al., 1996; Адонина и др., 2016). На основании маркерного анализа сделано предположение, что синтетическая форма Авродес имеет из перечисленных генов только три: *Lr28*, *Lr35* и *Lr51* (Давоян и др., 2012). В синтетической форме Авролата подтверждено наличие гена *Lr9* (Давоян и др., 2012). Из проанализированных 14 линий только у одной линии, 4991п17, идентифицирован диагностический фрагмент амплификации маркера SCS421, разработанного для идентификации гена *Lr28*. В остальных линиях искомые гены не установлены.

Идентификация гена *LrAsp5* не проводилась. В то же время транслокация T5BS·5BL-5SL выявлена у шести из полученных нами линий: 3379п16, 4581п16, 4623п16, 4626п16, 4635п16, 4665п16. Возможно, эти линии также могут иметь ген устойчивости *LrAsp5* от *Ae. speltoides*.

Заключение

Таким образом, использование синтетической формы RS7 позволило получить большое разнообразие интрогрессивных линий, устойчивых к листовой и желтой ржавчине и мучнистой росе. Выявлены линии с новыми транслокациями и замещениями от *Ae. speltoides* и *Ae. umbellulata*, которые могут нести новые гены устойчивости к грибным болезням.

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Conflict of interest. The authors declare no conflict of interest.
Received May 31, 2019. Revised September 11, 2019. Accepted September 12, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):836-845
DOI 10.18699/VJ19.557

The Fifth International Scientific Conference PlantGen2019

Identification of univalent chromosomes in monosomic lines of cotton (*Gossypium hirsutum* L.) by means of cytogenetic markers

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The lack of clear morphological markers of cotton chromosomes contributed to the development of an unconventional method for marking chromosomes using translocations. Today, tester translocation cotton lines represent the most complete set of cytological markers. The results of cytogenetic analysis of F_1 hybrids obtained from crosses of monosomic cotton lines with translocation lines with identified chromosomes are presented. Cytogenetic identification and numbering of univalent chromosomes in 25 monosomic lines of the cytogenetic collection of the National University of Uzbekistan allowed us to establish the following univalent occurrences: chromosome 2 in four monosomic lines, chromosome 4 in 15 lines, chromosome 6 in four lines, chromosome 7 of the A_t-subgenome in one line and chromosome 18 of the D_t-subgenome in one line. The remaining 21 lines were duplicates of three non-homologous chromosomes. All monosomic lines identified were characterized by differences in univalent sizes, meiotic index, number of tetrads with micronuclei, pollen fertility, frequency of monosomy in the progeny, and a complex of morphological characters associated with the monosomy of the chromosome identified. Despite differences in the genotypic environment and methods for producing monosomics in the two cotton collections, there is a surprising coincidence of data suggesting a higher frequency of chromosomes 2, 4 and 6 occurring as monosomics, while the other chromosomes of the set occur as monosomics at a much lower frequency, and eight nonhomologous chromosomes (5, 8, 13 of the A_t-subgenome and 14, 15, 19, 22 and 24 of the D_t-subgenome of cotton) never do.

Key words: cotton; *Gossypium hirsutum* L.; cytogenetic analysis; cotton cytogenetics; monosomic lines; chromosome identification.

For citation: Sanamyan M.F., Bobokhujayev Sh.U. Identification of univalent chromosomes in monosomic lines of cotton (*Gossypium hirsutum* L.) by means of cytogenetic markers. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):836-845. DOI 10.18699/VJ19.557

Идентификация унивалентных хромосом у моносомных линий хлопчатника *Gossypium hirsutum* L. с помощью цитогенетических маркеров

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Отсутствие четких морфологических маркеров хромосом хлопчатника способствовало разработке нетрадиционного метода маркировки хромосом с помощью транслокаций. Тестерные транслокационные линии хлопчатника на сегодняшний день представляют собой наиболее полный набор цитологических маркеров. Приведены результаты цитогенетического анализа гибридов F_1, полученных от скрещиваний моносомных линий хлопчатника с транслокационными линиями с идентифицированными хромосомами. Цитогенетическая идентификация и нумерация унивалентных хромосом у 25 моносомных линий цитогенетической коллекции Национального университета Узбекистана позволила установить, что четыре моносомные линии имеют унивалентные хромосомы по хромосоме 2, 15 линий – по хромосоме 4, четыре линии – по хромосоме 6, одна линия – по хромосоме 7 A_t-субгенома и одна линия – по хромосоме 18 D_t-субгенома хлопчатника. Остальная 21 линия была дубликатом трех негомологичных хромосом. Все идентифицированные моносомные линии характеризовались различиями в размерах унивалентов, величине мейотического индекса, числа тетрад с микроядрами, фертильности пыльцы, частоты воспроизводства моносомного состояния в потомстве и комплекса морфологических признаков, ассоциированных с моносомией по идентифицированной хромосоме. Несмотря на различия в генотипической среде и методах получения моносомиков в двух коллекциях хлопчатника, наблюдается удивительное совпадение дан-

ных по большей частоте появления моносомиков по хромосомам 2, 4 и 6, тогда как моносомики по другим хромосомам набора появляются с куда меньшей частотой, а по восьми негомологичным хромосомам (5, 8, 13 A_t-субгенома и 14, 15, 19, 22 и 24 D_t-субгенома) вообще никогда не выявляются.
Ключевые слова: хлопчатник; *Gossypium hirsutum* L.; цитогенетический анализ; транслокационные линии; моносомные линии; идентификация хромосом.

Introduction

The need for a karyological study of cultures with weakly morphologically differentiated chromosomes has contributed to the development of non-traditional methods for marking chromosomes using translocations. Work on the creation of translocation tester sets that were obtained in five plant species was widespread: maise (Burnham, 1954), barley (Burnham et al., 1954), pea (Lamm, Miravalle, 1959), rye (Sybenga, Wolters, 1972) and tomato (Gill et al., 1980). The problem of identifying individual chromosomes of a set in species such as horse beans, beans, and soybeans was partially solved by obtaining translocation lines for some non-homologous chromosomes of the genome (Sjodin, 1971; Ashraf, Bassett, 1986; Mahama et al., 1999).

As is known, cultivated cotton species *Gossypium hirsutum* L. is an allotetraploid ($2n$ = 52) and includes two subgenomes (the A_t-subgenome and the D_t-subgenome). Brown M.S. et al. (1980) received 62 translocation lines of *G. hirsutum* cotton using X-, γ, Bikini radiation and fast neutron irradiation of seeds or pollen of various varieties, as well as several lines. In 58 of these lines, two non-homologous chromosomes were involved, in three – three chromosomes, and in one – four. To identify and number chromosomes, studies were carried out to classify translocated chromosomes as subgenomes. As a result of identification, it was found that the 26th chromosome was not involved in any of the translocations and was determined by the exclusion method, since for all years of research it was not possible to obtain a translocation involving this chromosome (Stelly, 1993). All these translocations include the most complete set of cytological markers for studying cotton genomes (Menzel, Brown, 1978).

In decision the problem of identifying small chromosomes, high hopes were associated with the use of the differential staining method, however, attempts to obtain differential chromosome banding sufficient to identify non-homologous chromosomes did not lead to the desired result (Turkov et al., 1980; Escalant, Schwendiman, 1984; Wang, 1985). The staining of prometaphase cotton chromosomes using BrdU-Hoechst-Giemsa and a special analysis system made it possible to detect from 2 to 9 main blocks on the chromosome that corresponded to early replicating DNA (Muravenko et al., 1998; Muravenko, Zelenin, 2009).

Due to the fact that cultivated cotton *G. hirsutum* is an allotetraploid and includes two subgenomes, it is tolerant to the loss of individual chromosomes. However, the creation of a series of monosomic lines in the United States characterized by the loss of individual chromosomes ($2n$ = 51) has been went on for many years (Endrizzi, Brown, 1964; Endrizzi et al., 1985). So, until 1985, only 15 of the 26 non-homologous chromosomes of *G. hirsutum* were isolated and identified in the United States. To date, the cotton cytogenetic collection created in the USA is characterized by the absence of any types of deficiencies for three chromosomes (13, 19 and 24), whereas for five non-homologous chromosomes (5, 8 A_t-subgenome and 14, 15, 22 D_t-subgenome), in collection have deficiencies only individual chromosome arms (Saha et al., 2012). However, this does not prevent their use for the chromosome localization of marker genes and the production of a series of substituted lines that are created simultaneously with the participation of three tetraploid species (Saha et al., 2004, 2006, 2013).

For many years, studies on the induction of cotton plants by chromosomal aberrations using various methods of induced mutagenesis have been conducted at the National University of Uzbekistan (Sanamyan, 2003, Samanyan, Rakhmatullina, 2003). As a result, a unique cytogenetic collection of cotton was created, including monosomic, monotelodisomic, and translocation lines, which place second in the world in terms of the number of lines after a similar collection created in the USA (Sanamyan et al., 2010, 2014).

The aim of the work is the unified identification of univalent chromosomes in previously obtained monosomic cotton lines using a set of cotton tester translocation lines with identified chromosomes.

Materials and methods

The research material was hybrid monosomic cotton plants obtained by crossing monosomic lines of different origin from the cytogenetic cotton collection *G. hirsutum* of the National University of Uzbekistan (NUUz) (Sanamyan et al., 2014), with translocation lines with identified chromosomes of the American cytogenetic collection (Stelly, 1993). Monosomic cotton lines grow year-round in the cellophane envelopy greenhouse of NUUz, which are monitored and all agricultural activities are carried out. Cytogenetic markers of a set of lines with identified translocations were kindly provided by Professor David Stelly (Department of Soil and Crop Sciences, Texas A&M University, College Station, TX, USA) through the ARS-USDA exchange program.

Cytological analyzes examined metaphase I (MI) meiosis in pollen mother cells (PMCs) by fixed 2–3 mm buds in alcohol-acetic acid (7:3). Then, PMCs were stained with iron-acetocarmine. The metaphases of the first division of meiosis were analyzed on temporary pressed preparaty under a light microscope and the nature of chromosome pairing, the number of uni-, bi-, tri- and multivalents were taken into account.

All cytological observations were carried out using microscopes Laboval, AxioScopeA1 (Carl Zeiss, Germany) and Biomed (Leica, Switzerland) with a magnification of 10×, 100×, binocular lens 1.6× and GF 12.5 × 120 and 10× eyepiece. Microphotography was performed using a Mikroskop kamera AxioCamERc5s digital camera. When exposure was

used green filter 3C-11-3. The plants and their parts were photographed using a CanonA-610 digital camera.

Results and discussion

The cytogenetic analysis of F_1 hybrids obtained from crosses of cotton monosomic lines with the translocation lines identified in accordance with the international nomenclature allowed identification of univalent chromosomes of monosomic lines of the cytogenetic collection of cotton of NUUz. It is known that the identification of pollen in mother cells in the metaphase I of meiosis in hybrid translocation monosomics of quadrivalent and univalent indicates the non-homology of the univalent and chromosomes involved in translocation (Endrizzi, Brown, 1964). If trivalents are found in hybrid translocation monosomics in the PMCs, this indicates the homology of the univalent and one of the chromosomes in the translocation. In this case, crosses of this monosomic plant are carried out with other translocation lines, in which one of their chromosomes in translocations was the same as that of the first line. Analysis of chromosome associations in monosomic hybrids allows us to identify the univalent chromosome as a specific chromosome of the set.

The analysis was carried out for all hybrid offspring obtained from crosses of cotton monosomic and translocation lines with identified chromosomes, however, only those variants that shared common chromosomes were included in the table for pairing of chromosomes (Table 1).

As a result of the analysis of cotton monosomic lines using a series of translocation lines with identified chromosomes, it was possible to identify univalent chromosomes in a number of lines. Thus, homology of univalent chromosomes was established in four monosomic lines (Mo11, Mo16, Mo19 and Mo93) and one of the translocated chromosomes in crosses with six translocation lines – TT2L-6R, TT2L-3Lb, TT2R-3La, TT2R- 8Ra, TT2R-8Rb, and TT2R-14R, since 24 bivalents plus one trivalent were observed in monosomic translocation hybrids in the meiosis metaphase I (Fig. 1, see Table 1). Since six translocation lines share one common chromosome 2, then the univalent chromosome lines in monosome Mo11, Mo16, Mo19, and Mo93 are chromosome 2 of the A_t subgenome of cotton and, as four monosomic lines are duplicates. Molecular genetic analysis of four monosomic interspecific F_1 hybrids with the participation of the lines Mo11, Mo16, Mo19 and Mo93 confirmed these data (Sanamyan et al., 2016).

Plants of the initial primary monosomics of all four monosomic cotton lines with deficiencies on chromosome 2 were obtained by pollination of irradiated pollen at doses of 10–25 Gy. All of them were characterized by an medium size of univalents, a high meiotic index (from 92.50 ± 0.31 to 98.25 ± 0.22), an increased number of tetrads with micronuclei (up to 3.00 ± 0.20 %) and high pollen fertility (from 91.54 ± 0.48 to 96.41 ± 0.42 %), as well as a reduced frequency of transmission of the monosomic state in the progenies (from 19.35 to 44.44 %) and a reduced transmission frequency of $n-1$ gametes. Moreover, once in the progeny of the monosomic line Mo19, the appearance of a monotelodisomic plant was noted, which indicated cases of incorrect (transverse) division of the centromere univalent in this monosomic line.

Table 1. Cytogenetic analysis of F_1 hybrids obtained from crosses of monosomic lines with translocation lines of the test set

Subgenome	Chromosome	Hybrids
A_t-subgenome	2	Mo11×TT 2L-3Lb
		Mo11×TT 2R-3La
		Mo11×TT 2R-8Ra
		Mo11×TT 2R-8Rb
		Mo11×TT 2R-14R
		Mo16×TT 2L-3Lb
		Mo16×TT 2R-8Ra
		Mo16×TT 2R-8Rb
		Mo16×TT 2R-14R
		Mo19×TT 2L-6R
		Mo19×TT 2L-3Lb
		Mo19×TT 2R-8Ra
		Mo19×TT 2R-8Rb
		Mo19×TT 2R-14R
		Mo93×TT 2R-8Ra
		Mo93×TT 2R-8Rb
		Mo93×TT 2R-14R
	4	Mo7×TT 4L-19R
		Mo31×TT 4L-19R
		Mo31×TT 4R-15L
		Mo38×TT 4L-19R
		Mo58×TT 4L-19R
		Mo59×TT 4L-19R
		Mo60×TT 4R-15L
		Mo69×TT 4L-19R
		Mo70×TT 4L-19R
		Mo70×TT 4R-15L
		Mo71×TT 4R-15L
		Mo72×TT 4L-19R
		Mo72×TT 4R-15L
		Mo73×TT 4L-19R
		Mo73×TT 4R-15L
		Mo75×TT 4L-19R
		Mo75×TT 4R-15L
		Mo76×TT 4L-19R
		Mo76×TT 4R-15L
		Mo81×TT 4L-19R
		Mo89×TT 4L-19R
		Mo89×TT 4R-15L
	6	Mo13×TT 3L-6L
		Mo13×TT 6L-7L
		Mo13×TT 6L-10R
		Mo34×TT 3L-6L
		Mo34×TT 6L-14L
		Mo67×TT 6L-7L
		Mo95×TT 3L-6L
		Mo95×TT 6L-10R
	7	Mo27×TT 1L-7L
		Mo27×TT 7L-12R
		Mo27×TT 7R-11R
		Mo27×TT 7R-21R
D_t-subgenome	18	Mo48×TT 7L-18R

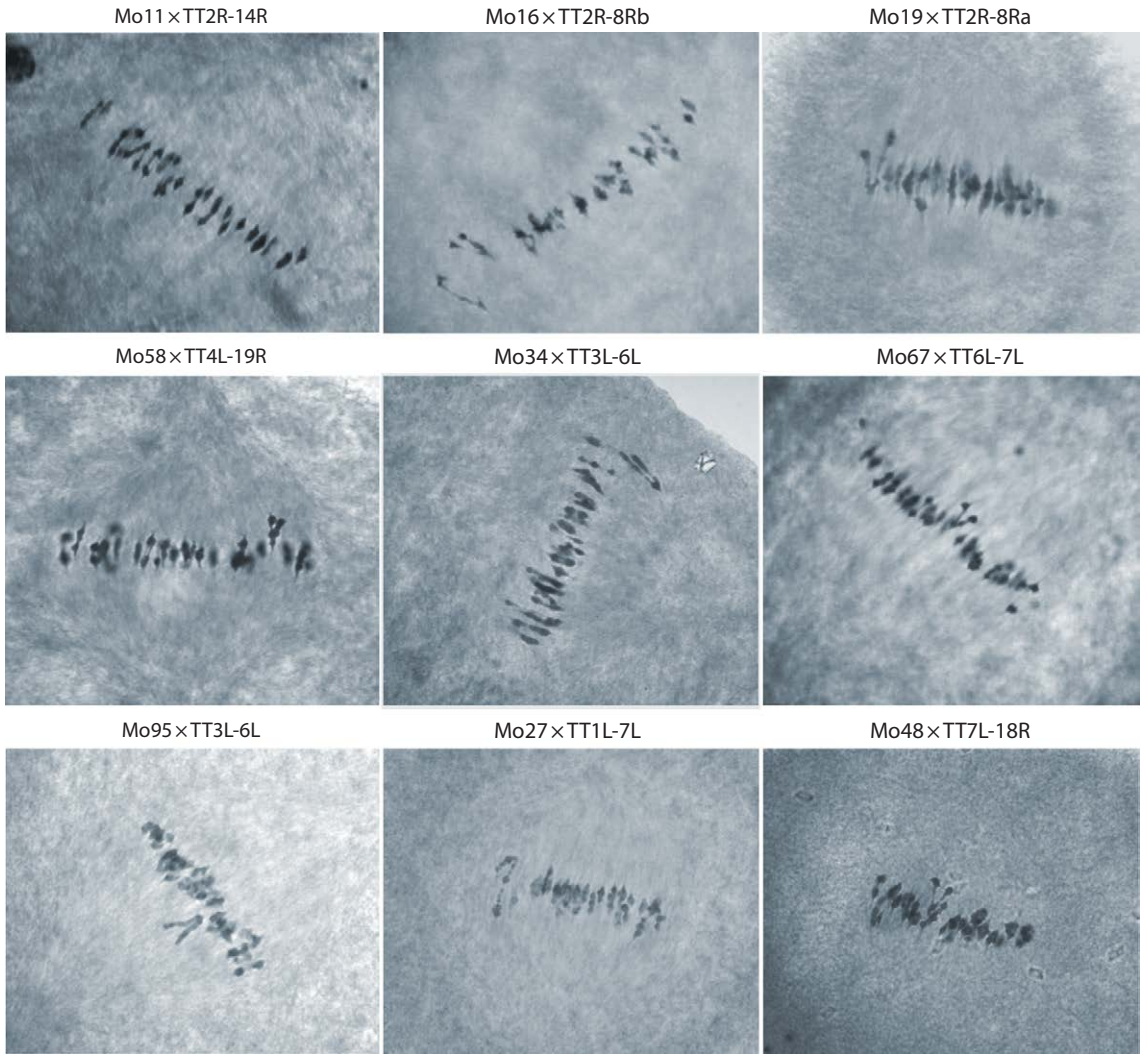

Fig. 1. Critical configuration of the chromosomes at the meiotic metaphase I in cotton monosome hybrids F_1, obtained from the crosses.

Monosomic lines on chromosome 2 were characterized by a similar set of characteristic phenotypic characters (Table 2, Fig. 2), low seed set (up to 54.84 %) compared with the original inbred line L-458 (89.81 %). This decrease in seed set occurred due to the presence of a large number of unfertilized ovules in the form of uluks in the monosomic bolls, the presence of which, along with a decrease in the number of seeds, led to a decrease in the size of the bolls.

When analyzing hybrids obtained by crossing seven monosomic lines of cotton (Mo31, Mo70, Mo72, Mo73, Mo75, Mo76, Mo89) with two TT4L-19R and TT4R-15L tester translocation lines, the homology of the univalents of these seven lines with one of the translocated chromosomes was established, since monosomic translocation hybrids in the metaphase I of meiosis showed 24 bivalents plus one trivalent (see Table 1, Fig. 1). In the TT4L-19R tester line, chromosomes 4 and 19 are involved in the translocation, and in the TT4R-15L line, chromosomes 4 and 15, therefore, one of these three chromosomes is homologous to the univalent chromosome in the monosomic lines Mo31, Mo70, Mo72, Mo73, Mo75, Mo76, Mo89. Since one common chromosome 4 is involved in both translocation lines, the mean univalent chromosomes of the monosomic lines Mo31, Mo70, Mo72, Mo73, Mo75, Mo76, Mo79 are chromosome 4 of the A_t-subgenome cotton, and these monosomic lines are duplicates. Molecular-genetic analysis of monosomic interspecific F_1 hybrids with the participation of the lines Mo31, Mo70, Mo72, Mo73, Mo75, Mo76, Mo89 confirmed these data (Sanamyan et al., 2016).

In the study of hybrids obtained by crossing eight monosomic cotton lines (Mo7, Mo38, Mo58, Mo59, Mo60, Mo69, Mo71 and Mo81) with one of two translocation lines – TT4L-19R or TT4R-15L, the homology of the univalents of these eight lines with one from translocated chromosomes was detected, since monosomic translocation hybrids in the meiotic metaphase I observed 24 bivalents plus one trivalent (see Table 1, Fig. 1). Since one common chromosome 4 is involved in both translocation lines, it can be assumed that the univalent chromosomes of the monosomic lines Mo7, Mo38, Mo58, Mo59, Mo60, Mo69, Mo71, and Mo81 are chromosome 4 of the A_t-subgenome cotton, and these monosomic lines are duplicates. The final cytological confirmation of this fact will be obtained after studying hybrids from the crossings of these eight monosomic lines with a different than the already studied translocation line involving chromosome 4. However, the lo-

Table 2. The origin and some characters of monosomic lines of cotton *G. hirsutum* L.

Monosomic line	Origin	Year of obtaining	Chromosome		Morphological characteristics
			Size	Identity	
Mo11	Pollen irradiation	1991	Medium	A 2	Small narrow leaf, shortened sympodial branches, small round bolls
Mo16		1991			
Mo19		1991			
Mo93		2007			
Mo7	Pollen irradiation	1990	Medium	A 4	Thick lush plant, elongated leaf blades, long bracts and pedicels, elongated ribbed bolls
Mo31		1993			
Mo38		1993			
Mo58	Desynapsis	1996			
Mo59		1996			
Mo60		1996			
Mo69		1997			
Mo70		1997			
Mo71		1997			
Mo72		1997			
Mo73		1997			
Mo75	Pollen irradiation	1999			
Mo76		2001			
Mo81		2003			
Mo89	Desynapsis	2003			
Mo13	Pollen irradiation	1991	Large	A 6	Sympodial branches, hard stem, small round bolls, late flowering
Mo34		1993			
Mo67	Heterozygous for translocation	1996			
Mo95	Pollen irradiation	2012			
Mo27	Pollen irradiation	1993	Medium	A 7	Short sympathies, thick bracts and leaves, small bolls
Mo48		1994	Small	D 18	Small leaves, long column and stigma, sympodial branches, round bolls

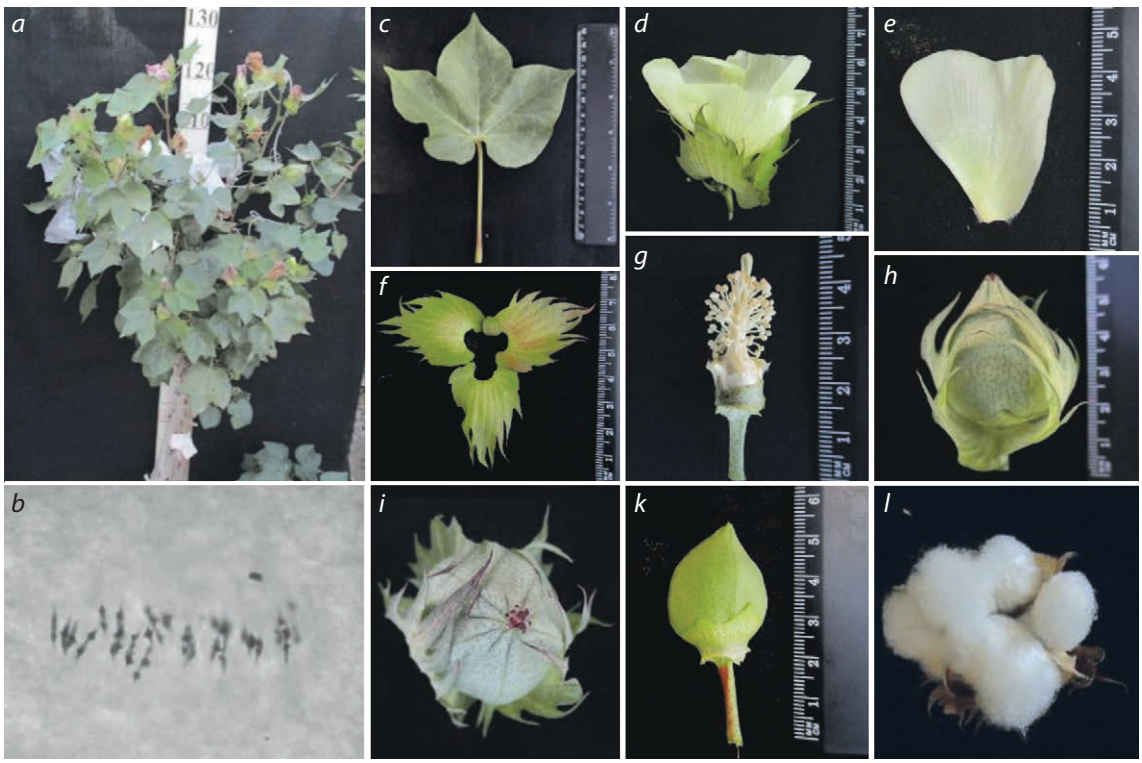

Fig. 2. Features of the cotton monosomic lines on chromosome 2:

a, bush; *b*, configurations of the chromosomes ($25^{II} + 1^{I}$); *c*, leaf; *d*, flower; *e*, petal; *f*, bract; *g*, staminate column; *h* and *i*, green bolls; *k*, boll with peduncle; *l*, open boll.

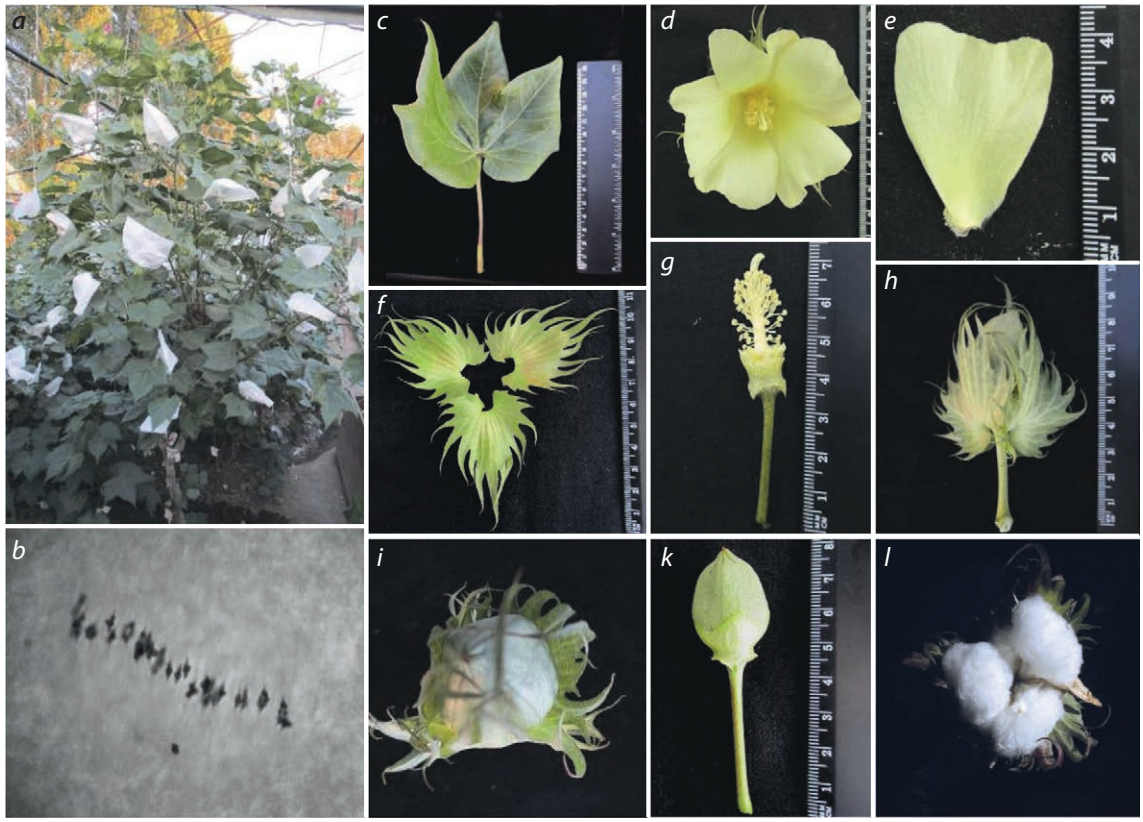

Fig. 3. Features of the cotton monosomic lines on chromosome 4:

a, bush; *b,* configurations of the chromosomes ($25^{II} + 1^{I}$); *c,* leaf; *d,* flower; *e,* petal; *f,* bract; *g,* staminate column; *h* and *i,* green bolls; *k,* boll with peduncle; *l,* open boll.

calization of chromosome-specific SSR markers on F_1 hybrids involving the lines Mo7, Mo38, Mo58, Mo59, Mo60, Mo69, Mo71, and Mo81 confirmed this data (Sanamyan et al., 2016).

Unfortunately, the third translocation line of the test set, TT4L-5, could not be used in a study to identify univalent chromosomes due to our discovery of two quadrivalents in "critical cells", apparently because of the homozygosity of both translocations simultaneously. Earlier M.S. Brown (1980) reported that two or more cytological aberrations were present in the initial plants of ten of the 62 translocation lines of cotton *G. hirsutum*, however only one translocation was obtained in a homozygous state later on their translocation lines.

The initial plants of six monosomic cotton lines (Mo7, Mo31, Mo38, Mo75, Mo76, Mo81) with deficiency of chromosome 4 were obtained as a result of pollination with irradiated pollen at doses of 10–25 Gy, while the original plants of nine monosomic lines (Mo58, Mo59, Mo60, Mo69, Mo70, Mo71, Mo72, Mo73, Mo89) were found in the progeny of desynaptic plants. All of the above lines were characterized by medium size of univalents, a high meiotic index (from 90.50 ± 0.72 to 98.46 ± 0.20), small number of tetrads with micronuclei (up to 2.07 ± 0.14 %) and high pollen fertility (from 90.67 ± 0.88 to 97.53 ± 0.35 %), as well as a reduced frequency of transmission of the monosomic state in the progeny (from 16.67 to 42.86 %), which led to a decrease in the frequency of transmission of haplo-deficient gametes.

Monosomic lines on chromosome 4 were characterized by similar phenotypic differences, which sharply distinguished them from other monosomic lines (Fig. 3, see Table 2), as well as a higher seed set (up to 72.22 %), with the exception of the Mo76 monosomic line (32.61 %).

When studying four monosomic cotton lines (Mo13, Mo34, Mo67, Mo95) using some of the four translocation lines – TT3L-6L, TT6L-7L, TT6L-10R, TT6L-14L, homology of univalent chromosomes was established for these four monosomic lines and one of the translocated chromosomes in the above translocations, since 24 bivalents plus one trivalent were observed in monosomic translocation hybrids in the meiosis metaphase I (see Table 1, Fig. 1). Since one common chromosome 6 is involved in four translocation lines, then the univalent chromosomes in monosomic lines Mo13, Mo34, Mo67, Mo95 are chromosome 6 A_t-subgenome cotton, and these monosomic lines are duplicates. Molecular-genetic analysis of four monosomic interspecific F_1 hybrids with the participation of the monosomic lines Mo13, Mo34, Mo67, Mo95 confirmed these data (Sanamyan et al., 2016).

The initial plants of three monosomic lines of cotton (Mo13, Mo34, Mo95) with a deficiency of chromosome 6 were obtained by pollination with irradiated pollen at doses of 20–25 Gy, while the original plant of the monosomic line Mo67 was found in the progeny of a plant heterozygous for translocation with a desynaptic effect. All these lines were characterized by a large univalent size, high meiotic index (from 94.13 ± 0.38 to 96.82 ± 0.49), a small number of tetrads with micronuclei (up to 2.07 ± 0.23 %) and reduced pollen fertility (from 88.46 ± 1.28 to 94.34 ± 0.51 %), as well as a

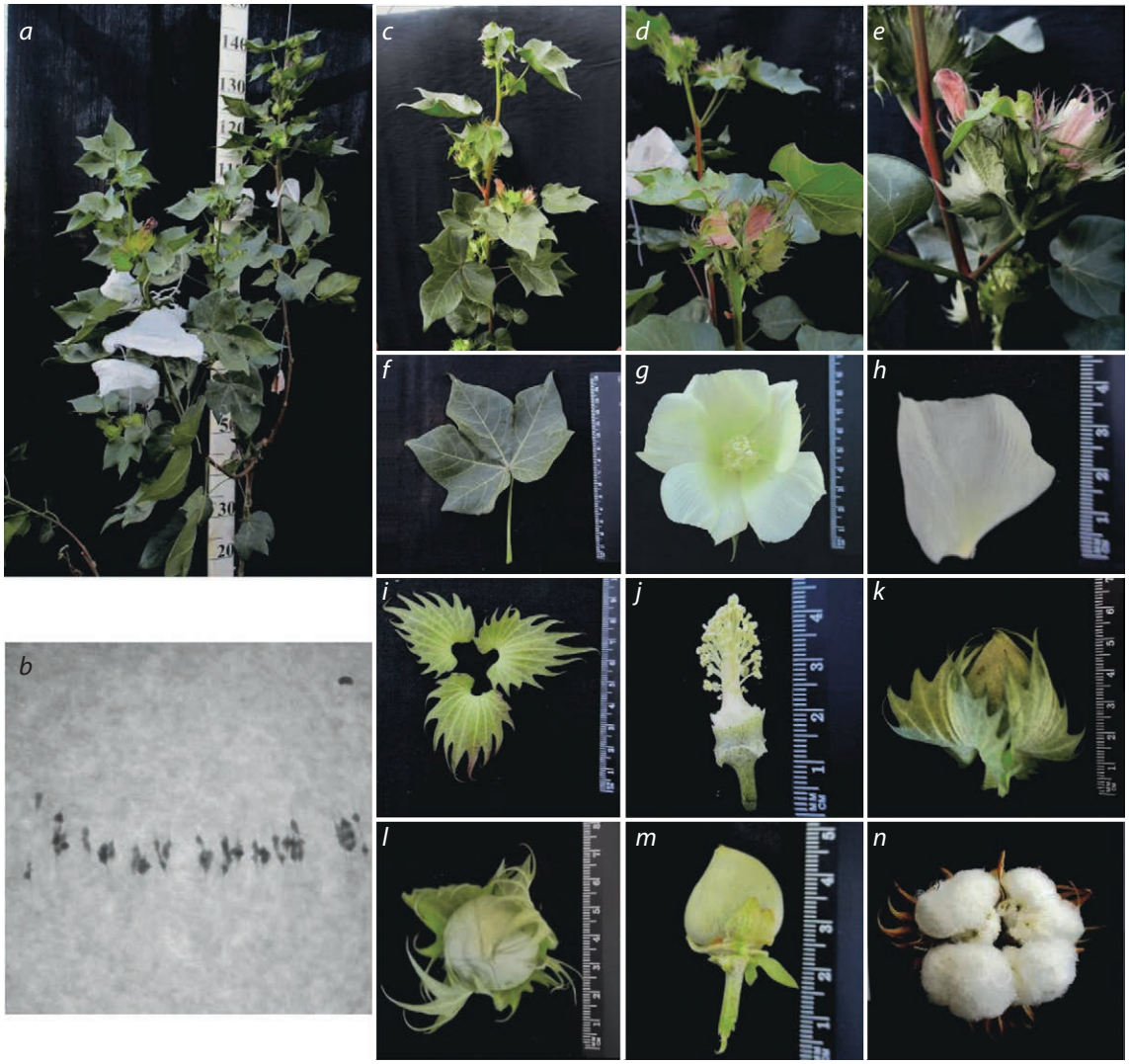

Fig. 4. Features of the cotton monosomic lines on chromosome 6:

a, bush; *b*, configurations of the chromosomes ($25^{II} + 1^{I}$); *c–e*, parts of the stem; *f*, leaf; *g*, flower; *h*, petal; *i*, bract; *j*, staminate column; *k* and *l*, green bolls; *m*, boll with peduncle; *n*, open boll.

low frequency of transmission of the monosomic state in the progeny (from 9.38 to 14.29 %), which significantly reduced the frequency of transmission of haplo-deficient gametes.

Monosomic lines with a deficiency of chromosome 6 were characterized by a whole complex of morphological characters associated with monosomy on this chromosome (Fig. 4, see Table 2), as well as lower seed set (from 38.78 to 57.45 %), compared with line L-458.

In the study of the Mo27 monosomic line in four variants of the crossings – Mo27×TT1L-7L, Mo27×TT7L-12R, Mo27×TT7R-11R and Mo27×TT7R-21R, homology of the univalent chromosome Mo27 and one of the translocated chromosomes was observed, since the monosome chromosome was observed 24 bivalents and one trivalent (see Fig. 1). In the TT1L-7L tester line, the involved chromosomes 1 and 7 are involved in translocation, in the TT7L-12R line, chromosomes 7 and 12, in the TT7R-11R line, chromosomes 7 and 11, and in the TT7R-21R line, chromosomes 7 and 21, therefore, one of these chromosomes is homologous to the univalent chromosome of the monosomic Mo27 line. Since one common chromosome 7 is involved in four-translocation lines, the univalent chromosome of the monosomic line Mo27 is chromosome 7 of the A_t-subgenome of cotton.

The initial plant of the monosomic cotton line Mo27 with a deficiency of chromosome 7 was obtained by pollination with irradiated pollen at a dose of 20 Gy. This line was characterized by an medium univalent size, a high meiotic index (95.81 ± 0.38), a small number of tetrads with micronuclei (1.77 ± 0.25 %) and reduced pollen fertility (89.88 ± 0.83 %), as well as a low transmission rate of the monosomic state in the progeny (22.23 %), which significantly reduced the frequency of transmission of haplo-deficient gametes. The monosomic line with a lack of chromosome 7 was also characterized by a complex of morphological characters associated with monosomy (Fig. 5, see Table 2), as well as reduced seed binding (65.10 %), compared with the L-458 line.

When studying the Mo48 monosomic line in one variant of crossing with the TT7L-18R translocation line in the meiosis metaphase I, 24 bivalents plus trivalent were found (see Fig. 1), which testified to the homology of the uni-

Fig. 5. Features of the cotton monosomic line on chromosome 7:

a, bush; *b*, configurations of the chromosomes ($25^{II}+1^{I}$); *c*, part of the stem; *d*, leaf; *e*, flower; *f*, bract; *g*, staminate column; *h* and *i*, green bolls; *k*, boll with peduncle; *l*, open boll.

valent chromosome in Mo48 and one of the translocated chromosomes in the translocation line. Since chromosomes 7 and 18 are involved in the translocation of the TT7L-18R test line, it can be assumed that the univalent chromosome in the monosomic line of Mo48 is homologous to one of the two chromosomes. Unfortunately, in the test set of lines with identified chromosomes, there is no second translocation line involving chromosome 18. Therefore, to determine which of the two chromosomes of this translocation, the homologous univalent chromosome of the Mo48 monosomic line was used earlier, we used chromosome-specific microsatellite SSR markers that were amplified by standard PCR. Molecular-genetic analysis of the monosomic interspecific hybrid F_1 (Mo48×Pima 3-79) revealed the presence of polymorphic alleles only from the species *Gossypium barbadense* L., which indicated the localization of the specific SSR marker BNL3280 chromosomes in the aforementioned hybrid (Sanamyan et al., 2016). Since this marker was previously located on the chromosome of the 18 D_t-subgenome of cotton, we can assume that the monosomic line – Mo48 of the collection of NUUs has a monosomy along the chromosome of the 18 D_t-subgenome.

The initial plant of the monosomic cotton line Mo48 with a deficiency of chromosome 18 was obtained by pollination with irradiated pollen at a dose of 25 Gy. This line was characterized by a small univalent size, a high meiotic index (95.68 ± 0.50), a small number of tetrads with micronuclei (0.86 ± 0.26 %) and a high pollen fertility (95.23 ± 0.74 %), and also low transmission rate of the monosomic state in the progeny (18.19 %), which significantly reduced the frequency of transmission of haplo-deficient gametes. The monosomic line with a deficiency of chromosome 18 was characterized by a set of morphological characters associated with monosomy (Fig. 6, see Table 2), as well as by high of seed set (85.64 %).

Conclusion

Thus, the use of translocation lines with identified chromosomes allowed us to bring the numbering of univalent chromosomes in the monosomic lines of our collection in accordance with the generally recognized nomenclature. Cytogenetic identification and numbering of univalent chromosomes in 25 monosomic lines of the cytogenetic collection of NUUs made it possible to establish that the four monosomic lines have univalent chromosomes on the chromosome 2, 15 lines on chromosome 4, the four lines on chromosome 6, the one line on chromosome 7 of A_t-subgenome and one line on chromosome 18 of D_t-subgenome of cotton. The predominant majority of monosomic lines were detected by the most frequently recorded cotton monosomics – chromosomes 2, 4, and 6.

A comparative analysis of the first 20 identified cotton monosomics obtained in the USA revealed similar trends, since the study revealed seven monosomics on chromosome 2, seven on chromosome 4, three on chromosome 6, and one on chromosomes 1, 17 and 18 (Brown, Endrizzi, 1964). The similarity of the data obtained in the study of different collections indicates that, despite differences in the genotypic environment and methods for producing monosomics, cotton has an amazing coincidence of data on the higher frequency of monosomics on chromosomes 2, 4 and 6, while monosomics on other the

Fig. 6. Features of the cotton monosomic line on chromosome 18:

a, bush; *b*, configurations of the chromosomes ($25^{II}+1^{I}$); *c*, leaf; *d*, flower; *e*, petal; *f*, bract; *g*, staminate column; *h*, green boll; *i*, boll with peduncle; *k* and *l*, open boll.

chromosomes of the set appear with a much lower frequency, and on eight non-homologous chromosomes (5, 8, 13 A_t-subgenome and 14, 15, 19, 22, and 24 D_t-subgenome of cotton) they were never detected at all (Saha et al., 2012). Apparently, the centromere regions of certain chromosomes are more prone to breakage and the genome as a whole remains tolerant of the loss of large A_t-subgenomic chromosomes without a large effect on viability and fertility, while the chromosomes of some small D_t-subgenomic chromosomes are not subject to any changes due to incompatibility with vitality.

A comparative analysis of the cytogenetic features of cotton monosomics from the two collections is not possible, since the literature contains only fragmentary information regarding misdivision of univalents and the frequency of transmission rate of the monosomic state in some monosomics of the American collection. However, the difficulties of creating a series of monosomic lines in tetraploid cotton and the obvious fact that certain chromosomes of the A_t-subgenome are more common in the monosomic state than the chromosomes of the D_t-subgenome do not diminish the value of the studies in view of the need for further development of molecular genetic studies and the creation of substituted cotton lines.

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Acknowledgements. This work was financially supported by the Committee on Science and Technology and the Ministry of Innovation of the Republic of Uzbekistan (grants F-5-31 and OT-A-KX-2018-379).

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

Received May 31, 2019. Revised August 21, 2019. Accepted August 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):846-855
DOI 10.18699/VJ19.558

The Fifth International Scientific Conference PlantGen2019

Создание и характеристика линии мягкой пшеницы с центрической транслокацией T2DL.2RL

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Создание интрогрессивных форм мягкой пшеницы с чужеродным генетическим материалом от культурных и диких видов трибы *Triticeae* является эффективным методом для расширения генофонда пшеницы, необходимого для селекционных работ. К настоящему времени получены многочисленные коллекции линий с интрогрессиями в виде замещений и модификаций хромосом, однако создание и изучение форм пшеницы с новыми ценными признаками остаются актуальным направлением современных научных разработок. Рожь *Secale cereale* L., чьи хромосомы несут гены, контролирующие ценные экономические и биологические характеристики и свойства, широко используется для получения новых форм. В данной работе охарактеризована линия пшеницы с транслоцированной хромосомой, которая была получена при беккроссировании дисомно-замещенной пшенично-ржаной линии 2R(2D)$_1$ сортом Новосибирская 67. С использованием флуоресцентной *in situ* гибридизации (FISH) и метода С-окрашивания изучен хромосомный состав кариотипов линий. Идентифицированы две центрические пшенично-ржаные транслоцированные хромосомы, образованные из двух длинных плеч хромосом 2D и 2R, T2DL.2RL. Остальные 40 хромосом пшеницы не подверглись структурным изменениям. Мейоз линий характеризовался стабильностью. Хромосомы T2DL.2RL формировали биваленты во всех мейоцитах, что подтверждает их гомологичность. По морфологическим признакам колоса линия T2DL.2RL не отличалась от сорта Новосибирская 67. Проведен сравнительный анализ показателей элементов продуктивности у линии с транслокацией T2DL.2RL и родительских форм, сорта Новосибирская 67 и дисомно-замещенной пшенично-ржаной линии 2R(2D)$_1$. По результатам сравнения, линия T2DL.2RL достоверно уступает сорту Новосибирская 67 по всем показателям с различной степенью достоверности. Показатели продуктивности линии 2R(2D)$_1$ превосходили либо не отличались от показателей линии с транслокацией T2DL.2RL, однако масса 1000 зерен была достоверно меньше. Обнаружено также влияние транслокации T2DL.2RL на признак «высота растения». Этот показатель был достоверно ниже, чем у Новосибирской 67, в условиях двух вегетаций. Следовательно, транслокация T2DL.2RL влияет на уменьшение высоты растений, а также вызывает отрицательный эффект на элементы продуктивности.
Ключевые слова: чужеродная интрогрессия; FISH; С-окрашивание; центрические транслокации; высота растений; продуктивность; рожь *Secale cereale* L.; мягкая пшеница *Triticum aestivum* L.

Для цитирования: Иванова Ю.Н., Соловей Л.А., Логинова Д.Б., Мирошникова Е.Е., Дубовец Н.И., Силкова О.Г. Создание и характеристика линии мягкой пшеницы с центрической транслокацией T2DL.2RL. Вавиловский журнал генетики и селекции. 2019;23(7):846-855. DOI 10.18699/VJ19.558

The creation and characterization of the bread wheat line with a centric translocation T2DL.2RL

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The development of bread wheat introgressions with alien genetic material from cultural and wild *Triticeae* species is an effective method for expanding the wheat gene pool necessary for breeding. To date, numerous collections of introgressions as substitutions and chromosome modifications have been obtained; however, the creation and study of wheat with new valuable traits still remain an important line of research. Rye *Secale cereale* L., whose chromosomes carry genes that control valuable economic and biological characteristics and properties, is widely used to produce new wheat forms. In this study, a wheat-rye translocation obtained by backcrossing the wheat-rye disomic-substitution line 2R(2D)$_1$ with the variety Novosibirskaya 67 was characterized. The chromosomal composition of karyotypes was studied using fluorescent *in situ* hybridization and C-banding. Two centric translocations,

derived from two long arms of chromosomes 2D and 2R, T2DL.2RL, were identified, the remaining 40 wheat chromosomes did not undergo modifications. Meiosis in the lines was stable. Chromosomes T2DL.2RL formed bivalents in all meiocytes, which confirmed their homology. The morphological characteristics of the spike in the T2DL.2RL line and Novosibirskaya 67 did not differ. A comparative analysis of productivity between the T2DL.2RL translocation line and the parental forms, Novosibirskaya 67 and the $2R(2D)_1$ line, was carried out. The T2DL.2RL line is inferior to Novosibirskaya 67 in all characters with different confidence levels. The productivity characters of the $2R(2D)_1$ line exceeded or did not differ from those of T2DL.2RL, however, the mass of 1000 grains was significantly lower. The results showed the effect of the T2DL.2RL translocation on the trait "plant height". This character was significantly lower than that of Novosibirskaya 67 in two vegetation periods. Consequently, the T2DL.2RL translocation reduces plant height and productivity.
Key words: alien introgression; FISH; C-banding; centric translocations; plant height; productivity; rye *Secale cereale* L.; bread wheat *Triticum aestivum* L.

For citation: Ivanova Yu.N., Solovey L.A., Loginova D.B., Miroshnikova E.E., Dubovets N.I., Silkova O.G. The creation and characterization of the bread wheat line with a centric translocation T2DL.2RL. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):846-855. DOI 10.18699/VJ19.558 (in Russian)

Введение

Мягкая пшеница *Triticum aestivum* L. – это стратегическая продовольственная культура мирового значения, возделываемая более чем на 200 млн га и обеспечивающая питанием треть населения земного шара (Rasheed et al., 2018). Значимым периодом в селекции пшеницы была «зеленая революция» (1967–1970 гг.), во время которой удалось достигнуть значительного прогресса в увеличении урожайности этой культуры в развивающихся странах. Успех «зеленой революции» стал возможен благодаря использованию генов карликовости, нечувствительности к фотопериоду и устойчивости к стеблевой ржавчине. Ее последствием было значительное сокращение генетического разнообразия в сортах мягкой пшеницы. Отсутствие аллельного разнообразия ограничивало улучшение таких признаков, как урожайность, качество зерна, а также сделало пшеницу более уязвимой к биологическим и экологическим стрессам. В связи с этим возникла необходимость в более эффективном использовании в селекционных программах уникального генетического разнообразия, собранного в коллекциях пшениц и ее родственных видов (Rasheed et al., 2018).

Дикие и культурные виды, а также местные сорта по-прежнему остаются неисчерпаемыми хранилищами генетического разнообразия, а отдаленная гибридизация является лучшим способом для передачи этого разнообразия (Jiang et al., 1994; Friebe et al., 1996; Feuillet et al., 2008; Mujeeb-Kazi et al., 2013). В качестве источника новых признаков широко применяют виды-доноры субгеномов мягкой пшеницы *Triticum monococcum*, *Aegilops tauschii* и *T. dicoccoides*, а также более отдаленные дикие виды родов *Triticum*, *Aegilops*, *Haynaldia*, *Thinopyrum* и вид культивируемой ржи *Secale cereale* L.

Использование форм пшеницы с интрогрессией чужеродного хроматина (амфидиплоиды, дополненные, замещенные и транслоцированные линии) для создания пребридингового материала имеет свои преимущества в сравнении с межвидовой и межродовой гибридизацией. Одно из них – это возможность объективно оценить эффект чужеродной интрогрессии в различных вариантах генотипической среды пшеницы (Jiang et al., 1994; Friebe et al., 1996; Rasheed et al., 2018). Известно, что негативные эффекты, наблюдаемые при интрогрессии генов, могут возникать не только в связи с присутствием фрагментов чужеродного генома, но и в результате влияния генотипической среды сорта-реципиента (Леонова, 2018).

Линии пшеницы с интрогрессией чужеродного материала в виде фрагментов хромосом различной величины, включенных в хромосомы пшеницы (транслокации), были получены разными способами: путем воздействия радиоактивного излучения, индукцией гомеологичного спаривания с использованием системы *Ph* локуса, разделением унивалентов по центромере, а также спонтанно (Zhang et al., 2007). Таким образом в геном мягкой пшеницы был передан генетический материал видов *Aegilops*, *T. timopheevii*, *Thinopyrum*, *S. cereale* (Friebe et al., 1996; Fu et al., 2012; Liu et al., 2013; Timonova et al., 2013; Leonova, Budashkina, 2017). В обзоре (Friebe et al., 1996) описаны 57 транслокаций. Десять из них являются Робертсоновскими, у 45 транслокаций чужеродные сегменты хромосом расположены дистально на хромосомах пшеницы, и 2 транслокации имеют интеркалярные вставки. Для широкого использования форм пшеницы с транслокациями важно место локализации чужеродного фрагмента на хромосоме пшеницы, синтения хромосомы донора хромосоме реципиента, обеспечивающая хорошую компенсационную способность хромосомы донора, а также нормальная передача интрогрессированного материала потомству, желательно по законам Менделя. Несмотря на то что в генбанках мира в большом количестве созданы и хранятся линии пшеницы с транслокациями, лишь малая часть коллекции используется в качестве пребридингового материала (Friebe et al., 1996; Леонова, 2018). Во многих случаях это объясняется отсутствием компенсационной способности чужеродной интрогрессии. Одно из требований для линий с транслокациями – включение небольшого фрагмента чужеродного хроматина в хромосому пшеницы, что предполагает целевой перенос необходимого участка хромосомы. Однако спонтанные пшенично-ржаные транслокации 1BL.1RS and 1AL.1RS являются примером генетически сбалансированных Робертсоновских транслокаций, которые стали самыми успешными среди пшенично-чужеродных транслокаций, использованных в селекции пшеницы (Jiang et al., 1994; Rasheed et al., 2018). По-видимому, передача адаптивных сцепленных локусов в полиплоидный геном мягкой пшеницы может быть более подходящим способом, чем передача единичных генов. Пшенично-ржаная транслокация

1RS.1BL несет признаки устойчивости к мучнистой росе, бурой, желтой и стеблевой ржавчине, увеличивает урожайность и не ухудшает качество зерна в определенных сортовых генотипах (Friebe et al., 1996; Belan et al., 2015). Транслокация 1RS.1BL входит в геномы многих современных коммерческих сортов яровой и озимой мягкой пшеницы (Степочкин и др., 2012; Belan et al., 2015). Созданы аллоплазматические рекомбинантные линии (*H. vulgare*) – *T. aestivum* с транслокацией 1RS.1BL, которые показали преимущество по сравнению с сортами-стандартами по устойчивости к бурой и стеблевой ржавчине, урожайности, качеству зерна. На их основе получены сорта яровой мягкой пшеницы Сигма, Уралосибирская 2 и Ишимская 11 (Pershina et al., 2018).

Работы по передаче чужеродного генетического материала в геном пшеницы не теряют своей актуальности, формы пшеницы с новыми ценными признаками создаются во многих лабораториях мира, а потенциал генофонда ржи как источник хозяйственно ценных признаков далеко не исчерпан (An et al., 2013; Ren et al., 2017; Schlegel, 2019). В качестве источника важных признаков используются линии с пшенично-ржаным замещением хромосом $2R(2D)_1$ и центрическими транслокациями, в структуру которых включено длинное плечо хромосомы ржи 2RL. Они характеризуются устойчивостью к гессенской мухе (Friebe et al., 1990), мучнистой росе, бурой, стеблевой и желтой ржавчинам (Heun, Friebe, 1990; McIntosh et al., 1995; Merker, Forsstrom, 2000; Hysing et al., 2007; Lei et al., 2011; Li et al., 2018). Присутствие 2RL увеличивает содержание арабиноксилана в зерне, который влияет на хлебопекарное и пищевое качество зерновых (Boros et al., 2002). Хромосома 2R увеличивает эффективность использования воды и укоренения растений при выращивании их в засушливых условиях (Ehdaie et al., 2003).

Целью данной работы было изучить хромосомный состав, мейотическую стабильность у линий с интрогрессией хромосомы ржи 2R в сорте Новосибирская 67, а также описать морфобиологические признаки колоса и провести оценку элементов продуктивности растений.

Материал и методы

Растительный материал. В работе были использованы: линии сортов мягкой пшеницы *T. aestivum* L., Саратовская 29 (С29), Новосибирская 67 (Н67), пшенично-ржаная дисомно-замещенная линия $2R(2D)_1$ ($2n = 42$) (Силкова и др., 2006). Ранее в ИЦиГ СО РАН получены дисомно-замещенные ($2n = 42$) пшенично-ржаные линии (Щапова, Кравцова, 1990). Все линии, кроме $2R(2D)_1$, $2R(2D)_2$, $2R(2D)_3$, созданы на генетической основе сорта Саратовская 29 (Силкова и др., 2006). У линии $2R(2D)_1$ процентное содержание локусов с аллелями, отличными от С29 и характерными для сорта Н67, составило 13.7 % (Силкова и др., 2006). Для создания замещенной линии 2R(2D) на сорте Н67 проведено беккроссирование линии $2R(2D)_1$ сортом Н67 (рис. 1). В потомстве BC_2 изучен хромосомный состав у 21 растения с помощью С-окрашивания, у трех растений 26-11, 26-12, 26-13 обнаружено по одной модифицированной хромосоме ржи, которая была идентифицирована как T2R.2DL (см. рис. 1) (Красилова и др., 2011). После последующего беккроссирования

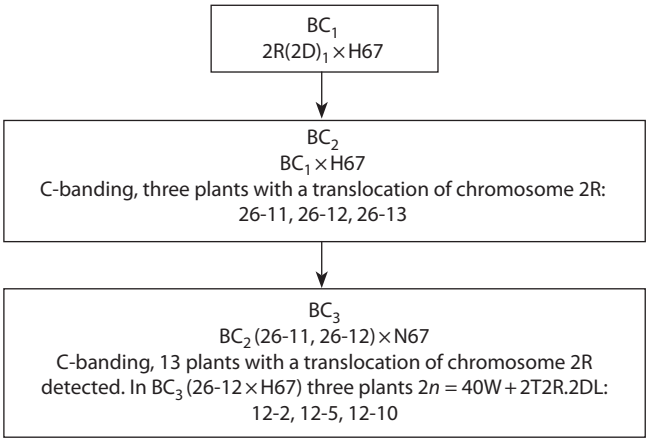

Fig. 1. Development of plants with translocation T2R.2DL.

Table 1. Plant material studied in the work

Plants			Plot no. (number of BC_3F_2)	
BC_2	BC_3	BC_3F_1, autumn–winter 2017	Summer 2018	Winter–spring 2019
26-12	12-2	16-1	1 (20)	13 (26)
		16-2	2 (20)	14 (29)
		16-4	3 (20)	15 (29)
	12-5	17-3	4 (20)	16 (28)
		17-4	5 (20)	17 (25)
		17-5	6 (20)	18 (27)
	12-10	18-3	7 (20)	19 (29)
		18-6	8 (20)	20 (15)
		18-9	9 (20)	21 (28)

этих растений проведен анализ кариотипов у потомков. В потомстве BC_3 (26-12 × Н67) выявлено три растения с двумя модифицированными хромосомами 2R (см. рис. 1). Потомство этих трех растений изучалось в настоящей работе (табл. 1).

Растения выращивали в условиях гидропонной теплицы вегетации осень–зима 2017 г., зима–весна 2019 г. (лаборатория искусственного выращивания растений ФИЦ ИЦиГ СО РАН), режим освещения день:ночь – 16:8, а также летом 2018 г. на экспериментальном поле Селекционно-генетического комплекса ФИЦ ИЦиГ СО РАН.

Флуоресцентная *in situ* гибридизация (FISH). Митотические и мейотические препараты для FISH готовили по описанной ранее методике (Silkova et al., 2018). Митотические препараты готовили из корешков вегетирующих растений BC_3F_1. Колосья для мейотического анализа фиксировали у растений BC_3F_2. Анализировали мейоциты на стадиях диакинеза, метафазы I, анафазы I и телофазы II. В работе использовали: пробу *Aegilops tauschii* pAet6-09, специфичную для центромерных повторов хромосом риса, пшеницы, ржи и ячменя (Zhang et al.,

2004); pAWRc, специфичную для центромерного повтора хромосом ржи (Francki, 2001), и геномную ДНК ржи. Образцы ДНК повторов pAet6-09 и pAWRc любезно предоставлены д-ром A. Lukaszewcki (Университет Риверсайд, Калифорния, США). Центромеро-специфичные пробы метили биотином 16-dUTP или дигоксигенином 11-dUTP при помощи полимеразной цепной реакции (ПЦР). Суммарную ДНК ржи метили ник-трансляцией с биотином 16-dUTP или дигоксигенином 11-dUTP. Пробы использовали совместно в различных пропорциях и смешивали с блокирующей пшеничной ДНК. Препараты заключали в среду Vectashield antifade solution (Vector Laboratories), замедляющую выцветание флуоресценции, содержащую 1 мкг/мл DAPI (4′,6-diamidino-2-phenylindol, Sigma-Aldrich, США) для окрашивания хроматина. Все препараты анализировали при помощи микроскопа Axio ImagerM1 (KarlZeiss, Германия). Изображения регистрировали камерой ProgRes MF (Meta Systems, Jenoptic) в ЦКП микроскопического анализа биологических объектов СО РАН и обрабатывали с использованием программного обеспечения Adobe Photoshop CS2.

С-дифференциальное окрашивание (C-banding) кариотипов BC_3F_2 проводили по ранее опубликованной методике (Badaeva et al., 1990). Митотические препараты готовили из корешков пророщенных зерновок. Препараты анализировали с помощью микроскопа Amplival (Karl Zeiss, Германия). Идентификацию индивидуальных хромосом геномов A, B, D и R осуществляли согласно обобщенной видовой идиограмме дифференциально окрашенных хромосом (Badaeva et al., 1990).

Анализ хозяйственно ценных признаков. Проанализированы следующие элементы продуктивности: высота растения, длина соломины, продуктивная кустистость, длина главного колоса, количество зерен главного колоса, масса зерен главного колоса, количество зерен с растения, масса зерен с растения, масса 1000 зерен. Статистический анализ проведен по *t*-критерию Стьюдента.

Результаты и обсуждение

Молекулярно-цитогенетический анализ состава хромосом и мейотического деления у линий с транслоцированной хромосомой

Для идентификации модифицированной хромосомы и анализа хромосомного состава у линий проведено окрашивание хромосом с использованием FISH и C-banding. FISH-анализ кариотипов с зондом общей ДНК ржи и центромеро-специфичными повторами pAWRc и pAet6-09 выявил две транслоцированные хромосомы (рис. 2).

На одном плече хромосомы локализован зонд общей ДНК ржи (см. рис. 2, *а*, *б*), а в центромерном районе – повторы pAWRc (*а*) и pAet6-09 (*б*). Следовательно, одно плечо вместе с центромерным районом у этих хромосом принадлежит хромосоме ржи 2R (*а*). Таким образом, в результате разрывов хромосом 2R и 2D в прицентромерном районе и последующего слияния плеч хромосом ржи и пшеницы была образована центрическая транслокация T2DL.2R.

С помощью окрашивания хромосом у кариотипов растений BC_3F_2 (см. табл. 1) методом C-banding выявлен полный набор хромосом пшеницы, кроме хромосомы 2D. Изученные кариотипы оказались идентичными. Модифицированная хромосома идентифицирована как центрическая пшенично-ржаная транслокация, состоящая из двух длинных плеч хромосом 2D и 2R – T2DL.2RL (рис. 3).

Известно, что центрические или Робертсоновские транслокации часто образуются в потомстве двойных пшенично-ржаных моносомиков (Lukaszewski, 1993; Marais G.F., Marais A.S., 1994; Liu et al., 2013). Механизм формирования центрических транслокаций выявлен в мейозе двойных моносомиков 1A-$1H^t$ (мягкая пшеница – *Elymus trachycaulus*) (Friebe et al., 2005). Хромосомы 1A и $1H^t$ не являются гомологами, поэтому в мейозе они не формировали бивалент и из-за аномального расхождения

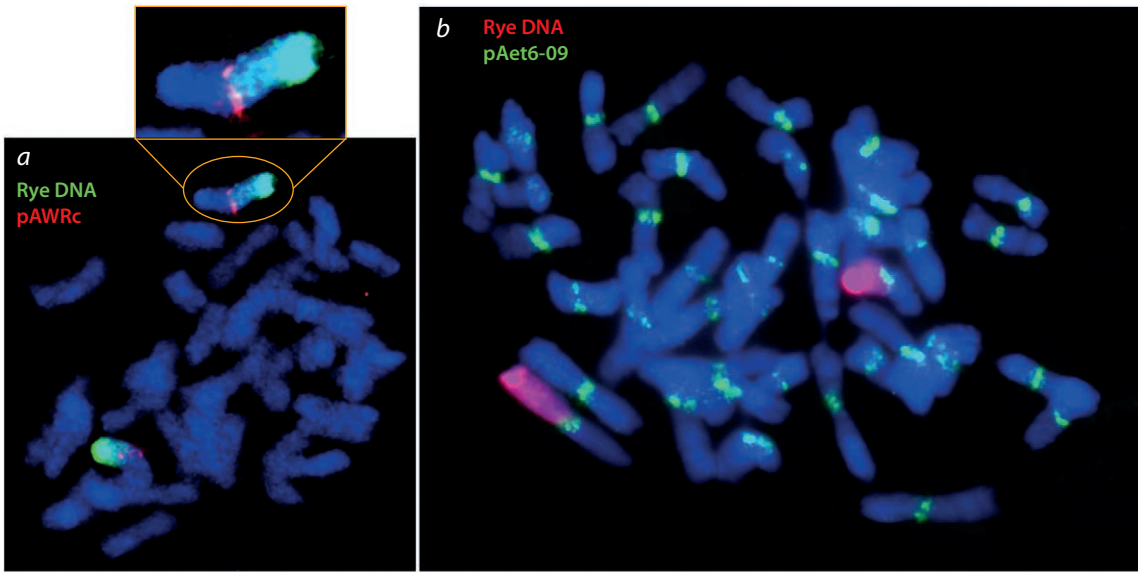

Fig. 2. Karyotypes of plants with centric wheat-rye translocation: (*a*) 16-1 and (*b*) 17-4. Rye DNA is stained (*a*) green and (*b*) red. The DNA of chromosomes is stained blue (DAPI).

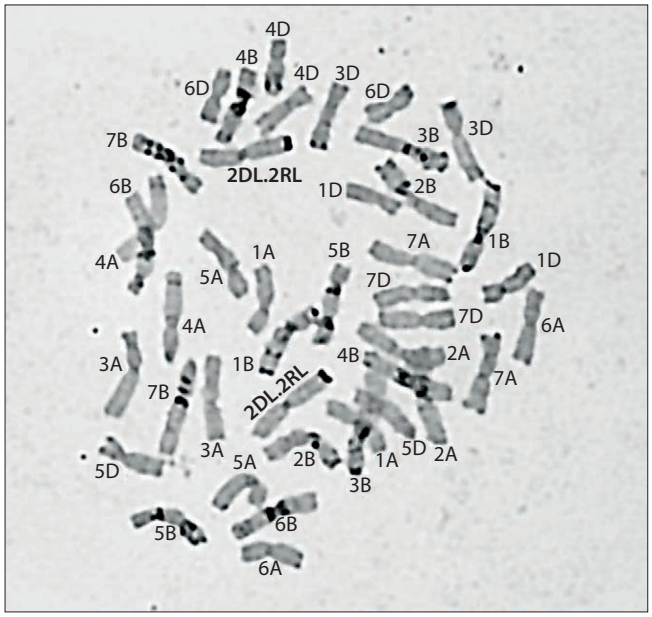

Fig. 3. C-banding of the chromosome karyotype of the translocation line T2DL.2RL.

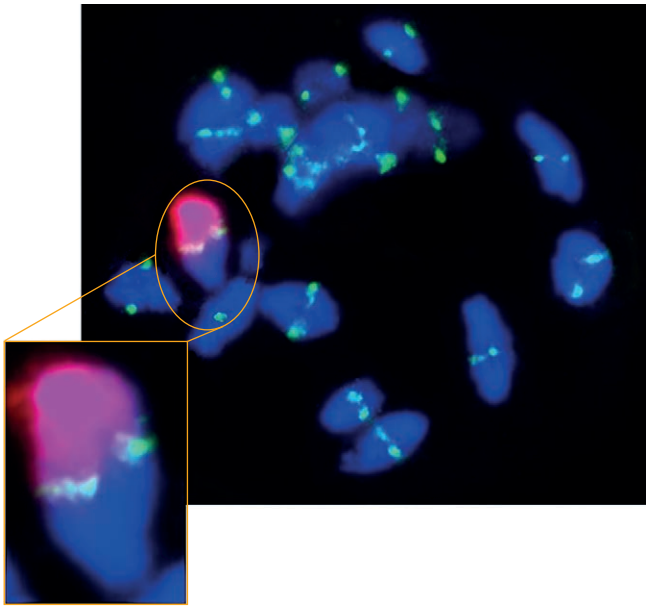

Fig. 4. Formation of a ring bivalent by translocated chromosomes at the stage of diakinesis.

Arms of chromosomes 2RL are stained red, arms of wheat chromosomes 2DL are blue, and centromeric regions are green.

в первом делении мейоза могли разрываться в центромерном районе. Объединение плеч хромосом происходило во время интеркинеза, так как уже во втором делении мейоза идентифицировалась транслокация T1A.1H^t. Хромосома 2R с измененной структурой в моносомном состоянии была обнаружена нами в кариотипах растений BC_2, которые были получены в результате беккросса растений BC_1, являющихся двойными моносомиками 2R-2D. Анализ мейоза у двойных моносомиков 2R-2D с использованием геномной *in situ* гибридизации показал, что в результате разрыва в центромере хромосомы 2R телоцентрики могут образовываться не только во втором, но и в первом делении мейоза (Силкова и др., 2014). Следовательно, механизм формирования центрической транслокации T2DL.2RL может быть аналогичным описанному в работе (Friebe et al., 2005).

Исследования по передаче чужеродного материала в геном мягкой пшеницы показывают, что созданные формы и линии могут характеризоваться низкой фертильностью и потерей интрогрессий (Jiang et al., 1994). Одной из причин является мейотическая нестабильность из-за генетической несовместимости генома мягкой пшеницы с чужеродными интрогрессиями. В связи с этим нами изучено поведение хромосом в мейозе у линий с транслокацией. Для анализа поведения непосредственно транслоцированных хромосом использовался FISH с зондами общей ДНК ржи и центромеро-специфичного повтора pAet6-09. Основной характеристикой стабильности мейоза является формирование бивалентов на стадии метафазы I. По результатам анализа, во всех мейоцитах на стадиях диакинеза (рис. 4) и метафазы I (рис. 5, *а*, *б*) биваленты формируются как хромосомами пшеницы, так и транслоцированными хромосомами (табл. 2). Гомологичная природа хромосом T2DL.2RL подтверждается тем, что они формировали биваленты во всех мейоцитах.

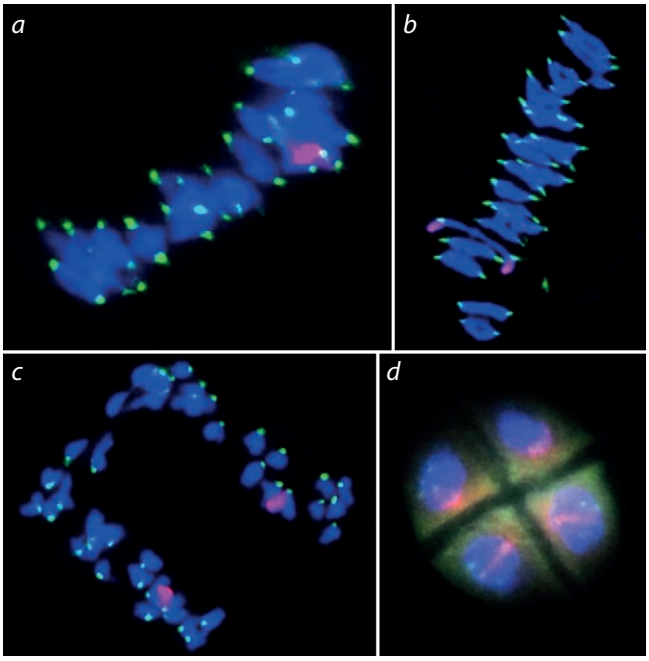

Fig. 5. Behavior of translocated chromosomes in meiosis. *a*, Formation of a ring bivalent at metaphase I. *b*, Formation of a rod bivalent at metaphase I. *c*, Correct segregation of the translocations at anaphase I. *d*, Translocated chromosomes are incorporated in each of the four microspores at the tetrad stage.

Arms of chromosomes 2RL are stained red, arms of wheat chromosomes 2DL are blue, and centromeric regions are green.

Хромосомы T2DL.2RL правильно распределялись между полюсами в первом делении (см. рис. 5, *в*), а в конце мейотического деления включались в микроспоры (см. рис. 5, *г*). Таким образом, линии характеризовались стабильностью.

Table 2. Frequency of ring and rod bivalents formed by translocated chromosomes T2DL.2RL in meiosis of lines

Plant designation, summer 2018	Number of meiocytes examined	Percentage bivalents	
		of ring	of rod
3-2	380	92.13	7.87
5-2	222	81.64	18.36
6-1	142	85.91	14.09
9-1	381	94.11	5.89
9-2	556	91.21	8.79

Fig. 6. Spikes of Novosibirskaya 67 variety, translocation line T2DL.2RL, and wheat-rye substitution line $2R(2D)_1$.

Морфологическая характеристика колоса растений линии T2DL.2RL

Колосья пшенично-ржаной замещенной линии $2R(2D)_1$, сорта Н67 и линии с транслокацией T2DL.2RL имеют одинаковые морфологические признаки: колос веретеновидный, безостый, белый; плотность колоса средняя; колосковая чешуя яйцевидная, среднего размера, со слабой нервацией; зубец короткий, тупой, слегка клювовидный; плечо широкое, прямое, в верхней части приподнятое; киль широкий, хорошо выраженный. Особенностью колоса у линии $2R(2D)_1$ было более плотное расположение колосков на вершине колоса, эти колоски характеризовались пониженной фертильностью (рис. 6).

Анализ элементов продуктивности

Родительские формы транслоцированных линий (BC_3) выращены в условиях гидропонной теплицы 2017 г., вегетация осень–зима. У растений были изучены элементы продуктивности: высота растения, длина главного колоса, продуктивная кустистость, количество зерен с растения, масса зерен с растения, масса 1000 зерен (табл. 3). Растения характеризовались низкорослостью (высота варьировала от 85.5 до 93 см), хорошей продуктивной кустистостью (формировали от трех до семи стеблей с вызревшим зерном в колосьях), высокими значениями массы 1000 зерен (33.14–37.89 г).

Сравнение элементов продуктивности у линии T2DL.2RL и сортов Н67 и С29, выращенных в полевых условиях 2018 г., показало, что транслоцированная линия достоверно уступает сорту Н67 по всем показателям, но не отличается от сорта С29 (табл. 4). Показатели продуктивности у растений линии T2DL.2RL также оказались ниже, чем у родительских растений, выращенных в условиях гидропонной теплицы (см. табл. 3).

По таким признакам, как число колосьев и число колосков в главном колосе, достоверных различий между транслоцированной линией и сортом Н67 не выявлено. В большей степени различались значения по количеству зерен с главного колоса и с растения, а также по массе зерен с растения и массе 1000 зерен. Сравнительный анализ

Table 3. Productivity components in parental plants (autumn–winter 2017)

Character	Plants								
	16-1	16-2	16-4	17-3	17-4	17-5	18-3	18-6	18-9
Plant height, cm	89	87	93	85.5	91	93	90.5	87	91
Productive tillering	6	3	6	4	5	3	7	3	7
Main spike length, cm	6	7	6	8.5	6	8	7.5	7	8
Total number of grains per plant	118	66	128	89	102	52	136	68	156
Total mass of grains, g	4.02	2.31	4.54	3.25	3.38	1.97	4.97	2.53	5.36
1000 grain mass, g	34.07	35.00	35.47	36.52	33.14	37.89	36.54	37.21	34.36

Table 4. Comparison of line T2DL.2RL and varieties Saratovskaya 29 and Novosibirskaya 67 in productivity components (summer 2018)

Character	T2DL.2RL	C29	H67
Plant height, cm	97.71 ± 2.37	126.30 ± 2.33***	121.36 ± 1.16***
Main spike length, cm	9.09 ± 0.35	9.10 ± 0.18	10.07 ± 0.24*
Number of spikes	4.60 ± 0.43	4.23 ± 0.34	4.73 ± 0.23
Number of ripe spikes	3.30 ± 0.35	3.03 ± 0.24	4.10 ± 0.23*
Number of spikelets in the main spike	15.68 ± 0.50	14.73 ± 0.16	15.90 ± 0.29
Number of grains in the main spike	23.16 ± 1.63	27.70 ± 1.37	37.23 ± 1.19***
Grain mass on the main spike, g	0.69 ± 0.08	0.86 ± 0.07	1.32 ± 0.07***
Total number of grains	56.53 ± 6.56	58.23 ± 4.25	135.10 ± 9.01***
Total mass of grains, g	1.48 ± 0.20	1.53 ± 0.14	4.61 ± 0.34***
1000 grain mass, g	24.65 ± 1.40	25.37 ± 1.14	33.71 ± 0.78***
Total number of plants examined	173	30	30

* $p \le 0.05$; *** $p \le 0.001$.

элементов продуктивности линии T2DL.2RL с сортами H67 и C29, а также с замещенной линией $2R(2D)_1$, проведенный в условиях гидропонной теплицы во время вегетации зима–весна 2019 г., показал, что линия T2DL.2RL достоверно уступает сорту H67 по всем показателям с различной степенью достоверности (см. табл. 4).

Наиболее достоверные отличия ($p \le 0.001$) линии T2DL.2RL от сорта H67 получены для признаков «количество колосков на главном колосе», «число зерен с растения», «масса зерен с растения» (табл. 5). Растения сорта C29 достоверно ($p \le 0.001$) превосходили растения линии T2DL.2RL только по четырем показателям: число зерен с главного колоса и с растения, масса зерен с главного колоса и с растения. Замещенная линия $2R(2D)_1$ с высокой достоверностью превосходила линии с транслокацией T2DL.2RL по признакам «длина главного колоса», «плотность колоса», «количество колосков в главном колосе», «масса зерен с главного колоса», однако масса 1000 зерен была достоверно меньше (см. табл. 5).

Таким образом, линия T2DL.2RL по элементам продуктивности достоверно уступала сорту H67 в обеих вегетациях, а также сорту C29 при выращивании в гидропонной теплице. Особенно эти различия были выражены у растений, выращенных в полевых условиях. Одним из факторов, отрицательно повлиявших на показатели продуктивности колоса (число и массу зерен), могут быть генетические особенности сортов H67 и C29, а также линии T2DL.2RL. Вероятно, в полевую вегетацию условия произрастания (температурный и водный режимы) растений на 8–11-м этапах органогенеза, когда происходит закладка генеративных органов, опыление, завязывание и налив зерновки (Батыгина, 2014), были неблагоприятными для сорта C29 и линии T2DL.2RL в сравнении с сортом H67. На продуктивность пшеницы может повлиять и реакция растений на длину светового дня (Шульгин и др., 2015). У сорта C29 обнаружен рецессивный ген *Ppd-D1b*, обусловливающий чувствительность к фотопериоду (Файт и др., 2014), а отсутствие плеча 2DS, на котором локализован ген *Ppd-D1*, могло повлиять на интенсивность процессов онтогенеза у линии T2DL.2RL, приведя к потере продуктивности. Растения пшеницы сорта H67, районированного в лесостепной зоне юга Западной Сибири, напротив, являются растениями длинного дня. Однако в условиях выращивания в гидропонной теплице с фиксированной продолжительностью светового дня, постоянной интенсивностью фотосинтетически активной радиации и одинаковым спектром излучения света достоверных различий по элементам продуктивности между сортами C29 и H67

Table 5. Comparison of line T2DL.2RL, varieties Novosibirskaya 67 and Saratovskaya 29, and substitution line 2R(2D) in productivity elements (spring 2019)

Character	T2DL.2RL	N67	S29	$2R(2D)_1$
Plant height, cm	106.03 ± 1.34	111.0 ± 1.31**	121.22 ± 1.51***	138.8 ± 1.38***
Productive tillering	4.75 ± 0.29	5.6 ± 0.34*	4.89 ± 0.3	4.8 ± 0.30
Main spike length, cm	8.42 ± 0.23	8.9 ± 0.15*	8.4 ± 0.11	9.5 ± 0.15***
Spike density	2.01 ± 0.02	2.1 ± 0.04**	1.7 ± 0.02***	2.2 ± 0.03***
Number of spikelets on the main spike	16.8 ± 0.42	18.9 ± 0.25***	14.4 ± 0.12***	20.4 ± 0.32***
Number of grains on the main spike	27.1 ± 0.81	30.5 ± 1.23*	35.7 ± 0.98***	30.5 ± 0.66**
Grain mass on the main spike, g	1.2 ± 0.04	1.4 ± 0.06**	1.8 ± 0.06***	1.3 ± 0.03**
Total number of grains	114.2 ± 5.38	163.6 ± 12.46***	154.0 ± 8.92***	133.3 ± 8.69
Total grain mass, g	4.8 ± 0.25	7.2 ± 0.58***	6.8 ± 0.4***	5.2 ± 0.32
1000 grain mass, g	42.2 ± 0.51	43.7 ± 0.42*	43.9 ± 0.6*	38.9 ± 0.62***
Total number of plants examined	236	23	28	28

* $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$.

не было получено, но показатели продуктивности у линии T2DL.2RL также были достоверно ниже.

На основании полученных результатов сделан вывод об отрицательном влиянии транслокации T2DL.2RL на признаки продуктивности. В других работах показано, что на изменение/сохранение агрономических признаков у пшеницы влияют конкретные хромосомы, образующие транслокацию, и происхождение родительских форм транслоцированных линий (May, Appels, 1984; Hysing et al., 2007). Растения линии с транслокацией T2BS.2RL характеризуются схожими с родительской линией показателями продуктивности (Hysing et al., 2007), а транслокация T2RS.2BL вызывает летальность проростков в определенных генотипах доноров пшеницы (May, Appels, 1984).

Отличительной характеристикой линий с транслокацией T2DL.2RL стала низкорослость. В полевых условиях средняя высота растений была на 23.65 и 28.6 см ниже растений сорта Н67 и С29 соответственно (см. табл. 4). В условиях выращивания в гидропонной теплице растения с транслокацией также имели достоверно более низкую высоту в сравнении с Н67 и С29 и с замещенной линией $2R(2D)_1$ (см. табл. 5). Высота растений линии $2R(2D)_1$ была достоверно выше по сравнению с сортами. Эти данные предполагают иную регуляцию признака «высота растения» у линий T2DL.2RL и $2R(2D)_1$, чем у сортов.

Признак короткостебельности у пшеницы и ржи контролируется генами карликовости. Наибольшее распространение в сортах пшеницы получили гены «зеленой революции» *Rht-B1b* (*Rht1*) и *Rht-D1b* (*Rht2*), локализованные на хромосомах 4B и 4D соответственно, а также ген *Rht8*, локализованный на хромосоме 2DS (Börner et al., 1996). Ген *Ppd-D1a*, обусловливающий нечувствительность растений к фотопериоду и локализованный тоже на 2DS, независимо от *Rht8* оказывает влияние на снижение высоты (Börner et al., 1993). Однако молекулярный анализ низкорослых образцов гексаплоидных тритикале с замещением хромосом 2R/2D не выявил амплификации праймеров к аллелям *Rht8c* и *Ppd-D1a*, на основании чего был сделан вывод о влиянии хромосомы 2D на уменьшение высоты растений (Коршунова, 2015). Участие хромосомы 2D в регуляции высоты растений показано также в работе по анализу агрономических признаков у озимой гексаплоидной тритикале (Bazhenov et al., 2015). У линий с замещением хромосомы 2R хромосомой 2D обнаружен обратный эффект: снижение высоты растений (Bazhenov et al., 2015).

Заключение

Таким образом, увеличение высоты растений у линии $2R(2D)_1$ может быть следствием замещения хромосом 2R/2D. В данном случае хромосома ржи 2R не компенсирует отсутствие хромосомы 2D, возможно потому, что на хромосоме 2R расположен рецессивный ген карликовости *dw2* (Börner et al., 1996). Снижение высоты растений у линии с транслокацией T2DL.2RL, вероятно, может быть вызвано другими неизвестными в настоящее время генами, находящимися на хромосомах 2DL и 2RL или других хромосомах пшеницы. Для выявления истинной причины необходимы дальнейшие исследования.

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Acknowledgements. Works at the Shared Accession Center for Microscopic Analysis of Biologic Objects, SB RAS, and the Laboratory of Artificial Plant Growth, ICG SB RAS, were supported by State Budgeted Project 0324-2018-0018 for ICG FISH analysis of chromosome sets and chromosome behavior in meiosis was supported by the Russian Science Foundation, project 16-16-00011. The C-banding of mitotic chromosomes was supported by the Belarusian Republican Foundation for Fundamental Research, project B15CO-030.

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

Received July 30, 2019. Revised September 27, 2019. Accepted September 30, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):856-864
DOI 10.18699/VJ19.559

The Fifth International Scientific Conference PlantGen2019

Effect of the host-specific toxin SnTOX3 from *Stagonospora nodorum* on ethylene signaling pathway regulation and redox-state in common wheat

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The fungus *Stagonospora nodorum* Berk. is the causative agent of Septoria nodorum blotch (SNB) of wheat. The most important factors of *Stagonospora nodorum* virulence include numerous fungal necrotrophic effectors (NEs) encoded by *SnTox* genes. They interact with the matching products of host susceptibility genes (*Snn*). SnTox-*Snn* interactions are mirror images of classical gene-for-gene interactions and lead to the development of disease. We have studied the SnTox3-*Snn3* interaction, resulting in the development of infection on leaves and formation of extensive lesions. The mechanism of SnTox3 action is likely to be linked to the regulation of redox metabolism and the influence on ethylene synthesis in the wheat plants, although the molecular mechanisms are not fully unveiled. To characterize the SnTox3-*Snn3* interaction, we used *S. nodorum* isolates differing in the expression of the NEs genes *SnTox3* (SnB (Tox3$^+$), Sn4VD (Tox3$^-$)) and two soft spring wheat (*Triticum aestivum* L.) cultivars, contrasting in resistance to the SNB agent and differing in the allelic composition of the susceptibility locus *Snn3-B1*: Kazakhstanskaya 10 (susceptible) and Omskaya 35 (resistant). We carried out a comparative assessment of the transcriptional activity patterns of genes responsible for ethylene biosynthesis (*TaACS1*, *TaACO*) and signaling pathway (*TaEIL1*, *TaERF1*) by real-time PCR and estimated the redox state of wheat plants infected with different isolates of *S. nodorum* by spectrometry. The induction of ethylene biosynthesis and signaling has been shown to result from gene-for-gene interaction between *Snn3-B1* and SnTox3. The results of plant redox status estimation showed that ethylene inhibited accumulation of hydrogen peroxide in SnTox3-sensitive genotypes by regulating the operation of various pro-/antioxidant enzymes at the transcriptional and posttranslational levels. Our results suggest that NE SnTox3 influences ethylene biosynthesis and signaling, thereby regulating redox metabolism in infected wheat plants as necessary for successful host colonization at the initial phases of infection, which ultimately leads to extensive lesions due to fast pathogen reproduction.

Key words: *Stagonospora nodorum*; *Triticum aestivum*; polymerase chain reaction; real-time polymerase chain reaction; necrotrophic effectors; ethylene; redox-metabolism; gene-for-gene interaction; nonspecific resistance.

For citation: Veselova S.V., Burkhanova G.F., Nuzhnaya T.V., Rumyantsev S.D., Maksimov I.V. Effect of the host-specific toxin SnTOX3 from *Stagonospora nodorum* on ethylene signaling pathway regulation and redox-state in common wheat. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):856-864. DOI 10.18699/VJ19.559

Влияние хозяин-специфичного токсина SnTOX3 патогена *Stagonospora nodorum* на сигнальный путь этилена и редокс-статус растений мягкой яровой пшеницы

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Важнейший фактор вирулентности возбудителя септориоза пшеницы *Stagonospora nodorum* Berk. – многочисленные некротрофные эффекторы (НЭ) гриба (SnTox), взаимодействующие с продуктами генов восприимчивости хозяина (*Snn*). Взаимодействия SnTox-*Snn* осуществляются по типу ген-на-ген и ведут к развитию болезни. В настоящей работе изучено взаимодействие SnTox3-*Snn3*, результатом которого является развитие инфекции на листьях с образованием обширных зон поражения. Предположительно, механизм действия SnTox3 связан с регуляцией редокс-метаболизма и влиянием на синтез этилена у растений пшеницы, однако молекулярные механизмы до конца не раскрыты. Для характеристики взаимодействия SnTox3-*Snn3* в работе были использованы изоляты *S. nodorum*, различающиеся по экспрессии гена *SnTox*: SnБ (Tox3$^+$) и Sn4ВД (Tox3$^-$), и два сорта мягкой яровой пшеницы (*Triticum aestivum* L.), контрастные по устойчивости к возбудителю септориоза и различающиеся по аллельному составу локуса восприимчивости *Snn3-B1*: Казахстанская 10 (восприимчи-

вая) и Омская 35 (устойчивая). Проведена сравнительная оценка характера транскрипционной активности генов биосинтеза (*TaACS1*, *TaACO*) и сигнального пути этилена (*TaEIL1*, *TaERF1*) методом полимеразной цепной реакции (ПЦР) в реальном времени и оценен редокс-статус растений пшеницы, инфицированных различными изолятами *S. nodorum* с помощью спектрофотометрических методов. Показано, что индукция биосинтеза и сигнального пути этилена происходила в результате взаимодействия по типу ген-на-ген *Snn3-B1*-SnTox3. Результаты оценки редокс-статуса растений показали, что этилен подавлял накопление пероксида водорода в чувствительных к SnTox3 генотипах за счет регуляции работы различных ферментов про-/антиоксидантной системы на транскрипционном и посттрансляционном уровнях. Таким образом, полученные результаты предполагают, что НЭ SnTox3 влиял на биосинтез и сигнальный путь этилена с целью регуляции редокс-метаболизма инфицированных растений пшеницы для успешной колонизации хозяина на начальных этапах инфицирования, что впоследствии приводило к обширным зонам поражения за счет быстрого размножения патогена.
Ключевые слова: *Stagonospora nodorum*; *Triticum aestivum*; полимеразная цепная реакция; полимеразная цепная реакция в реальном времени; некротрофный эффектор; этилен; редокс-метаболизм; взаимодействие ген-на-ген; неспецифическая устойчивость.

Introduction

Wheat, a staple crop, has been attacked by various kinds of leaf spot diseases in recent decades, and Septoria nodorum blotch (SNB) ranks among the most injurious ones. It is caused by the fungal pathogen *Stagonospora nodorum* Berk. Yield losses inflicted by this pest reach up to 30 % in susceptible wheat cultivars under permissive environmental conditions (Bertucci et al., 2014). Studies of SNB have been intensively conducted over the past three decades, but there is still no clear understanding of the mechanisms that underlie wheat resistance/susceptibility to infection, on the one hand, and pathogen virulence, on the other hand (Fraaije et al., 2002; Bertucci et al., 2014; Winterberg et al., 2014; Phan et al., 2016; Shi et al., 2016).

It has been shown that among the most important factors of virulence of *S. nodorum* are the numerous necrotrophic effectors (NEs), formerly referred to as host-specific (selective) toxins (Phan et al., 2016; McDonald, Solomon, 2018). The interaction in the wheat–*S. nodorum* pathosystem is of the gene-for-gene type (McDonald, Solomon, 2018). These relationships are confirmed by the fact that products of the pathogen virulence genes (=host-specific toxins) (SnTox) cause compatibility, i. e. disease expansion, when interacting with products of the host plant susceptibility genes (*Snn*) (Phan et al., 2016). The effect of each SnTox-*Snn* interaction is incomplete and is complemented by other interactions. To date, eight SnTox-*Snn* interactions are known, while only three genes encoding NEs (*SnToxA*, *SnTox1*, and *SnTox3*) have been cloned from the pathogen, and only two susceptibility genes (*Tsn1* and *Snn1*) have been cloned from wheat (Phan et al., 2016; Shi et al., 2016).

The genetics of the relationship between wheat and *S. nodorum* is very complex, race-specific resistance explaining only about 40 % of phenotypic manifestations (Shi et al., 2016). In addition, it was shown in the last four years that some of the characterized SnTox-*Snn* interactions causing susceptibility are aimed at manipulating nonspecific plant defense pathways associated with redox metabolism, secondary metabolism and pathogenicity-related proteins (Winterberg et al., 2014; Phan et al., 2016; Shi et al., 2016).

The *Snn3-B1*-SnTox3 interaction plays a significant role in SNB development (Shi et al., 2016; McDonald, Solomon, 2018). It is assumed that the result of this interaction is the development of infection on leaves with the formation of extensive lesions, which is associated with the influence of SnTox3 on the generation of reactive oxygen species (ROS); unfortunately, the mechanism underlying this effect remains obscure (Winterberg et al., 2014). Nevertheless, a recent study shows that SnTox3 induces methionine accumulation and ethylene synthesis in wheat plants within 24 h after infection (Winterberg et al., 2014).

One of the earliest plant responses to the penetration of a pathogen is known to be local ROS generation, which plays an important role in the development of systemic resistance (Barna et al., 2012). Currently, the mechanisms regulating apoplastic ROS synthesis during immune response are intensively studied but still insufficiently understood. Recent studies have shown that the pro-/antioxidant state of plants is under the strict control of plant hormones involved in the formation of defense reactions during stress (Barna et al., 2012). They include ethylene, whose role in biotic stress is complex and depends on the type of pathogen and plant species (Vleesschauver et al., 2010; Barna et al., 2012). Earlier, we showed the negative role of ethylene in the development of wheat plant resistance to *S. nodorum* (Veselova et al., 2016). Unfortunately, the mechanisms of action of plant hormones, including ethylene, on ROS generation under biotic stress are poorly known (Barna et al., 2012).

In this regard, the aim of this work was a comparative assessment of the transcriptional pattern of genes involved in ethylene biosynthesis and signaling pathway genes and of the redox state of wheat plants infected with *Stagonospora nodorum* isolates differing in NE *SnTox3* expression.

Materials and methods

The objects of the study were two cultivars of soft spring wheat (*Triticum aestivum* L.) contrasting in resistance to *S. nodorum* Berk.: susceptible cv. Kazakhstanskaya 10 (Kaz10) and resistant cv. Omskaya 35 (Om35). The pathogen objects were two isolates of the fungus *S. nodorum*: Sn4VD (Republic of Belarus) and SnB (Republic of Bashkortostan). Fungi were grown on potato-glucose agar (PGA). Plants were hydroponically grown on 10 % solution of Hoagland–Arnon nutrient medium in a KS-200 SPU growth chamber (Russia) at

20/24 °C (night/day) at the irradiance 146 W/m^2 FAR (Osram lamps L 36W/77) and the 16-h photoperiod for seven days. The assessment of seedling resistance of cultivars was carried out by the lawns method, as described in (Veselova et al., 2016). The resistance/susceptibility of cultivars was assessed from the lesion area seven days after inoculation with *S. nodorum* isolates. The development of SNB symptoms on wheat leaves was photographed with an SP-800UZ Image Stabilization camera (Olympus, Indonesia). The lesion area was measured with ImageJ program (rsbweb. nih.gov/ij/download.html) and expressed as percent leaf area infected. The degree of damage was also assessed according to the International scale based on the percentage of the affected area of plant organs: RR (0–5 %) – caltivars with very high and high resistance; R (up to 10–15 %) – resistant caltivars; M (up to 25 %) – slightly susceptible caltivars; S (up to 40–65 %) – susceptible caltivars; SS (over 65 %) – caltivars with very high and high susceptibility.

DNA was isolated from wheat seedlings and 7-day fungus culture by the phenol-detergent method (Maniatis et al., 1984). *SnTox3* gene (FJ823644) identification in *S. nodorum* isolates was performed by PCR with gene-specific primers (5′→3′): F–CGAGCTGATATCCCGTTTGA; R–GGGACAGTGACAATAGGTAAGG (Winterberg et al., 2014); primers for the housekeeping gene *tubulin* (S56922) (Fraaije et al., 2002) being used as an internal control for the presence of fungal DNA. Analysis of *SnTox3* gene expression in different isolates of *S. nodorum* during inoculation of wheat plants was performed with the same primers using semi-quantitative PCR. Total RNA was isolated with Trizol reagent (Sigma, Germany) according to manufacturer's recommendations from leaves of susceptible wheat cv. Kaz10 and resistant cv. Om35 fixed in liquid nitrogen after their inoculation with a pathogen. To obtain cDNA based on RNA from the studied samples, reverse transcription reaction was performed using reverse transcriptase in accordance with manufacturer's protocol (Synthol, Russia). PCR with the cDNA template was performed in a TP4-PCR-01-Tertsik type PCR machine (DNK-Tekhnologia, Russia).

The allelic state of the *Snn3-B1* locus was determined by PCR with primers for the *Xcfd20* and *Xgwm234* microsatellite markers (Bertucci et al., 2014). The sequences of primers *Xcfd20* (5′→3′): F–TGATGGGAAGGTAATGGGAG; R–ATCCAGTTCTCGTCCAAAGC; of primers *Xgwm234* (5′→3′): F–GAGTCCTGATGTGAAGCTGTTG; R–CTCATTGGGGTGTGTACGTG (Bertucci et al., 2014). In all cases, PCR products were resolved in 7 % PAAG stained with ethidium bromide using the Gene Ruler DNA Ladder (Fermentas). Gels were photographed using a GelDoc XR documenting system (Bio-Rad, USA).

To reveal the effect of SnTox3 on the biosynthesis and signaling pathway of ethylene, part of the wheat seedlings were treated with 1.5 mM solution of ethephone (ET) (2-chloroethylphosphonic acid), an ethylene-releasing compound (Sigma, Germany) (Veselova et al., 2016), 24 h before inoculation with various *S. nodorum* isolates, while the remainder of the wheat seedlings were treated with 50 μM aminoethoxyvinylglycine (AVG), ethylene biosynthesis inhibitor (Sigma, Germany). After the treatment, the vessels were closed and kept in the dark. The content of hydrogen peroxide (H_2O_2) and the activities of peroxidase enzymes (PO), oxalate oxidase (OXO), and catalase were measured 24 and 72 h after inoculation with *S. nodorum* isolates as previously described (Veselova et al., 2018).

Total RNA was isolated from control and experimental wheat leaves with Trizol reagent according to manufacturer's (Sigma, Germany) recommendations. Prior to the isolation, the leaves were fixed in liquid nitrogen 24 h after their inoculation with *S. nodorum*. Analysis of the expression of genes for oxidoreductases and genes involved in the biosynthesis and signaling pathways of ethylene was performed by quantitative real-time PCR with an iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, USA) and SYBR Green I intercalating dye (Sintol, Russia). To normalize the expression results of the studied genes, primers for the *RLI* gene for constitutively expressed RNA inhibitor protein (RNase L inhibitor-like) (AY059462) were used (Gimenez et al., 2011). Changes in the expression of the gene of interest were estimated by the level of normalized gene expression calculated with the iCycler iQ5 Real-Time Detection System Software (Bio-Rad, USA). Primers for the genes encoding NADPH oxidase (*TaRboh*, AY561153) (Giovanini et al., 2006), superoxide dismutase (SOD) (*TaSod*, JX398977.1) (Giovanini et al., 2006), anionic peroxidase (*TaPrx*, TC151917) (Maksimov et al., 2014), aminocyclopropane synthase (ACC synthase – *TaACS1*, U35779) (Subramaniam et al., 1996), aminocyclopropane oxidase (ACC oxidase – *TaACO*, KF900072) (primer sequences (5′→3′): F–TGTCCATCGCCTCCTTCTA; R–CGAACACGAACCTTGGGTAT; transcription factor of the ethylene signaling pathway EIN3-LIKE1 (ETHYLENE INSENSITIVE3- LIKE1 (EIL1) – *TaEIL1*, KU030837, *Arabidopsis* orthologue gene *AtEIN3*) (Liu et al., 2016) and the transcription factor of the primary response to ethylene ERF1 (ETHYLENE RESPONSE FACTOR1 – *TaERF1*, EF583940) (Dong et al., 2010) were used in this study.

All experiments were carried out three times with three biological and three analytical replications ($n = 9$ in total), except for the measurements of infected area, which were performed in not less than 30 biological replications ($n = 90$ altogether). The Figures 1–3 and Tables 1–3 report mean values and their confidence intervals calculated from their standard errors. Significance of differences between experimental variants was estimated by Student's *t*-test at the confidence level $p \leq 0.05$.

Results

***SnTox3* gene and *Snn3-B1* susceptibility locus.** Two *S. nodorum* isolates, SnB and Sn4VD, were tested for the presence/absence of the *SnTox3* gene by PCR. The gene was found in both (Fig. 1, *a*).

However, analysis of the transcriptional activity of this NE gene showed no expression in the avirulent Sn4VD isolate and accumulation of *SnTox3* transcripts after inoculation of the susceptible cv. Kaz10 and the resistant cv. Om35 with the virulent isolate SnB (see Fig. 1, *b*). PCR diagnostics of the allelic state of the *Snn3-B1* locus was performed in two soft spring wheat cultivars, Kaz10 and Om35. For this purpose, specific primers for two *Xcfd20* and *Xgwm234* microsatellite markers flanking the *Snn3-B1* locus were used (Bertucci et al., 2014; Shi et al., 2016). The null allele was not found in these varieties. However, the cultivars differed in the allelic

composition of the *Snn3-B1* locus (see Fig. 1, *c*). In particular, the *Xcfd20* marker was represented by two alleles in both cultivars, and *Xgwm234* was represented by one allele in Om35 and by two in Kaz10 (see Fig. 1, *c*).

Cultivar/isolate combinations. It was shown previously that cv. Om35 was the most resistant among several cultivars of soft spring wheat, whereas Kaz10 was the least resistant to the hemibiotrophic fungus *S. nodorum* (Veselova et al., 2016). Those observations were made using isolate SnB. In this work, the following cultivar/isolate combinations were studied: Kaz10/SnB (S/Tox3$^+$), Kaz10/Sn4VD (S/Tox3$^-$), Om35/SnB (I/Tox3$^+$), Om35/Sn4VD (I/Tox3$^-$), where S is the Tox3-sensitive cultivar, I is the Tox3-insensitive cultivar, Tox3$^+$ is the isolate expressing the toxin gene, Tox3$^-$ is the isolate not expressing the toxin gene.

A complete compatibility reaction was detected in the S/Tox3$^+$ cultivar/isolate combination (Kaz10/SnB), where inoculation with the pathogen led to the formation of large lesions covering up to 80 % of the total leaf area (Table 1). Resistance reactions were observed in the remaining cultivar/isolate combinations (Om35/SnB, Kaz10/Sn4VD, Om35/Sn4VD) (see Table 1). Pretreatment of both Tox3-sensitive (Kaz10) and Tox3-insensitive (Om35) plants with ET increased their susceptibility only to the SnB isolate (Tox3$^+$) but did not affect the susceptibility to the Sn4VD isolate (Tox3$^-$) (see Table 1). Pretreatment of Tox3-insensitive plants (Om35) with ethylene biosynthesis inhibitor AVG did not affect their defense response, regardless of the isolate that was used for plant inoculation: SnB (Tox3$^+$) or Sn4VD (Tox3$^-$) (see Table 1). Treatment of Tox3-sensitive plants (Kaz10) with AVG increased their resistance to the SnB isolate but did not affect the resistance to the Sn4VD isolate (see Table 1).

The biosynthesis and signaling pathway of ethylene in infected plants. Analysis of the transcriptional activity of ethylene biosynthesis genes (*TaACS* for ACC synthase and *TaACO* for ACC oxidase) and signaling pathway genes (*TaEIL1* and *TaERF1*) showed an increase in the mRNA contents of these genes during the compatibility reaction developed in susceptible plants (Kaz10/SnB) and in plants treated with ET (Kaz10/SnB + ET, Om35/SnB + ET) (Table 2). In Tox3-sensitive plants (Kaz10), the transcripts contents

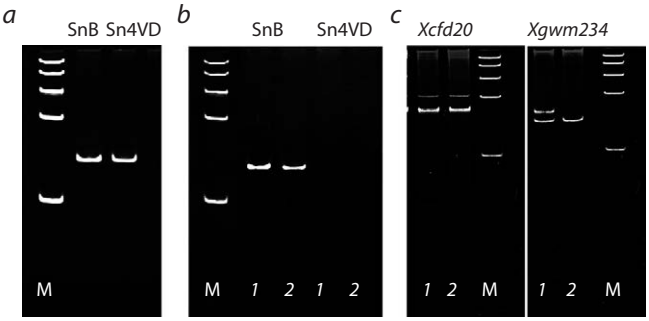

Fig. 1. Identification of the *SnTox3* gene in two *S. nodorum* isolates: SnB and Sn4VD (*a*); analysis of transcriptional activity of the *SnTox3* gene in infection of two soft spring wheat cultivars (*b*); identification of alleles of the *Snn3-B1* locus in these cultivars using primers for SSR markers (*Xcfd20* and *Xgwm234*) by PCR (*c*).

Lanes: *1* – Kazakhstanskaya 10; *2* – Omskaya 35; M – DNA molecular weight ladder 100–1000 bp.

of the ethylene biosynthesis genes and the gene coding for *TaERF1* (transcription factor involved in the primary response to ethylene) increased about three to four fold, and the mRNA content of the *TaEIL1* gene (coding for the main regulatory factor of the ethylene signaling pathway) increased 14–18 times on the 24th hours after inoculation (see Table 2). However, in Tox3-insensitive plants (Om35/SnB + ET cultivar/isolate combination), the accumulation of mRNAs of the genes responsible for biosynthesis and signaling pathway of ethylene was lower than in other cultivar/isolate combinations, which led to a compatibility reaction (see Table 2).

When the incompatibility reaction developed in Tox3-insensitive plants in the Om35/SnB, Om35/Sn4VD cultivar/isolate combinations, suppression or absence of the accumulation of transcripts of genes involved in ethylene biosynthesis and signaling pathway was found regardless of ET or AVG treatments (see Table 2). When the incompatibility reaction developed in Tox3-sensitive plants, either inoculated with Sn4VD isolate (Tox3$^-$) (Kaz10/Sn4VD, Kaz10/Sn4VD + AVG, Kaz10/Sn4VD + ET) or with SnB isolate (Tox3$^+$) (Kaz10/SnB + AVG), there was no accumulation of mRNAs of the *TaACS*, *TaACO*, or *TaERF1* genes and the accumula-

Table 1. Reaction of two wheat cultivars with different allelic states of the *Snn3-B1* locus to inoculation with *S. nodorum* SnB (Tox3$^+$) and Sn4VD (Tox3$^-$) isolates

Kazakhstanskaya 10			Omskaya 35		
Cultivar/isolate	Damage area, %	Group*	Cultivar/isolate	Damage area, %	Group*
Kaz10/SnB	80.0 ± 3.0	SS	Om35/SnB	10.9 ± 2.5	R
Kaz10/SnB+ET**	94.9 ± 2.3	SS	Om35/SnB+ET	57.2 ± 2.6	S
Kaz10/SnB+AVG**	18.6 ± 2.2	M	Om35/SnB+AVG	9.1 ± 1.9	R
Kaz10/Sn4VD	8.4 ± 2.2	R	Om35/Sn4VD	1.6 ± 0.5	RR
Kaz10/Sn4VD+ET	8.1 ± 1.8	R	Om35/Sn4VD+ET	1.9 ± 1.1	RR
Kaz10/Sn4VD+AVG	6.5 ± 2.6	R	Om35/Sn4VD+AVG	1.6 ± 0.5	RR

* RR (0–5 %) – cultivars with very high and high resistance; R (5–15 %) – resistant cultivars; M (15–25 %) – slightly susceptible cultivars; S (25–65 %) – susceptible cultivars; SS (65–100 %) – cultivars with very high and high susceptibility.

** Plants were treated with either ethephon (ET) or ethylene biosynthesis inhibitor (AVG) 24 h before inoculation with *S. nodorum*.

Table 2. Transcriptional analysis of ethylene biosynthesis and signaling pathway genes measured in Kazakhstanskaya 10 and Omskaya 35 wheat cultivars contrasting in SNB resistance 24 h after inoculation with *S. nodorum* isolates

Variant of treatment	Gene			
	TaACS	*TaACO*	*TaEIL1*	*TaERF1*
		Kazakhstanskaya 10		
Kaz10	100	100	100	100
Kaz10/SnB	311 ± 60	237 ± 30	1400 ± 200	356 ± 70
Kaz10/SnB+AVG	143 ± 23	77 ± 3	389 ± 0	100 ± 20
Kaz10/SnB+ET	350 ± 34	223 ± 10	1810 ± 270	450 ± 34
Kaz10/Sn4VD	128 ± 28	110 ± 10	322 ± 10	102 ± 10
Kaz10/Sn4VD+AVG	117 ± 9	63 ± 15	390 ± 60	146 ± 10
Kaz10/Sn4VD+ET	121 ± 10	65 ± 6	375 ± 20	107 ± 7
		Omskaya 35		
Om35	100	100	100	100
Om35/SnB	86 ± 10	47 ± 10	77 ± 6	92 ± 10
Om35/SnB+ET	169 ± 8	190 ± 15	325 ± 54	262 ± 35
Om35/SnB+AVG	102 ± 20	54 ± 10	115 ± 20	178 ± 40
Om35/Sn4VD	97 ± 10	143 ± 10	133 ± 20	54 ± 2
Om35/Sn4VD+ET	125 ± 6	95 ± 7	156 ± 10	199 ± 2
Om35/Sn4VD+AVG	108 ± 20	206 ± 20	129 ± 20	134 ± 5

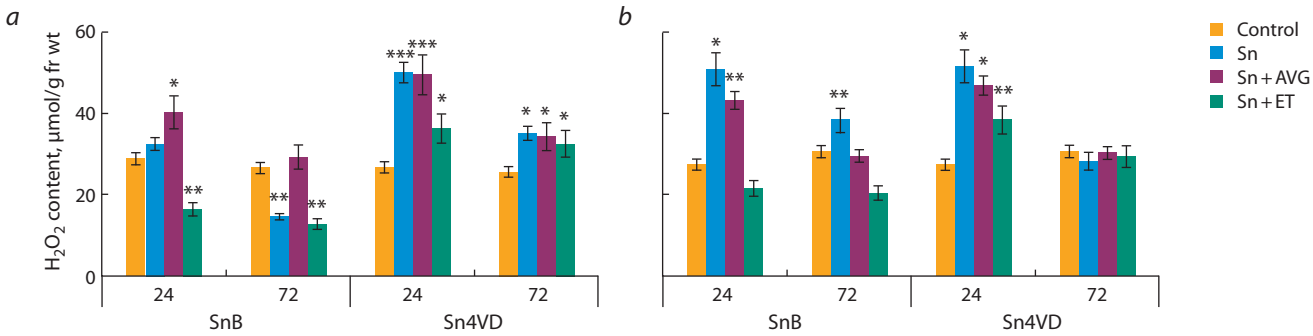

Fig. 2. Hydrogen peroxide contents in leaves of cultivars Kazakhstanskaya 10 (*a*) and Omskaya 35 (*b*) 24 and 72 h after inoculation with *S. nodorum* SnB (Tox3$^+$) and Sn4VD (Tox3$^-$) isolates.

Control – uninfected plants; Sn – infection with *S. nodorum* isolates; Sn + AVG – infection + AVG treatment; Sn + ET – infection + ET treatment. Asterisks show statistically significant differences from the control group, and different numbers of asterisks indicate differences between the variants significant at $p \leq 0.05$.

tion of *TaEIL1* mRNA was low as compared to control plants (see Table 2).

Redox state of infected plants. We studied the components of the pro-/antioxidant system in order to elucidate the role of SnTox3 in the regulation of redox metabolism in the host plant via the biosynthesis and signaling pathway of ethylene in suppressing defense reactions in infected plants. The compatibility reactions in susceptible plants (Kaz10/SnB) and in plants treated with ET (Kaz10/SnB+ET, Om35/SnB+ET) were characterized by a decrease in H_2O_2 content (Fig. 2, *a*, *b*) due to elevated activity of catalase (CAT) (Fig. 3, *e*, *f*), reduced peroxidase (PO) (see Fig. 3, *a*, *b*) and oxalate oxidase (OXO) (see Fig. 3, c, *d*) activities, and lack of transcript accumulation of the genes encoding oxidoreductases NADPH oxidase (*TaRbohF*), SOD (*TaSod*), and PO (*TaPrx*) (Table 3). However, in Tox3-insensitive plants (Om35/SnB + ET cultivar/isolate combination), the suppression reaction of oxidative burst was less pronounced than in other combinations leading to a compatibility reaction (see Fig. 2, 3, Table 3).

The incompatibility reactions in the Om35/SnB, Om35/Sn4VD, Kaz10/Sn4VD cultivar/isolate combinations and in AVG-treated plants from all combinations were characterized by an increase in H_2O_2 generation (see Fig. 2, *a*, *b*) due to a decrease or absence of increase in CAT activity, a drastic increase in the activity of PO and OXO (see Fig. 3), and the accumulation of *TaRbohF*, *TaSod*, and *TaPrx* transcripts at the early stage of infection (24 h) (see Table 3), which led to the development of a hypersensitive-type response and arrest

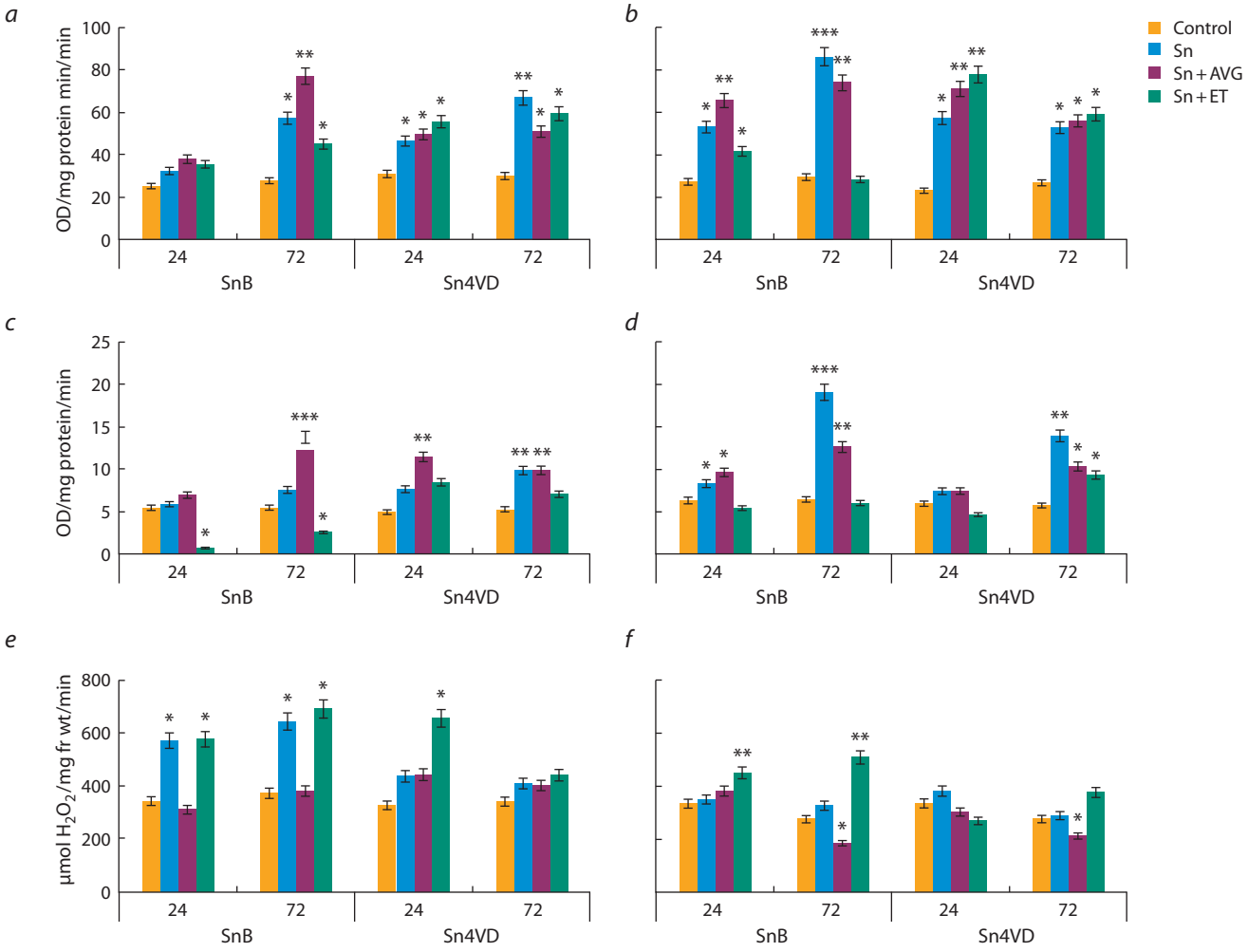

Fig. 3. Enzyme activities in leaves 24 and 72 h after inoculation with *S. nodorum* SnB (Tox3$^+$) and Sn4VD (Tox3$^-$) isolates: (*a*, *b*) peroxidase; (*c*, *d*) oxalate oxidase; (*e*, *f*) catalase; (*a*, *c*, *e*) cv. Kazakhstanskaya 10; (*b*, *d*, *f*) Omskaya 35.

Designations follow Fig. 2.

Table 3. The results of transcriptional analysis of oxidoreductase genes registered 24 h after inoculation with *S. nodorum* isolates in wheat cultivars Kazakhstanskaya 10 and Omskya 35 characterized by different levels of resistance to the disease

Variant of treatment*	Pathogen isolate					
	SnB (Tox3$^+$)			Sn4VD (Tox3$^-$)		
	Gene					
	TaRboh	*TaSod*	*TaPrx*	*TaRboh*	*TaSod*	*TaPrx*
	Kazakhstanskaya 10					
Control	100	100	100	100	100	100
Sn	78 ± 3	88 ± 10	71 ± 3	160 ± 21	144 ± 8	223 ± 20
Sn + AVG	111 ± 20	169 ± 20	163 ± 30	145 ± 40	110 ± 3	210 ± 50
Sn + ET	15 ± 1	81 ± 7	104 ± 20	152 ± 37	114 ± 2	251 ± 50
	Omskya 35					
Control	100	100	100	100	100	100
Sn	126 ± 10	264 ± 20	365 ± 26	160 ± 20	196 ± 9	264 ± 20
Sn + AVG	136 ± 20	242 ± 40	311 ± 31	129 ± 10	178 ± 18	170 ± 20
Sn + ET	83 ± 10	118 ± 9	136 ± 15	132 ± 30	159 ± 10	181 ± 34

* Treatment options are designated as in Fig. 2.

of pathogen growth (see Table 1). Treatment with ET did not affect the nature of the response to inoculation with the Sn4VD isolate in either Tox3-sensitive or insensitive plants (see Fig. 2, 3, Table 3).

Discussion

At present, it is known that *S. nodorum* produces eight NEs associated with the virulence of pathogen isolates (Phan et al., 2016; Shi et al., 2016). The main NEs are SnToxA, SnTox1, SnTox3 toxins, which are considered to be the key factors of the virulence of pathogen strains and isolates, as shown in experiments with mutant *S. nodorum* strains and different wheat lines sensitive and insensitive to these effectors (Phan et al., 2016; Shi et al., 2016).

In our study, high transcriptional activity of the *SnTox3* gene was found in the virulent isolate SnB (Fig. 1, *b*). The avirulent isolate Sn4VD did not express this gene (see Fig. 1, *b*), which may be indicative of SnTox3 inactivation in this isolate (Tan, Oliver, 2017). Here we studied two cultivars of soft spring wheat contrasting in resistance to *S. nodorum* and differing in the allelic composition of the *Snn3-B1* locus (see Fig. 1, *c*). This difference is presumed to be associated with their sensitivity and insensitivity to NE SnTox3 (Shi et al., 2016). In the referred study, two BG220 and Sumai3 wheat lines carrying different alleles of the *Snn3-B1* locus showed different degrees of sensitivity to NE SnTox3. Despite the fact that the *Snn3-B1* null allele was not detected in cv. Om35, this cultivar was insensitive or weakly sensitive to SnTox3. This may indicate a large deletion in the locus between the *Xcfd20* and *Xgwm234* markers, which is in agreement with the literature data (Shi et al., 2016). Thus, out of 17 Sumai3 mutants insensitive to SnTox3, the *Snn3-B1* null allele was detected only in 5 of them: two lines harbored null alleles for three microsatellite markers, *Xgwm234*, *Xmag705* and *Xcfb306*, and three lines had a null allele for one microsatellite marker *Xcfb306* (Shi et al., 2016). Thus, four different cultivar/isolate combinations were selected with two isolates of *S. nodorum* SnB ($Tox3^+$) and Sn4VD ($Tox3^-$) and two cultivars of spring common wheat with different genotypes to study the role of SnTox3 in the development of infection.

On the one hand, the main function of NE SnTox3 is the formation of lesion zones on the wheat leaves of sensitive genotypes by hijacking host's nonspecific signaling defense pathways and manipulating them for pathogen growth and propagation (Winterberg et al., 2014). Furthermore, SnTox3 has been shown to increase ethylene synthesis in infected plants (Winterberg et al., 2014).

To elucidate the role of SnTox3 in ethylene biosynthesis and signaling pathways, we treated part of wheat plants of two cultivars, Kaz10 and Om35, with ethylene chemical precursor ET and ethylene biosynthesis inhibitor AVG. The results showed that both the elevated susceptibility of the ET-treated plants to *S. nodorum* and elevated resistance of the AVG-treated plants depended on the pathogen genotype: $Tox3^+$ in SnB or $Tox3^-$ in Sn4VD (see Table 1). This observation suggests that NE SnTox3 acts as a virulence factor and affects the plant defensive system by regulating ethylene biosynthesis and signaling pathways. It has been shown that ethylene production by some pathogens is closely associated with their virulence (Ma K.-W., Ma W., 2016). For instance, the XopD effector of the pathogenic bacterium *Xanthomonas euvesicatoria* manipulates the ethylene signaling pathway, affecting transcription factor ERF4 (Ma K.-W., Ma W., 2016), and the necrotrophic fungus *Cochliobolus miyabeanus* induces the ethylene signaling pathway in rice to produce and secrete ethylene as an effector to accelerate infection (Shen et al., 2018).

In our work, the analysis of the transcriptional activity of the genes controlling the biosynthesis and signaling pathway of ethylene also showed that the activation of genes involved in this pathway in infected plants depended on the pathogen isolate genotype and the sensitivity of the wheat genotype to NE SnTox3 (see Table 2). These results suggest that the biosynthesis and signaling pathway of ethylene are induced in the gene-for-gene interaction between *Snn3-B1* and SnTox3.

Interestingly, in SnTox3 insensitive plants (Om35/SnB + ET cultivar/isolate combination), ET treatment increased plant sensitivity to this NE (see Table 3), suggesting that such a reaction could result from a mutation in genes regulated by the *Snn3-B1*-SnTox3 interaction (Shi et al., 2016). However, the activation of genes for ethylene biosynthesis and the signaling pathway in the Om35/SnB + ET combination was weaker than in case of the compatible interaction in plants sensitive to SnTox3 (Kaz10/SnB, Kaz10/SnB + ET) (see Table 2). This suggests that resistant plants possess a mechanism for efficient suppression of ethylene biosynthesis and signaling pathway to induce defense responses that are inhibited by ethylene. For example, ethylene inhibited salicylic acid (SA) biosynthesis and suppressed the expression of the SA-mediated signaling pathway marker genes *PR-1* and *PR-2* in *Arabidopsis* plants infected with *Pseudomonas syringae* (Chen et al., 2009), as well as in wheat plants infected with *S. nodorum* (Veselova et al., 2016). Silicon blocked ethylene production by the pathogen *C. miyabeanus*, which improved the resistance of rice plants (Shen et al., 2018). Thus, the obtained results prove the influence of SnTox3 on the biosynthesis and signaling pathway of ethylene in the course of *Snn3-B1*-SnTox3 interaction according to the gene-for-gene type with ultimate suppression of the defense reactions of wheat plants to facilitate colonization.

Our previous studies showed that ethylene provided comfortable conditions for the penetration and development of *S. nodorum* in wheat plant tissues at the initial stage of infection due to the regulation of redox metabolism and reduction of H_2O_2 generation (Veselova et al., 2016, 2018). On the contrary, the accumulation of ROS in wheat plants at the initial stage of infection with the pathogen *S. nodorum* determined the resistance of the cultivar, inducing the expression of the genes encoding pathogenicity-related proteins (Veselova et al., 2016, 2018). The change in the redox state of infected wheat plants in our experiments completely depended on the activation or inhibition of the biosynthesis and signaling pathway of ethylene, and this effect was due to the *Snn3-B1*-SnTox3 interaction of the gene-for-gene type (see Fig. 2, 3). Our results demonstrate that ethylene suppresses H_2O_2 accumulation in plants sensitive to SnTox3 via increasing CAT activity, reducing PO and OXO activities, and lowering the transcript contents of genes encoding NADPH oxidase and SOD, in consistency with literature data (Golemiec et al., 2014; Ma et al., 2017) and our earlier results (Veselova et al., 2018).

Formerly, it was shown that NE SnTox3 regulated genes involved in redox metabolism and the formation of necrosis (Winterberg et al., 2014), but the mechanisms effecting the influence of SnTox3 on ROS generation remain obscure. It is known from the literature that specific effectors of pathogens can induce hypersensitive response in plant cells and suppress the oxidative burst in plants during the infection process in various ways (Jwa, Hwang, 2017). For example, two cytoplasmic effectors of *Phytophthora sojae* interact with catalases to regulate H_2O_2 concentration. The Pep1 effector of *U. maydis* interacts with POX12 maize peroxidase *in vivo* and suppresses early immune responses in maize (Hemetsberger et al., 2012).

Conclusion

The results of our work suggest that the pathogen effector SnTox3 influences biosynthesis and signaling pathway of ethylene in order to regulate the redox metabolism of infected wheat plants in the way promoting successful colonization of the host at initial stages of infection, which subsequently gives rise to extensive damage lesions due to fast pathogen reproduction.

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Acknowledgements. This work was supported by State Project AAAA-A16-116020350027-7 and the Russian Foundation for Basic Research, project 18-04-00978. The authors are grateful to the staffs of the "Biomika" Shared Access Center (Branch of Biochemical Methods and Nanobiotechnology, "Agidel" Resource Center for Collective Use) and the "KODINK" Complex of Equipment for the Study of Nucleic Acids for access to the equipment.

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

Received June 1, 2019. Revised July 8, 2019. Accepted July 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):865-872
DOI 10.18699/VJ19.560

The Fifth International Scientific Conference PlantGen2019

Induced resistance to the greenbug aphid *Schizaphis graminum* Rond. in species of the genus *Triticum*

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The greenbug aphid *Schizaphis graminum* Rond. causes a significant loss of the grain harvest. Therefore, to improve plant resistance to aphids is one of the topical tasks. The problem of creating varieties resistant to phloem-feeding insects is quite urgent, since the mechanisms procuring the resistance of plants to insects are not fully understood. Nevertheless, modern literature describes some mechanisms associated with changes in the redox state of colonized plants. Besides, attention is being increasingly focused on the study of mechanisms that underlie inducible resistance to aphids in wheat and are regulated by hormonal signaling systems. To detect connections among the redox status, indicators of resistance (antibiosis and endurance) of wheat plants to the pest, and induction of the jasmonate (JA) and salicylate (SA) signaling pathways, we studied accessions of three species of wheat plants – *Triticum aestivum* L., *T. monococcum*., and *T. timopheevii* Zhuk.– infested with *S. graminum* greenbugs by physiological, biochemical, and molecular methods. Analysis of antibiosis and endurance showed that *T. timopheevii* k-58666 and *T. monococcum* k-39471 were resistant to *S. graminum*, the latter accession being the most enduring. High hydrogen peroxide contents and high peroxidase activities were detected in the resistant plants. We investigated the expression of genes encoding PR proteins, including markers and regulators of the salicylate (*TaRboh*, *TaPAL*, *Tapr1*, *TaPrx*) and jasmonate (*TaPI*, *TaLOX*, *TaPrx*) signaling pathways. At the early stage of infestation in the susceptible *T. aestivum* variety Salavat Yulaev, the expression of only jasmonate-dependent genes was activated in response to plant damage. In the resistant *T. timopheevii* accession k-58666, expression of only salicylate-dependent genes was activated, while the aphid reproduction was practically absent. In the resistant *T. monococcum* accession k-39471, expression was activated in both the salicylate-dependent and jasmonate-dependent gene groups. We assume that the oxidative burst in the resistant forms of wheat was induced via the activation of the SA signaling pathway, which was of crucial importance in the further cascade of chemical reactions leading to the development of resistance.

Key words: *Schizaphis graminum* Rond.; *Triticum aestivum* L.; *T. monococcum* L.; *T. timopheevii* Zhuk.; polymerase chain reaction; plant immunity; redox metabolism; hormonal signaling systems; gene expression.

For citation: Rumyantsev S.D., Veselova S.V., Burkhanova G.F., Maksimov I.V. Induced resistance to the greenbug aphid *Schizaphis graminum* Rond. in species of the genus *Triticum*. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):865-872. DOI 10.18699/VJ19.560

Индуцированная устойчивость представителей рода *Triticum* к обыкновенной злаковой тле *Schizaphis graminum* Rond.

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Обыкновенная злаковая тля *Schizaphis graminum* Rond. наносит значительный ущерб посевам зерновых культур, поэтому повышение устойчивости растений к тлям – одна из первостепенных задач. Проблема создания устойчивых сортов к насекомым, питающимся флоэмным соком, стоит достаточно остро, так как механизмы, характеризующие устойчивость растений к насекомым, до конца не изучены. Тем не менее в современной литературе описывают механизмы, связанные с изменением редокс-статуса растений, заселенных тлей. Кроме того, в последнее время все большее внимание уделяется изучению механизмов индуцируемой устойчивости пшеницы к тлям, регулируемой гормональными сигнальными системами. Для обнаружения связи между редокс-статусом, показателями устойчивости растений пшеницы (антибиозом и выносливостью) к вредителю и индукцией жасмонат (ЖАК)- и салицилат (СК)-сигнальных путей у представителей трех видов растений пшеницы, *Triticum aestivum* L., *T. monococcum* L. и *T. timopheevii* Zhuk., заселенных обыкновенной злаковой тлей *S. graminum*, были проведены исследования с помощью физиологических, биохимических и молекулярно-генетических методов. Тесты на антибиоз и выносливость показали, что устойчивыми по отношению к *S. graminum* были образцы *T. timopheevii* – к-58666 и *T. monococcum* – к-39471, последний из которых оказался наиболее выносливым. Устойчивые образцы отличались высоким содержанием перекиси водорода и повышенной активностью пероксидазы. В нашей работе исследована экспрессия генов, кодирующих

PR-белки, маркеры и регуляторы СК- (*TaRboh*, *TaPAL*, *TaPr1*, *TaPrx*) и ЖАК-сигнальных путей (*TaPI*, *TaLOX*, *TaPrx*). При ранних ответных реакциях после заселения тлей у восприимчивого сорта Салават Юлаев (*T. aestivum*) активировалась экспрессия только ЖАК-зависимых генов, что отражало реакцию растений на повреждение. У устойчивого образца *T. timopheevii* к-58666 повышалась транскрипционная активность СК-зависимых генов, при этом тля практически не размножалась. У устойчивого образца *T. monococcum* к-39471, проявившего наибольшую выносливость, увеличивалась экспрессия как СК-, так и ЖАК-зависимых генов. Мы предполагаем, что окислительный взрыв у устойчивых форм пшеницы индуцировался благодаря запуску СК-сигнального пути, что имело решающее значение в дальнейшем каскаде химических реакций, ведущих к развитию устойчивости.
Ключевые слова: *Schizaphis graminum* Rond.; *Triticum aestivum* L.; *T. monococcum* L.; *T. timopheevii* Zhuk.; полимеразная цепная реакция; фитоиммунитет; редокс-метаболизм; гормональные сигнальные системы; экспрессия генов.

Introduction

Greenbug aphids (*Schizaphis graminum*) damage wheat crops significantly (Morkunas et al., 2011; Radchenko, 2012). Today, improvement of the resistance of grain crops to aphids is among the primary concerns for both science and practice. One of the approaches to this problem is the breeding of resistant plant genotypes. However, there are few resistant genotypes of *Triticum aestivum* L., and thus the study of wild wheat species is of great importance. It has been shown that most *T. monococcum* L. and *T. timopheevii* Zhuk. accessions are resistant to a broad spectrum of pests (Radchenko, 2012). However, knowledge of the molecular mechanisms of plant resistance is required to create a resistant variety.

Plant protection against phloem-feeding insect is provided by mechanisms of both specific gene-for-gene resistance and nonspecific resistance associated with plant hormonal signaling systems (Morkunas et al., 2011; Radchenko, 2012). Presently, increasing attention is focused on mechanisms of induced (termed active by N.I. Vavilov) resistance of wheat to aphids. The induced nonspecific resistance of plants developed against phytophagous insects is regulated by hormonal signaling pathways, including those depending on jasmonate (JA), ethylene, and salicylate (SA), which affect gene expression, synthesis of protective proteins and various enzymes, redox metabolism of plants, activity of peroxidases in the apoplast, plant cell wall strength, etc. (War et al., 2012). Hormonal signaling pathways do not act independently of each other but form a complex network of interactions playing an important role in the fine-tuning of plant protective reactions (Morkunas et al., 2011). It has been shown that infestation by aphids induces both JA- and SA-dependent protective responses in plants (Morkunas et al., 2011; Kerchev et al., 2012). Unfortunately, the mechanisms underlying the crosstalk of these signaling pathways in the response of plants to aphid damage are not fully understood. The activation of the JA-dependent protective pathway was observed in both susceptible and resistant plants, whereas the induction of the SA-dependent way was faster and stronger only in resistant genotypes (Morkunas et al., 2011).

However, it has been demonstrated that both the JA and SA signaling pathways cause the accumulation of reactive oxygen species (ROS), in particular, hydrogen peroxide (H_2O_2), in plants infested by aphids (Morkunas et al., 2011). ROS are known as the second messenger in a broad spectrum of plant reactions to environmental stress, and the redox state of plants infested by aphids is an important indicator of resistance in a variety (Morkunas et al., 2011; Koch et al., 2016). Oxidative burst during aphid colonization is considered to be a typical reaction implicated in plant resistance to the pest (Koch et al., 2016). The involvement of ROS in the early signal response and in the regulation of the protective response along with both the JA- and SA-dependent signaling pathways has been shown, but the molecular mechanism underlying these processes is not known yet (Kerchev et al., 2012).

In this regard, the aim of this work was to investigate links among the redox state, indicators of resistance of wheat plants (antibiosis and endurance) to the pest, and the induction of JA and SA signaling pathways in three wheat species: *Triticum aestivum*, *T. monococcum* and *T. timopheevii*, infested with greenbug aphids (*S. graminum* Rond.).

Materials and methods

Objects of research. The greenbug aphid *Schizaphis graminum* Rond. was cultivated on young seedlings of soft spring wheat, *Triticum aestivum* L., cultivar Salavat Yulaev (SY) (bred at the Bashkir State Agrarian University, Russia) grown in isolated flasks filled with soil preheated at 180 °C and placed into a KS-200 SPU climatic chamber (Russia) at the 16 : 8 h light/dark schedule, 20/24 °C (night/day) and light intensity 146 W/m^2 (lamps Osram L 36W/77, Germany). The tested accessions included the susceptible soft spring wheat (*T. aestivum*) variety Salavat Yulaev (SY), *T. monococcum* accession k-39471, and *T. timopheevii* accession k-58666, all obtained from the N.I. Vavilov All-Russia Institute of Plant Genetic Resources (St. Petersburg).

Biochemical parameters. Three-day-old wheat seedlings hydroponically grown in isolated plastic vessels (10 % Hoagland–Arnon solution) under the conditions described above were settled with aphids. For this, equal numbers of aphids of different ages were flipped into each vessel with plants so that the load be 10 aphids per plant (Radchenko, 2008). The vessels were covered with plastic insulators tightened with porous nonwoven fabric. The concentrations of hydrogen peroxide (H_2O_2) and the activities of peroxidase (PO) were estimated on days 1, 3, and 10 after the infestation as described in (Veselova et al., 2016).

Antibiosis test. First instar nymphs were placed under the insulators on three-day-old seedlings, one per plant. The numbers of dead and living aphids, imagoes and larvae, were counted after 14 days (Radchenko, 2008). Mortality

was expressed as percentage of the total number of aphids. The propagation coefficient was calculated by the formula: K = average fertility of females during the experiment/duration of the experiment, days (Radchenko, 2008).

Test of plant endurance. The length of a seedling was measured from the level of the raft to the tip of the leaf at the age of three days, and then each plant was settled with 20 wingless females and insulated. The constant number of aphids was maintained by removing hatching larvae at 48-h intervals for two weeks. At the end of the experiment, the heights of the first and second leaves of plants uninhabited and infested by aphids were measured again, and the results were compared with the original measurement (Radchenko, 2008). Endurance was expressed as percentage of leaf growth inhibition compared to the intact control.

Transcriptional gene activity. Total RNA was isolated from both control and experimental wheat plants fixed in liquid nitrogen with Trizol reagent (Sigma, Germany) on days 3 and 10 after infestation by aphids in accordance with manufacturer's recommendations. The nucleic acid concentration was assessed by the A_{260}/A_{280} ratio with a Spec Plus spectrophotometer (Bio-Rad, United States). cDNA was obtained by reverse transcription with M-MuLV reverse transcriptase (Sintol, Russia). With the cDNA as the template, PCR was performed with primers flanking conservative gene sites of the studied PR proteins in a TP4-PCR-01-Tertsik thermocycler (DNA-Technology, Russia). We used primers to genes encoding PR1 protein (*TaPr1*, AF384143) (Veselova et al., 2016), proteinase inhibitors (*TaPI*, EU293132.1), anionic peroxidase (*TaPrx*, TC151917), NADPH oxidase (*TaRboh*, HE674332) (Veselova et al., 2019), phenylalanine ammonia lyase (*TaPAL*, X99725) (Ding et al., 2011), and lipoxygenase (*TaLOX*, BJ223744) (Diallo et al., 2014). To calculate the normalized levels of genes expression, primers to the gene for constitutively expressed protein RNase inhibitor were used (RNase L inhibitor-like) (*TaRLI*, AY059462) (Veselova et al., 2016). Primers to these genes were matched to *T. aestivum* L. Homologous genes were obtained from *T. monococcum* and *T. timopheevii* in pilot experiments with these primers. It is known that carriers of the primary (for example, *T. monococcum*, *Ae. tauschii* Coss., *T. spelta* L., *T. compactum* Host., *T. durum* Desf., *T. dicoccum* (Schrank.) Schuebl.) and secondary (*T. timopheevii*, *T. araraticum* Jakubz., *Ae. speltoides* Tausch., *Ae. sharonensis* Eig, *Ae. longissima* Schw. et Musch.) gene pools contain genomes with high or partial homology to soft wheat *T. aestivum* genomes A, B, and D (Chaudhary et al., 2014).

Statistical analysis. All experiments were carried out in three biological and analytical replications and were repeated three times (n = 9 in total), except for the tests for antibiosis and endurance, which included at least ten biological replications (n = 30). The Figures 1–4 and Tables 1–2 present the arithmetic mean values and their confidence intervals calculated from standard errors. The reliability of the differences between the variants of the experiment was assessed by Student's t test at the confidence level $p \leq 0.05$.

Results

Indicators of resistance of a plant accession. Two types of plant resistance to aphids were studied: antibiosis and endurance. Aphids fed on SY showed the lowest mortality and the highest propagation rate in the antibiosis test (see Fig. 1, *a*, *b*); i. e., this variety was susceptible to greenbug aphid. The mortality of aphids on *T. timopheevii* k-58666 was about 3–4 times higher and the propagation rate 4–5 times lower than on the susceptible variety (see Fig. 1, *a*, *b*). However, *T. monococcum* k-39471 had the highest effect on aphid viability, as the aphids colonizing it had the highest mortality and the lowest propagation rates (see Fig. 1, *a*, *b*). *T. monococcum* k-39471 was the most resistant among the studied accessions.

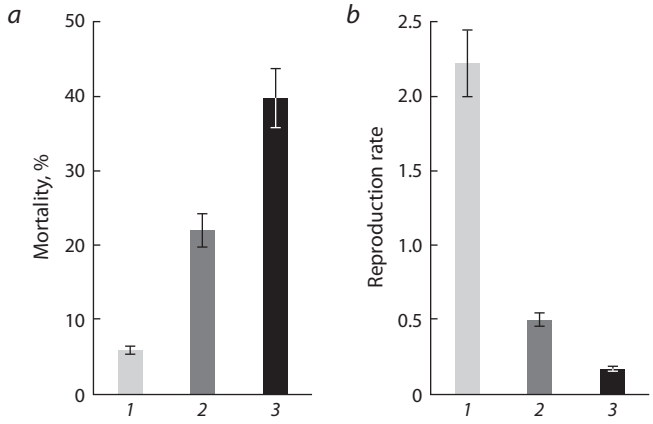

Fig. 1. Mortality (*a*) and propagation (*b*) of greenbug aphid *S. graminum* settled on different species of wheat *Triticum* spp.

1 – Triticum aestivum variety Salavat Yulaev; *2 – T. timopheevii* accession k-58666; *3 – T. monococcum* accession k-39471.

In our experiments, SY exhibited poor endurance to *S. graminum*, manifesting itself as strong inhibition of the growth of the 1st and 2nd leaves (Table 1). The k-58666 accession of *T. timopheevii* showed intermediate endurance to greenbug aphid (see Table 1). *T. monococcum* k-39471 was the most endurant accession, as the infestation by aphids did not inhibit the growth of the 1st leaf, and the inhibition of the growth of the 2nd leaf was the least (see Table 1).

The redox state of plants infested by aphids. Colonization of the susceptible variety SY with aphids led to a decrease in the content of H_2O_2 at the initial stages of feeding: on days 1 and 3 (Table 2). In contrast, resistant plants of *T. timopheevii* k-58666 showed elevated H_2O_2 levels at the early stages of response to infestation by aphids (see Table 2). Accession *T. monococcum* k-39471, resistant to *S. graminum*, showed drastic H_2O_2 accumulation on the 1st day after settling of aphids and reduction of H_2O_2 generation on the 3rd day of feeding (see Table 2). In SY and *T. timopheevii* k-58666, the contents of H_2O_2 remained at the level of control plants on day 10 after the settling with aphids but greatly decreased in the resistant *T. monococcum* accession k-39471 (see Table 2).

Triticum timopheevii k-58666 and *T. monococcum* k-39471, resistant to *S. graminum*, showed elevated PO activities and H_2O_2 generation at early response stages (days 1–3) after settling with aphids (see Table 2). In resistant genotypes, the increase in PO activity was found to continue on day 10

Table 1. Resistance of different *Triticum* spp. species settled with greenbug aphid *S. graminum*

Wheat species	Growth inhibition, %	
	first leaf	second leaf
Triticum aestivum – SY	25.8 ± 6.1	45.0 ± 3.7
T. timopheevii – k-58666	12.3 ± 6.6	22.6 ± 4.2
T. monococcum – k-39471	0.1 ± 2.4	7.6 ± 2.8

Table 2. The effect of greenbug aphid *S. graminum* on H_2O_2 content and peroxidase activity in three wheat *Triticum* spp. accessions at different stages of feeding

Variant	Time after settling, days		
	1	3	10
H_2O_2 content, μmol/g fr wt			
SY	25.2 ± 5.6	26.8 ± 1.7	26.3 ± 2.7
SY + *S. graminum*	12.6 ± 1.3	19.0 ± 2.4	27.0 ± 1.1
k-58666	27.8 ± 5.3	28.9 ± 2.8	22.9 ± 1.7
k-58666 + *S. graminum*	58.7 ± 5.5	48.6 ± 4.5	28.0 ± 3.3
k-39471	28.2 ± 3.6	28.7 ± 3.1	28.8 ± 1.1
k-39471 + *S. graminum*	53.7 ± 5.3	20.4 ± 3.0	12.0 ± 1.2
Peroxidase activity, OD/mg protein min			
SY	30.4 ± 0.7	29.0 ± 0.4	30.3 ± 1.8
SY + *S. graminum*	26.1 ± 1.0	25.8 ± 0.6	28.8 ± 1.2
k-58666	29.6 ± 0.4	31.4 ± 0.8	28.7 ± 1.7
k-58666 + *S. graminum*	35.7 ± 1.2	36.0 ± 0.3	57.1 ± 2.2
k-39471	23.6 ± 0.5	32.1 ± 1.1	30.2 ± 1.2
k-39471 + *S. graminum*	43.2 ± 1.4	60.5 ± 2.6	90.7 ± 3.4

after aphid settling, especially in *T. monococcum* k-39471 (see Table 2). On the contrary, increased activity of PO and low concentration of H_2O_2 were found in the susceptible wheat variety SY both on the first days and on day 10 of aphid feeding (see Table 2).

Transcriptional activity of genes associated with redox metabolism. The contents of mRNA of the *Rboh* gene increased in the resistant accession *T. timopheevii* k-58666 and in *T. monococcum* k-39471 during the early response, on day 3 after aphid settling, but no increase was detected in the susceptible variety SY (Fig. 2, *a*). On the contrary, the content of *Rboh* transcripts increased in the susceptible variety SY at a later response stage, on day 10, whereas it decreased by about one-half in the resistant accessions (see Fig. 2, *a*). The content of *Prx* mRNA increased slightly in the susceptible variety SY. In *T. timopheevii* k-58666, this parameter decreased throughout the experiment (see Fig. 2, *b*). A significant (eightfold) increase in *Prx* gene mRNA content was found in *T. monococcum* k-39471 on day 3 after aphid infestation, and a threefold increase was detected on day 10 (see Fig. 2, *b*).

Transcriptional activity of genes regulated by the JA-dependent signaling cascade. In the susceptible variety SY, elevated levels of transcripts of the *LOX* and *PI* genes, which are regulated by the JA signaling cascade, were observed on days 3 and 10 after aphid settling (Fig. 3, *a*, *b*). In the resistant *T. timopheevii* accession k-58666 the transcriptional activity of the genes in question was close to that in control plants and even decreased by day 10 (see Fig. 3, *a*, *b*). The resistant *T. monococcum* accession k-39471 showed a significant increase in the mRNA contents of the *LOX* and *PI* genes on day 3 after aphid settling, by factors of 2.5 and 6.4, respectively (see Fig. 3, *a*, *b*). On day 10 of feeding, this accession showed a significant accumulation of transcripts only for the LOX gene, by a factor of 3.7 times compared to the intact plants (see Fig. 3, *a*).

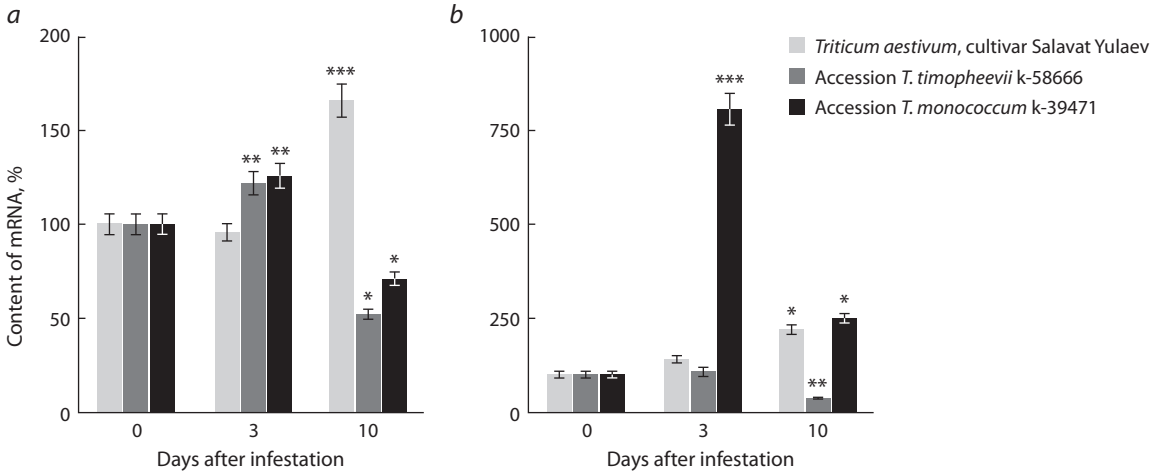

Fig. 2. Effect of greenbug aphid *S. graminum* on the contents mRNAs of *Rboh* (*a*) and *Prx* (*b*) genes in three *Triticum* spp. species at different stages of feeding.

*A*sterisks show statistically significant differences from the control group, and different numbers of asterisks mean differences between the variants significant at $p \le 0.05$.

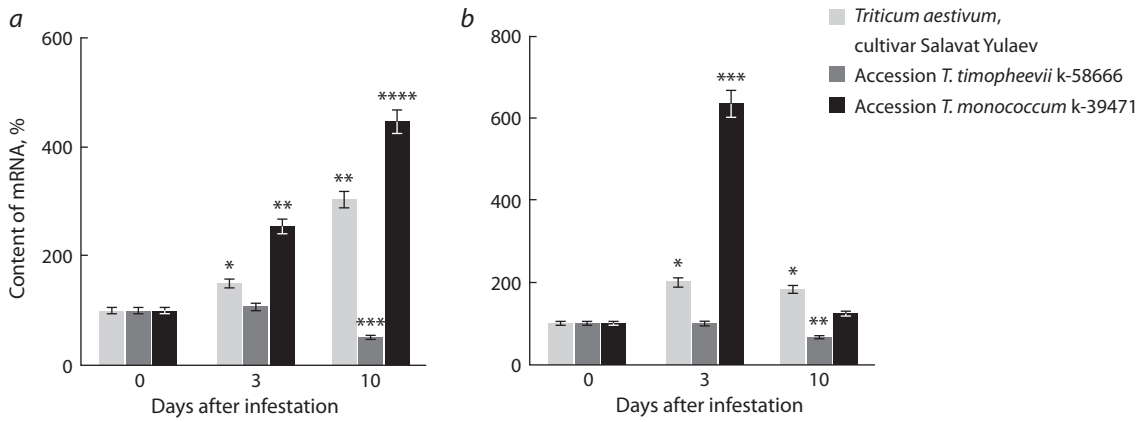

Fig. 3. Effect of greenbug aphid *S. graminum* on the mRNA contents of the *LOX* (*a*) and *PI* (*b*) genes of three *Triticum* spp. accessions at different stages of feeding.

Designations follow Fig. 2.

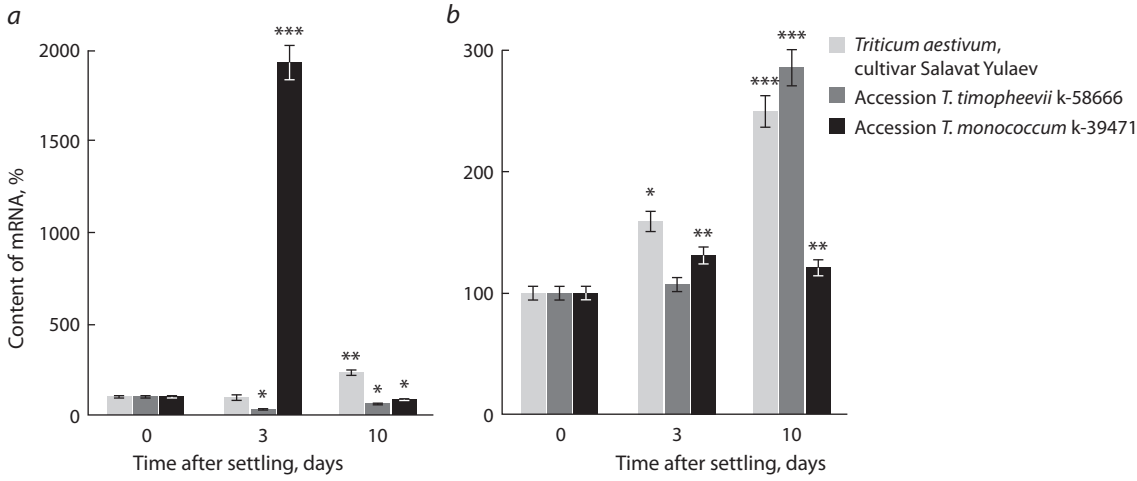

Fig. 4. Effect of greenbug aphid *S. graminum* on the mRNA contents of the *Pr1* (*a*) and *PAL* (*b*) genes of three *Triticum* spp. accessions at different stages of feeding.

Designations follow Fig. 2.

Transcriptional activity of genes regulated by SA-dependent signaling cascade. In susceptible SY, twofold accumulation of transcripts of the *Pr1* gene was detected only on day 10 after aphid settling (Fig. 4, *a*). Resistant *T. timopheevii* k-58666 showed a slightly lowered transcription level of the main gene of the SA signaling pathway throughout the experiment (see Fig. 4, *a*). In contrast, a significant 20-fold increase in the mRNA of the *Pr1* gene was discovered in the resistant *T. monococcum* accession k-39471, which showed the greatest endurance, on day 3 after aphid settling (see Fig. 4, *a*). The pattern of changes in the content of *PAL* gene transcripts was similar in susceptible SY and resistant *T. timopheevii* k-58666: the mRNA level increased by 20–50 % on day 3 after aphid settling and almost threefold on day 10 (see Fig. 4, *b*). The resistant *T. monococcum* accession k-39471 showed a slight increase in the transcriptional activity of the studied gene, by 20–30 % compared to the intact control throughout the experiment (see Fig. 4, *b*).

Discussion

The study of the mechanisms of plant resistance to phloem-feeding insects requires distinguishing early responses found in plants during the first five days after colonization by aphids and long-term responses observed after 10 days of ingestion by the pest (Koch et al., 2016). Early responses of resistant plants are characterized by increased generation of ROS and activation of basal immunity (Morkunas et al., 2011). The protective role of oxidative burst against aphid feeding consists of their signaling function in the development of systemic resistance and the direct damaging effect of high H_2O_2 concentrations on aphids, leading to the suppression of pest survival (Morkunas et al., 2011; War et al., 2012; Koch et al., 2016). Long-term responses are ROS detoxication and growth recovery, which should be induced by early responses of plants (Koch et al., 2016).

Our results showed that *T. monococcum* k-39471 was the most resistant to greenbug aphid *S. graminum* among

the accessions studied. This observation coincides with the literature data on the resistance of numerous *T. monococcum* accessions to different types of aphids (Radchenko, 2012). *Triticum timopheevii* k-58666 also showed medium resistance to the pest in our experiments. In the literature, there is inconsistent information about the resistance of *T. timopheevii* to different types of cereal aphids; however, this wheat species generally shows resistance to the pest and is poorly populated by aphids (Radchenko, 2012).

In the resistant accessions *T. timopheevii* k-58666 and *T. monococcum* k-39471, an increase in H_2O_2 generation during early responses after pest colonization and decrease at the later stage were observed (see Table 2). Thus, it led to high mortality of aphids (see Fig. 1) and a sufficient increase in plant endurance (see Table 1). In susceptible plants of wheat variety SY, suppression of the oxidative burst during early responses after the infestation by pests was observed. The content of H_2O_2 during long-term responses after aphid settling did not differ from control plants (see Table 2), which led to high fertility of the insect (see Fig. 1) and poor plant endurance (see Table 1). An oxidative burst in barley plants populated by the barley aphid *Diuraphis noxia* and *Arabidopsis* plants populated by the peach aphid *Myzus persicae* was detected only in resistant but not in susceptible forms of plants (Lei, Zhu-Salzman, 2015).

In our experiments, the high H_2O_2 generation in resistant wheat forms was probably due to the elevated transcriptional activity of the *Rboh* and *Prx* genes (see Fig. 2). The same was observed earlier by the example of the wheat – *Staganospora nodorum* pathosystem (Veselova et al., 2018). In addition, experiments with mutant *Arabidopsis* plants with the silenced *RbohD* gene showed that NADPH oxidase played an important role in the generation of ROS in plants attacked by aphids (Kerchev et al., 2012).

The oxidative burst induces an immune response in plants, but excessive amounts of ROS inhibit photosynthesis and growth and can cause host cell damage. Therefore, resistant plants have mechanisms of ROS detoxication, which involves antioxidant enzymes such as PO, catalase, and others (Koch et al., 2016). In our experiments, PO activity increased in the resistant accessions *T. timopheevii* k-58666 and *T. monococcum* k-39471 during early responses after aphid settling. The PO activation was accompanied by an H_2O_2 content increase. The PO activity during long-term responses was even more significant than at the early stage (see Table 2). These results suggest the involvement of H_2O_2 and PO in the synthesis of toxic phenolic compounds and the reorganization of plant cell walls via lignification, which may be the cause of reduced pest viability (Morkunas et al., 2011; War et al., 2012; Koch et al., 2016). In our experiments, this kind of response was detected in the resistant accessions (see Fig. 1). In susceptible SY, enhanced activity of PO during early and long-term responses was not detected after aphid settling (see Table 2).

Thus, the activation of peroxidases may play a crucial role in the development of plant resistance to aphids via detoxifying large amounts of ROS (War et al., 2012; Koch et al., 2016). In addition, the H_2O_2 molecule has a signaling function in protection of plants from aphids. It acts as a second messenger in hormonal signaling pathways in the development of systemic resistance (Kerchev et al., 2012). The primary factor inducing the protective response of plants to infestation by aphids is considered to be mechanical injury, which induces JA-dependent activation of proteinase inhibitors and lipoxygenases (Morkunas et al., 2011). Next, the plant responds to the chemical determinants present in aphid saliva and induces a protective response similar to that of plants to biotrophic pathogens, which triggers the SA-dependent signaling cascade (Morkunas et al., 2011). Genes encoding protective proteins *Pr1* and *PAL* are SA-dependent (Van Loon et al., 2006). It is worth noting that the *Rboh* gene is also regulated by the SA-dependent signaling cascade (Kerchev et al., 2012). The *PI* and *LOX* genes are regulated by JA (Van Loon et al., 2006). The *Prx* gene, encoding anionic PO, is induced during JA- and SA-dependent defense reactions (Van Loon et al., 2006).

In our work, the contents of transcripts of the SA-dependent *Rboh* and *PAL* genes increased in plants of the resistant accession *T. timopheevii* k-58666 at both the early and late stages of feeding (see Fig. 2, *a*, 3, *b*). The increase was accompanied by enhanced H_2O_2 generation during early responses and high PO activity (see Table 2). These results suggest that phenylalanine ammonia lyase (encoded by the *PAL* gene), the main enzyme of the phenylpropanoid pathway, is involved in the synthesis of lignin and phenolic compounds, including SA. It is worth noting that lignification of plant cell walls occurs only when apoplastic peroxidases were activated together with elevated H_2O_2 content (Herrero et al., 2013). In addition, the induction of the SA-signaling pathway is likely to be a joint mechanism for antibiosis and aphid repulsion in resistant plant forms (Morkunas et al., 2011).

The resistant accession *T. monococcum* k-39471 showed significantly elevated concentrations of mRNAs of all genes under study, which were regulated by both the SA-dependent and JA-dependent signaling cascades, during early responses after the plants were attacked by aphids (see Fig. 2–4). The transcript contents of some genes decreased during long-term responses after aphid infestation, but the concentration of *LOX* gene mRNA continued to increase (see Fig. 2–4). The most important function of lipoxygenases is the oxidation of linolenic acid as the first step of the JA synthesis pathway and further activation of the JA-dependent signaling system protecting plants from pests. This leads to the synthesis of protease inhibitors and enzymes that participate in the production of lignin, suberin, and cutin and to the generation of volatile organic compounds acting as insect repellents (Wasternack, Strnad, 2018). Proteinase inhibitors bind to digestive enzymes in the insect intestine and inhibit their activity, thereby aggravating protein digestion, which, in turn, causes amino acid deficiency, slow development, and insect hunger (War et al., 2018). However, just the induction of SA-dependent genes (*Pr1*, *PAL*, *Rboh*, and *Prx*) induced the oxidative burst during early responses after the infestation by aphids and triggered the cascade of subsequent protective responses of plants against aphids. Probably, this was a result of the inhibition of catalase activity due to the direct binding of SA to catalase (Mohase, van der Westhuizen, 2002). It is known that some aphid species increase the activity of this enzyme to reduce the oxidative burst and secure favorable conditions for their life (Lei, Zhu-Salzman, 2015). We also showed earlier that the decreased reduced activity of catalase was among the

factors ensuring high H_2O_2 levels in the resistant accessions *T. timopheevii* k-58666 and *T. monococcum* k-39471 (Rumyantsev et al., 2018).

In the susceptible variety SY, no significant increase in the contents of transcripts of genes regulated by SA-dependent signaling cascades during early responses after the aphid invasion was detected except for a small increase in the content of the *PAL* mRNA (see Fig. 2, 3), whereas the content of H_2O_2 was reduced. However, an increase in the content of transcripts of genes (*LOX* and *PI*) regulated by the JA-signaling cascade (see Fig. 4) was observed, which most likely reflected the plant's response to the damage (Morkunas et al., 2011). In the susceptible variety, both JA- and SA-sensitive genes were activated during long-term responses after the aphid invasion; however, the lack of oxidative burst appears to have prevented the start of the cascade of protective reactions.

Conclusions

To sum up, the oxidative burst is induced in resistant wheat varieties due to the triggering of the SA-signaling pathway, which is crucial in the subsequent cascade of chemical reactions leading to the development of resistance. Time is another factor important in the development of protective reactions and in the crosstalk between the SA- and JA-dependent signaling systems in plants. Induction of the JA-signaling pathway alone did not lead to the development of resistance, whereas the SA-signaling pathway, either by itself or along with JA-signaling cascade, induced resistance in *T. timopheevii* k-58666 and *T. monococcum* k-39471, respectively.

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Acknowledgements. This work was supported by State Budgeted Projects AAAA-A16-116020350027-7 and AAAA-A19-119021190011-0. The authors are grateful to the staffs of the "Biomika" Shared Access Center, Branch of Biochemical Methods and Nanobiotechnology, "Agidel", and USU "KODINK" for access to the equipment.

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

Received June 1, 2019. Revised July 26, 2019. Accepted July 29, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):873-878
DOI 10.18699/VJ19.561

The Fifth International Scientific Conference PlantGen2019

Endophytic *Bacillus* bacteria with RNase activity in the resistance of potato plants to viruses

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Viral diseases annually cause significant crop losses and significantly reduce the quality of products, including potatoes, some of the most important crops. Currently, viruses cannot be controlled with chemical pesticides, since known antiviral compounds are teratogenic and hazardous to people's health. Biocontrol agents based on endophytic microorganisms may be an alternative to them. Many strains of *Bacillus* produce ribonucleases (RNases). Our laboratory possesses a collection of bacteria that produce various metabolites and have RNase activity. The results showed that the inoculation of potato with *B. subtilis* 26D and *B. thuringiensis* increased the grain yield by 32–43 %. In addition, the treatment of potato plants with *Bacillus* spp. significantly reduced the infection of potato plants with virus M. The prevalence of the disease in potato plants was significantly reduced from 60 % in the control to 18 % (*B. subtillis* 26D) and 25–33 % (*B. thuringiensis*) in the inoculated plants. Similarly, the infection index decreased from 14 in the control to 1 in the inoculated plants. The further study of molecular mechanisms related to bacterial induction of plant defense reactions in response to viral infections will lead to a better understanding of stress resistance problems. The endophytic microorganisms studied in this report may become the basis for the creation of biological agents for plant protection.
Key words: ribonuclease; phytopathogenic RNA-viruses; *Solanum tuberosum*.

For citation: Burkhanova G.F., Sorokan A.V., Cherepanova E.A., Sarvarova E.R., Khairullin R.M., Maksimov I.V. Endophytic *Bacillus* bacteria with RNase activity in the resistance of potato plants to viruses. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):873-878. DOI 10.18699/VJ19.561

Эндофитные бактерии *Bacillus* spp. с РНКазной активностью и устойчивость картофеля к вирусам

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Вирусные заболевания ежегодно вызывают существенные потери урожая и заметно снижают качество продукции, в том числе важнейшей сельскохозяйственной культуры – картофеля. Антивирусных препаратов для растениеводства, которые были бы безопасны для человека и животных, не существует, и в этих условиях перспективным методом защиты растений является использование биопрепаратов на основе эндофитных микроорганизмов, продуцирующих РНКазы. В работе проанализирована способность ряда эндофитных штаммов *Bacillus* spp. продуцировать РНКазы и влиять на пораженность растений вирусом М, широко распространенным в средней полосе России, и урожайность раннеспелого сорта картофеля Удача в полевых условиях. Обнаружено, что обработка штаммами бактерий *B. subtilis* 26Д и *B. thuringiensis* снижала степень инфицированности растений вирусом М с 60 % в контроле до 18–30 % на участках, обработанных микроорганизмами. Аналогичным образом снизился индекс развития вирусной инфекции: с 14 % в контроле до 1–7 % у инокулированных растений. Кроме того, бактерии с высокой РНКазной активностью вызывали прибавку урожая картофеля до 40 %. Предполагается, что изученные бактерии способны не только повышать рост и урожайность картофеля, но и, благодаря своей РНКазной активности, подавлять распространение вируса М. Дальнейшее изучение молекулярных механизмов, связанных с индукцией эндофитными бактериями защитных реакций растений в ответ на вирусные инфекции, приведет к лучшему пониманию фитоиммунитета растений. Более того, эндофитные штаммы *Bacillus* spp. с высокой РНКазной активностью могут стать основой для биопрепаратов комплексной защиты растений.
Ключевые слова: бактерии; РНКазы; вирусы; картофель.

Introduction

Viral diseases cause the significant loss (up to 30 %) of potato (*Solanum tuberosum*) yield and a marked deterioration of its quality annually. For instance, cultivated plants are affected by at least 450 different viruses, more than 40 of them infect potatoes, significantly reducing their productivity and deteriorating the quality of tubers which is known as the cultivar degeneration (Makarova et al., 2017). The main directions of

plant protection against viral infections are: the rehabilitation of seeds by isolating and cultivating the apical meristem *in vitro*, the generation of transgenic plants resistant to viral infections using genes of specific and nonspecific defense, use of plant protection preparations of a chemical and biological nature against viral vectors, the application of inductors of plant resistance, etc. (Nicaise, 2017). Genome-editing technologies, such as genetic transformation (Prins et al., 2008) and CRISPR/Cas9 system (Romay, Bragard, 2017), are more efficient to control viral diseases. For instance, overexpression of genes encoded antiviral proteins (interferon, ribonucleases), proteins toxic to insects or viral proteins (capsid protein) were shown to enhance resistance against viruses (Stasevski, Ilinskaya, 2009; Chung et al., 2013).

Despite of a huge amount of applied experimental works on the development of biocontrol agents on the basis of rhyzosphaeric and endophytic microorganisms, there are very few data about their antiviral activity, influence on distribution and severity of plant viruses. Biocidal activity of *Bacillus* strains resulted from the synthesis of specific insecticidal proteins (Cry and Vip of *B. thuringiensis*), bacteriocines and lipopeptides (Rodríguez et al., 2018). This perspective tends to favour the view that *Bacillus* can protect plants against viral diseases by affecting insect, bacterial and fungal phytopathogens, nematodes, which are vectors of viral particles. It's worth noting that currently the search of endophytic microorganisms which inhabit in the internal tissues of plants and less influenced by environmental factors and more integrated in plant metabolism than rhyzosphaeric and fillosphaeric microorganisms is of great interest.

A lot of *Bacillus* species can inhabit internal plant tissues (Burkhanova et al., 2017) and produce ribonucleases (Ulyanova et al., 2016; Ilinskaya et al., 2018). Thus, 73 % of *Bacillus* which were isolated from Cucurbitaceae produce nucleases (Khalaf, Raizada, 2018). Synthesis of secreted enzymes including RNases, which participate in mobilization of organic phosphates is one of the mechanisms of adaptation to changing environmental conditions. Low concentrations of RNases stimulate plant growth and resistance to a broad spectrum of stress factors, high levels of them show antiviral properties by destroying viral RNA. Microbial RNases are potential therapeutic agents which are suggested for the treatment of human viral diseases (Mahmud et al., 2017). In this regard, the identification of the biological properties of endophytic *Bacillus* spp. is relevant for the development of biological products with complex (antiviral, immunizing and growth stimulating) activity for the environmentally safe protection of potato plants from diseases and pests.

The aim of this work was to evaluate the RNase activity of a number of endophytic bacteria strains from the collection of the Laboratory of Biochemistry of Plant Immunity of the Institute of Biochemistry and Genetics UFRC (http://ibg.anrb.ru/wp-content/uploads/2019/04/Katalog-endofit.doc) and their effect on resistance of potato plants of early-ripening variety Udacha to phytopathogenic viruses (potato virus M) and potato productivity under the field conditions.

Materials and metods

Bacterial strains *B. subtilis* 26D, *B. thuringiensis* var. *thuringiensis* (B-5689) and *B. thuringiensis* var. *kurstaki* (B-5351) courtesy of the limited liability company "Bashincom". Isolates *B. subtilis* Stl-7, *B. subtilis* Stk-18, *B. subtilis* Stk-22 were obtained from surface-sterilized leaves tissues of potato varieties, which were cultivated on the territory of Iglinskiy district of Bashkortostan Republic. Identification of the all strains was carried out through DNA sequencing of 16S RNA gene fragments. Nucleotide sequence analysis was carried out by using international database GenBank. Bacterial culture was growth on Lysogeny broth basal medium (0.5 g/l NaCl) in TS 1/20 thermostat (SPU, Russia) at a temperature 28 °C. 16-hours old cultures were used for endophytic properties, antiviral activity and influence on potato productivity estimation.

Activity of extracellular RNases culture medium was estimated using the method reported by Hole et al. (2004). Strains were growth on LB agar with addition of yeast RNA (6 g/l) (Sigma, USA) at 30 °C. The plates were than incubated at required temperatures until growth was clearly visible (48 h). After incubation the plates were flooded with 3 ml of the precipitant (perchloric acid) and left to stand for 5 min. The plates were then visualized for transparent halos formed around the grown colonies, against an opaque background. RNase activity was shown as the distance between colony edge and halo edge. Quantitative estimation of extracellular RNase activity in liquid cultural medium was carried out using spectrophotometer UNICO 2800 (USA). The absorbance at 260 nm was measured according to (Margulis et al., 2012). Rate of reaction was evaluated as tangent of the slope of the rectilinear ascending part of the curve of light absorption versus reaction time, and expressed as an increase in absorption for 1 min per 1 mg of protein.

Endophyticity of the tested strains was evaluated by counting the colony-forming units (CFU) of microorganisms in plant tissues 7 days after inoculation of sterile test-tube potato plants (Udacha variety) cultivated for 25 days at 16-h illumination (Osram L 36W/77 bulbs, Germany) in the KS200 climate chamber (Smolensk SKTB SPU, Russia) on the agarised Murashige–Skoog medium. For this purpose, 100-mg samples of experimental plants were superficially sterilized in the following order: 70 % ethanol (1 min) → 0.1 % Diacide-1 (3 min) → distilled water. The samples were homogenized in sterile mortars with 2 ml sterile water added. Two consecutive 10-fold dilutions of the resultant homogenate were then performed. Aliquots (100 μL) were spread over the surface of potato-glucose agar by a microbiological loop until complete drying. Petri dishes were then incubated at 28 °C in the TS-1/20 SPU thermostat (Smolensk SKTB SPU, Russia) for 24 h. CFU were counted in second and third dilutions, and their number was recalculated per 1 g of plant wet weight.

The study was carried out at the experimental fields of the Ufa Federal Research Center (Birsk experimental station, 55°24′27″ N, 55°36′39″ E). Fields were located on gray forest soils (northern forest-steppe). The plants of the original Udacha variety under study were planted in three repeats of 30 plants for each line. Three plots were used as replicates for each treatment (*B. subtilis* 26D, *B. thuringiensis* var. *thuringiensis* B-5689, *B. thuringiensis* var. *kurstakii* B-55351) as well as for the untreated control treatment (water-sprayed). 2-weeks seedlings were sprayed with different strains of *Bacillus* suspensions (10^6 cells/plant). Spraying was duplicated after flowering in the same man-

ner. Viral spreading was estimated using diagnostic sets for immune-chromatographic detection of viral particles of X, Y, S, M and PLRV according to the recommendations of manufacturer (LLC Agrodiagnostica, Russia). Phenotypic observations and diagnostics of viral infection were carried out according to methodic instructions (Methodology…, 1995) before flowering. Data on the productivity of potato was prepared according to Dospechov B.A. (1985). The experiments were performed three times in three replicates. Data presented are mean values with standard errors (±SE). Statistical analyses were performed with Microsoft Excel 2013 for Windows (Microsoft Corporation, 2013).

Results

Screening of bacterial strains in collection of the Laboratory of Biochemistry of Plant Immunity of the Institute of Biochemistry and Genetics UFRC RAS and isolates collected from the field population showed the presence of RNase activity in all *Bacillus* strains under investigation (Table 1, Figure). The maximal halo was observed on culture medium of the isolated from potato leaves *B. subtilis* Stl-7, minimal – in *B. thuringiensis* var. *kurstaki* B-5351 medium. Thus, isolated from Colorado potato beetle *Enterobacter* spp. which were earlier identified using specific primers, didn't express any RNase activity (see Table 1).

The significant RNase activity was observed in culture medium of *B. thuringiensis* var. *kurstaki* B-6066, *B. subtilis* Stl-7, *B. subtilis* 26D, *B. thuringiensis* var. *thuringiensis* B-5689 (see the Figure). These measures suggested the usability of the method of Hole et al. (2004) for screening, since the large of halo correlated with enzyme activity in liquid medium.

Bacteria *B. subtilis* 26D and *B. thuringiensis* var. *kurstaki* B-5351 were found in potato plant tissues in the amount of 10^5 CFU/g wet weight. The CFU number of *B. thuringiensis* var. *thuringiensis* B-5689 in potato plant tissues was diminished more than two orders of magnitude. Thus, it was shown that the strains *B. subtilis* 26D and *B. thuringiensis* var. *kurstaki* B-5351 have a greater ability to actively invade and colonize plant tissues as compared to *B. thuringiensis* var. *thuringiensis* B-5689.

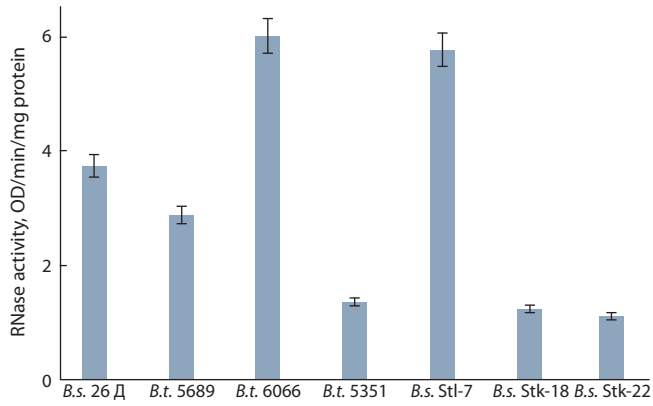
It's worth noting that application with strains, which have high RNase activity and ability to colonize internal plant tissues can promote plant resistance to viral diseases. Thus, the influence of endophytic strains *B. subtilis* 26D, *B. thuringiensis* var. *kurstaki* B-5351 and *B. thuringiensis* var. *thuringiensis* B-5689 on potato plants (Udacha variety) were investigated at the field conditions.

The presence of X, Y, S, M viruses and PLRV was preliminary estimated using immune-chromatographic sets manufactured by LLC Agrodiagnostica (Russia). High incidence of M virus was observed (Table 2). 60 % of plants growing on non-treated plots were infected with M virus. About 14 % on average leaves on each plant showed visible manifestation of disease symptoms.

The lowest number of plants with simptoms of viral desease was detected on the plots which were treated with *B. subtilis* 26D. Treatment with *B. thuringiensis* var. *thuringiensis* B-5689 (low endophytic rate, but high RNase activity) decreased at twice the rate of plants with virose symptoms. Disease severity was no more than 1 % of leaves on each plant.

Table 1. RNase activity *in vitro* of strains from collection

Strains	Halo, mm
B. subtilis 26D	4.5
B. subtilis Stl-7	6.5
B. subtilis Stk-18	3.0
B. subtilis Stk-22	4.5
B. thuringiensis var. *kurstaki* B-6066	4.0
B. thuringiensis var. *thuringiensis* B-5689	5.0
B. thuringiensis var. *kurstaki* B-5351	2.0
Enterobacter sp. BeP	0
Enterobacter sp. m10	0
Enterobacter sp. m9	0



RNase activity of cell filtrate strains of *Bacillus* spp.

B. thuringiensis var. *kurstaki* B-5351 (low RNase activity, but high endophytic rate) prevented the spread of the virus under the same conditions. However, the rate of disease symptoms manifestation in this case was higher than in plots which were treated with strains which displayed high RNase activity *in vitro*. Probably, spreading of infection was limited by through insecticidal properties of *B. thuringiensis* var. *kurstaki* B-5351 (Sorokan et al., 2018).

It's important that *B. subtilis* 26D and *B. thuringiensis* var. *thuringiensis* B-5689 treatment significantly increased potato productivity. It could be attributable to their direct antiviral activity and plant growth-stimulating properties.

Discussion

It is known that many bacteria, especially from the genus *Bacillus*, have a wide range of enzymes with RNase activity, as well as nucleases and other proteins responsible for RNA interference (Aguiar-Pulido et al., 2016). For example, bacteria *B. amyloliquefaciens*, *B. intermedius*, and *B. licheniformis* can produce extracellular ribonucleases called baRNases, binases,

Table 2. Content of CFU of *Bacillus* spp. in internal tissues of potato plants Udacha cv, their virus M infection rates and yield

Parameter	Control (H_2O)	*B. subtilis* 26D	*B. thuringiensis*	
			var. *thuringiensis* B-5689	var. *kurstaki* B-5351
CFU/g (fresh weight)	0	$5 \cdot 10^5$	$5 \cdot 10^3$	$1 \cdot 10^5$
Plants affected by virus M, %	60	18	33	25
Development of the virus M, % of affected leaves/plant	14	1	1	7
Yield, 100 kg/ha	182.45 ± 12.38	241.91 ± 22.67	261.03 ± 23.59	205.58 ± 15.60
Increase, % of control	–	32	43	12

and balifases, respectively (Ulyanova et al., 2011, 2016). Recent studies demonstrated that *B. subtilis* and *B. thuringiensis* contain bacterial RNases effectively inactivate RNA-containing viruses – baRNase and binase (Ulyanova et al., 2011). It has been established that *B. cereus* ZH14 produces a new type of extracellular ribonuclease which are active against tobacco mosaic virus (Zhou, Niu, 2009). Genetic transformation using bacterial RNase gene may be a promising approach for the engineering of plants with resistance to viral infection (Zhang, 2001; Cao et al., 2013). Soil treatment with *Pseudomonas putida* A3 prior to sowing reduced TMV infection in tobacco plants in comparison with the soil treatment with this PGPR after sowing (Guo et al., 2011). PGPR *P. putida* A3 was shown to destroy virus particles in the juice from tobacco leaves infected with TMV (Yang et al., 2012). Thus, in these works it was demonstrated not only the ability of PGPR of the genera *Pseudomonas* to suppress viral infection indirectly by stimulating the nonspecific plant defense mechanisms but also their direct viricidal activity (Guo et al., 2011; Yang et al., 2012). According to our data, strains with high *in vitro* RNase activity significantly reduced the intensity of the development of M virus symptoms on Udacha potato plants in the field compared to strains with low RNase activity.

It was shown that enhanced expression of PR-8 and NPR-1 defense genes contributes significantly to *B. amylolequifaciens*-induced multiple reduction in rhisomania infection caused by the BNYVV virus (Desoignies et al., 2013). The application of *B. subtilis* BS3A25 strain have been found to reduce cucumber mosaic virus (CMV) infection by inhibiting the development of its vector *Aphis gossipi* (Sudhakar et al., 2011).

It is of interest to develop antiviral biocontrol agents based on PGPR isolates having high RNase activity or the preparation of RNase itself to protect plants from viral infection, taking into account that the majority of phytoviruses are RNA viruses (Sharipova et al., 2015) as well as the generation of genetically modified plants expressing RNase genes (Trifonova et al., 2004). It has been found that potato plants expressing *Serratia marcescens* nuclease display enhanced resistance to pathogens (Trifonova et al., 2018).

Strains under investigation also displayed insecticidal effect against Colorado potato beetle (Sorokan et al., 2018) and wheat aphid (Veselova et al., 2019), representing about 20 % from all aphids species which damaged potato plants (Ekaterinskaja et al., 2016) and act as carriers for viral particles. In addition to developing increased resistance against viral infection, bacterial barnases can participate in plant protection against other diseases, for example, tobacco plants from the late blight desease, as evidenced by high resistance of transgenic plants producing barnase (Natsoulis, Boeke, 1991). Earlier we demonstrated that *B. subtilis* 26D effectively decreased disease severity of the late blight (Maksimov et al., 2015), which could contribute to the limitation of virus spreading in potato crops. It is important to note that effective suppression of viral diseases requires the constant presence of antiviral compounds in plant tissues. It makes endophytic microorganisms producing RNases promising viral biocontrol agents. These data can be a basis of approach to protection of plants from viral infection by using the "RNase enhanced" endophytic bacteria *Bacillus* spp., as an antiviral agent.

Conclusion

According to the data obtained, we can say that the use of biocontrol agents based on bacteria of the genus *Bacillus* reduced the natural viral infectious background, which depended on the endophytic properties of strains under investigation and the ability of bacteria to produce extracellular RNases. Thus, we suggest the development of biocontrol agents with complex (antiviral, insecticidal, fungicidal, bactericidal and growth stimulating) activity for environmentally safe system of plant protection from diseases and pests.

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Acknowledgements. The work was carried out by a joint international grant from the Russian Science Foundation and the Department of Science and Technology (DST) of the Government of India No. 19-46-02004.

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

Received June 5, 2019. Revised August 26, 2019. Accepted August 26, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):879-886
DOI 10.18699/VJ19.562

The Fifth International Scientific Conference PlantGen2019

Phytopathological screening and molecular marker analysis of wheat germplasm from Kazakhstan and CIMMYT for resistance to tan spot

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Tan spot caused by the fungus *Pyrenophora tritici-repentis* is an important leaf spot disease in wheat growing areas throughout the world. The study aims to identify wheat germplasm resistant to tan spot based on phytopathological screening and molecular marker analysis. A collection of 64 common wheat germplasms, including cultivars and breeding lines from Kazakhstan and CIMMYT, was assessed for tan spot resistance in greenhouse conditions and characterized using the *Xfcp623* molecular marker, diagnostic for the *Tsn1* gene. All wheat cultivars/lines varied in their reaction to tan spot isolate race 1, ranging from susceptible to resistant. Most accessions studied (53 %) were susceptible to Ptr race 1. Spring wheat cultivars were more susceptible to race 1 than winter wheat cultivars. As a result of genotyping, an insensitive reaction to Ptr ToxA was predicted in 41 wheat cultivars (64 %). The *tsn1* gene carriers identified included 27 Kazakhstani and 14 CIMMYT cultivars/lines, demonstrating insensitivity to Ptr ToxA. The majority of the *Tsn1* genotype were sensitive to race 1 and showed susceptibility to the pathogen in the field. Disease scores from seedling stage positively correlated with field disease ratings. Of particular interest are 27 wheat accessions that demonstrated resistance to spore inoculation by Ptr race 1, were characterized by insensitivity to ToxA and showed field resistance to the pathogen. The results of this study will contribute to wheat breeding programs for tan spot resistance with Marker Assisted Selection using the closely flanking markers.
Key words: wheat; molecular markers; *Pyrenophora tritici-repentis*; tan spot; *Tsn1*; ToxA.

For citation: Kokhmetova A.M., Atishova M.N., Kumarbayeva M.T., Leonova I.N. Phytopathological screening and molecular marker analysis of wheat germplasm from Kazakhstan and CIMMYT for resistance to tan spot. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):879-886. DOI 10.18699/VJ19.562

Фитопатологический скрининг и молекулярный анализ гермоплазмы пшеницы из Казахстана и CIMMYT на устойчивость к пиренофорозу

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Пиренофороз, возбудитель *Pyrenophora tritici-repentis*, является важным заболеванием листовых пятнистостей в регионах выращивания пшеницы по всему миру. Цель исследования – идентифицировать и отобрать гермоплазму пшеницы, устойчивую к пиренофорозу *P. tritici-repentis*, с использованием молекулярных маркеров. Коллекция из 64 образцов мягкой пшеницы, включающая зарегистрированные сорта и элитные селекционные линии пшеницы из Казахстана и CIMMYT, была подвергнута оценке устойчивости к *P. tritici-repentis* в теплице и охарактеризована с помощью молекулярного маркера *Xfcp623*, диагностического для гена *Tsn1*. Все сорта/линии пшеницы различались по реакции на изолят расы 1 Ptr, проявляя широкий спектр реакции – от восприимчивой до устойчивой. Большинство исследованных образцов (53 %) оказались восприимчивы к изоляту расы 1 Ptr. Сорта яровой пшеницы были более восприимчивы к расе 1, чем сорта озимой пшеницы. В результате генотипирования нечувствительная реакция к токсину Ptr ToxA была предсказана у 41 сорта пшеницы (64 %). Идентифицированные носители гена *tsn1* включали 27 казахстанских и 14 сортов/линий CIMMYT, демонстрировавших нечувствительность к Ptr ToxA. Большинство образцов с генотипом *Tsn1* были чувствительны к расе 1 и показали восприимчивость к патогену в полевых условиях. Оценки заболеваемости на стадии проростков положительно коррелировали с оценками в полевых условиях. Особый интерес представляют 27 образцов пшеницы, которые проявили устойчивость к инокуляции спор расы 1

P. tritici-repentis, характеризовались нечувствительностью к токсину ToxA и демонстрировали устойчивость к патогену в полевых условиях. Полученные результаты внесут вклад в программы селекции пшеницы на устойчивость к пиренофорозу на основе Marker Assisted Selection с использованием тесно фланкирующих маркеров.
Ключевые слова: пшеница; молекулярные маркеры; *Pyrenophora tritici-repentis*; пиренофороз; *Tsn1*; ToxA.

Introduction

Ensuring food security is the most important priority of Kazakhstan's economic strategy. Wheat production is limited to a number of biotic stresses, including leaf spot diseases. Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechsler (anamorph), is an important foliar blight disease in temperate and warmer wheat growing areas throughout the world (Duveiller et al., 1998), including Kazakhstan (Koyshibayev, 2018). Under conditions favorable for tan spot development, yield losses can rise beyond 50 % (Rees et al., 1988). This pathogen infects leaf, stem and head tissue resulting in reduced photosynthetic area, increased transpiration and reduced accumulation of organic matter and causes considerable reduction in yield and quality of wheat. Modern industrial farming technologies contribute to the development of the disease: minimal tillage of the soil with the preservation of stubble on its surface, monoculture and cultivation of wheat cultivars with insufficient resistance to pathogen. The infected seeds, plant residues of the previous growing season, infected self-seeding plants and wild cereals susceptible to this disease could serve as a source of infection to winter wheat seedlings in autumn. Integrated disease control strategies, such as the cultivation of resistant varieties, combined with desired crop rotations and management practices, are the most effective, environmentally friendly and cost-effective tools to combat wheat tan spot (Mikhailova et al., 2012).

Inheritance to tan spot resistance has both quantitative and qualitative nature, while the toxins resistance genes and quantitative trait loci (QTL) are race specific and they control the processes that reduce the sensitivity to toxins (Mikhailova et al., 2012; Faris et al., 2013; Liu et al., 2017). To date according to Catalogue of Gene Symbols eight major resistance genes (*Tsr1*, *Tsr2*, *Tsr3*, *Tsr4*, *Tsr5*, *Tsr6*, *TsrHar*, *TsrAri*) located on chromosomes 2BS, 3A, 3BL, 3DS, and 5BL have been identified (McIntosh et al., 2013). Numerous genetic studies performing quantitative trait loci analysis, have reported resistance to tan spot as a polygenic trait however the major racespecific genes frequently underlines these QTLs. Additional QTLs have been identified and located on chromosome arms 1AL, 2AS, 3AS, 4AL, 5AL, 1BS, 2BL, 3BS, 2DS, 2DL, 6A, 7A, and 7DS (Chu et al., 2010; Singh et al., 2010, 2016; Xiao-Chun et al., 2010; Kalia et al., 2018). Recent studies using associative mapping allowed the detection of loci that determine resistance to different Ptr races (Gurung et al., 2011; Kollers et al., 2014; Juliana et al., 2018).

In order to increase effectiveness of wheat breeding subject to resistance to tan spot, it is essential to understand the genetic basis of resistance to the disease. *Pyrenophora tritici-repentis* induces on susceptible cultivars two different symptoms, necrosis and chlorosis. Genetically, both symptoms are under independent host control. Based on the ability to induce necrosis and chlorosis symptoms, 8 races of Ptr were identified (Lamari, Bernier, 1989a; Strelkov, Lamari, 2003). It has been found that the fungus produces a number of host-selective toxins (HSTs) known as Ptr ToxA, Ptr ToxB and Ptr ToxC, etc., which interact directly or indirectly with the products of the dominant plant genes *Tsn1*, *Tsc2* and *Tsc1*, respectively (Ballance et al., 1989). Recent studies on cloning and characterization of the *Tsn1* gene have shown that the pathogen utilizes HST-toxins to weaken resistance mechanisms of the host and cause the disease. However, in addition to host-HST interactions, a wide range of QTLs responsible for race-non-specific resistance and recessively inherited genes of "qualitative" resistance have been identified (Faris, Friesen, 2005; Singh et al., 2016). Molecular markers for the HST resistance genes and for race non-specific QTLs intended for use in marker assisted selection (MAS) have been developed (Faris et al., 2012; https://maswheat.ucdavis.edu/protocols). Diversity Arrays Technology (DArT) WPT-3049 (2.9 cM) and WPT-0289 (4.6 cM) markers were closely linked to Tsr1 and Tsr6, respectively (Singh et al., 2016).

There are a number of studies on the racial composition of *P. tritici-repentis* in Asia and Kazakhstan. The greatest diversity was observed in Azerbaijan, where races 1, 2, 3, 5, 7, and 8 were identified, and in Syria, where races 1, 3, 5, 7, and 8 were detected; the little variation was found in the virulence of isolates from Kazakhstan (race 1 and race 2) (Lamari et al., 2005). It has been revealed that race 1 is the most widespread race in Central Asia and Kazakhstan (87 %), and races 2, 3 and 4 were minor (Zhanarbekova et al., 2005; Maraite et al., 2006). It was found that races 1 and 2 dominate in the North Caucasus region of Russia, and races 1 and 8 *P. tritici-repentis* dominate in Kazakhstan (Kokhmetova et al., 2016). Studies on the racial composition of *P. tritici-repentis* in Kazakhstan indicate the necessity to identify the wheat germplasm resistant to the prevailing races of the disease among promising lines and wheat varieties cultivated in Kazakhstan. The results of previous studies indicate the possibility of postulating recessive alleles of genes for resistance to *P. tritici-repentis* toxins using molecular markers (Kokhmetova et al., 2017, 2018).

Conventional phytopathological methods are not always effective for identification of pathogen resistance genes. The situation is complicated by the fact that different leaf spot pathogens occur together in the field, which make more difficult disease evaluation. In this case, the use of molecular markers associated with disease resistance will be effective for identification of disease resistance factors. The presence of effective molecular markers closely linked to the genes of resistance to toxins makes it possible to conduct molecular screening of wheat breeding material. The main objectives of this study were (i) phytopathological and molecular screening elite cultivars and wheat breeding lines for resistance to tan spot, (ii) identification of resistance sources effective against tan spot.

Materials and methods

A collection of 64 common wheat germplasms, including 46 registered cultivars and elite wheat breeding lines from Ka-

Table 1. *Pyrenophora tritici-repentis* isolate (29A-11) served as representative of race 1

Isolate	Glenlea	6B-662	6B-365	Race*	Source	Location
29A-11	N (ToxA)	R	C (ToxC)	1 (nec+chl+)	Winter wheat	Almaty region of Kazakhstan

* Based on Lamari et al., 2005: N (ToxA) – disease inducing factor (Ptr ToxA), causing necrosis in Glenlea; C (ToxC) – disease inducing factor (Ptr ToxC), causing chlorosis in 6B-365; R – stand for resistance to toxins of *P. tritici-repentis*; nec+ – presence of necrosis; chl+ – presence of chlorosis.

zakhstan and 18 entries from CIMMYT was used in the work (see Table 2). Evaluation of the resistance to *P. tritici-repentis* on adult plant stage was carried out under field conditions of Southeast Kazakhstan, Kazakh Research Institute of Agriculture and Plant Growing (KRIAPG), Almalybak (43°13′ N, 76°36′ E, and 789 m asl), Almaty region, in the 2017–2018 crop seasons. The experiment was conducted in a completely randomized design with three replications. The field evaluation (from naturally ocurring infections) to tan spot resistance was assessed three times according to the scale for appraising the foliar intensity of diseases (Saari, Prescott, 1975) in the modification for tan spot (Kremneva, Volkova, 2007).

The standard wheat differentials included Glenlea and Salamouni cultivars, as well as 6B662 and 6B365 lines. Seedling resistance of the wheat cultivars were assayed in the greenhouse conditions at the two-leaf seedling stage. Three seedlings of each differential line and tested wheat cultivars were produced in plastic cones filled with soil and grown in the greenhouse at an average temperature of 21 °C with a 16-h photoperiod. The seedlings were inoculated with spore suspension with 4,000 spores per ml of each isolate individually until run off. Inoculated seedlings were moved to a mist chamber at 21 °C with a 16-h photoperiod for 24 h. Thereafter, the plants were moved to a growth chamber at 22 °C with a 16-h photoperiod for 24 h (light for 16 h and darkness for 8 h). The plants were rated for disease, using rating system based on lesion type; 1–2 represent resistance, and 3–5 represent susceptibility (Lamari, Bernier, 1989a).

The samples of *P. tritici-repentis* were randomly collected from hexaploid wheat in Almaty region of Kazakhstan. The Petri dishes with leaf pieces with lesions were incubated at 20 °C for 12–18 h under fluorescent lights (~80 μmol·m^{-2}·s^{-1}), followed by an additional 12–18 h in the dark at 15 °C (Lamari et al., 1995). Two to three isolates from each field were tested. All *P. tritici-repentis* isolates were typed by their respective race through inoculating them individually on the wheat differential set developed by Lamari, Bernier (1989b) and Strelkov, Lamari (2003). It was found, that *P. tritici-repentis* isolate 29A-11 was related to race 1, since it was produced necrosis on Glenlea and chlorosis on 6B-365 and possible characterized by the production of two toxins: ToxA and ToxC (Table 1).

P. tritici-repentis isolate 29A-11, representing race 1 was used for inoculation. Culture of *P. tritici-repentis* isolate 29A-18 race 1 was grown on V4 agar (150 ml V4 juice in a ratio of 4:3:2:1 parts beet juice, parsley, tomato and carrot, respectively, 20 g agar, 1.5 g $CaCO_3$ and 850 ml distilled water) in the dark at 20–22 °C for 6 days (Mikhailova et al., 2012). The plates were filled with sterile distilled water; the mycelium flattened the base of a sterile test tube and excess water poured off. To induce conidiophore production, the plates were incubated under continuous light at room temperature for two days followed by 1 day in the dark in an incubator at 16 °C to induce conidia production. The plates were filled with distilled water and the conidia were suspended in the distilled water by gentle brushing the mycelium to dislodge the conidia from the conidiophores. In order to reduce surface tension 2–3 drops of Tween 20 were added per liter of spore suspension. Spore concentration was measured and adjusted to 4,000 spores per ml. Wheat accessions were screened for toxin ToxA reaction. Plants were infiltrated at the second leaf stage as desciebed in Liu et al. (2006) and were scored as sensitive or insensitive 3 days later based on presence or absence of necrosis, respectively.

Genomic DNA was extracted at two-leaf seedling stage for each individual plant using the CTAB method (Riede, Anderson, 1996). DNA concentration was measured using a spectrophotometer SmartSpecTMPlus (BioRAD). The DNA concentration for each sample was adjusted to 30 ng/μl. Samples were genotyped using the SSR marker *Xfcp623* designed to detect alleles of the *Tsn1* gene. The primer sequence and PCR conditions are given by Faris et al. (2010). The amplification products were separated on 2%-agarose gels, to determine the length of the amplification fragment 100 bp DNA Ladder (Ferments, Lithuania) was used. Gels were visualized on GelDoc BIO-PRINT MEGA for documentation of allele types in cultivars. Wheat entries 6B662 and Glenlea served as positive and negative controls, respectively.

Results

Wheat germplasm reaction to race 1 of *Pyrenophora tritici-repentis*. Seedlings and adult plant response of wheat germplasm to *P. tritici-repentis* are presented in Table 2.

The reaction of wheat cultivars and lines representing the range of lesion types to race 1 using rating system (Lamari, Bernier, 1989a) based on two phenotypically distinct symptoms: tan necrosis (N) and chlorosis (C) was carried out. Evaluation to race 1 showed that lesion type varied greately amongst wheat cultivars. It was found that 30 entries out of 64 (46.9 %) had average disease reaction type, less than 2 and considered as resistant to this isolate (see Table 2). A type of symptom consisting of small dark spots without any surrounding chlorosis or tan necrosis (rating 1–2, R) was observed in about 19 % of the accessions tested. A type symptom consisting of minute dark spots with very little chlorosis or tan necrosis (rating 2, R-N), showed 19 (30.6 %) accessions. The chlorosis or tan necrosis symptoms (rating 3–4, S-N, S-C, S-NC), were observed in 34 (55.1 %) of the accessions tested.

Fifteen of the 64 entries were sensitive to production of chlorosis and this indicate that they may be sensitive to Ptr ToxB or Ptr ToxC, but resistant to Ptr ToxA. Relatively large proportion of resistant cultivars was presented in entries from CIMMYT (72.22 %). The number of Kazakhstani samples resistant to race 1 was significantly less (36.96 %). So, the majority of studied Kazakhstani and CIMMYT entries were

Table 2. The reaction of wheat accessions to race 1 *P. tritici-repentis* and the allelic state of the *Tsn1* gene

Accession	Origin	Growth habit	Response to race 1		Response to Ptr ToxA	Allelic state of molecular marker	Field evaluation Ptr, %
			Lesion type	Reaction			
Sapaly	KZ	Winter	3	S-C	I	–	10
Reke	KZ		2	R-N	I	–	15
Taza	KZ		2	R	I	–	10
Zhenis	KZ	Spring	2	R-N	I	–	10
Karabalykskaya 101	KZ		3	S-N	I	–	5
Akmola 40	KZ		4	S-N	I	–	5
Celinnaya Jubileynaya	KZ		3	S-C	I	–	10
Kazakstanskaya 3	KZ		4	S-N	S	–	15
Shortandinskaya 2007	KZ		3	S-N	I	–	10
Ishimskaya 92	KZ		2	R	I	–	0
Karagandinskaya 22	KZ		2	R-N	I	–	10
Karabalykskaya 90	KZ		3	S-N	I	–	15
Kazakstanskaya 19	KZ		3	S-N	I	–	10
Kazakstanskaya 20	KZ		3	S-N	I	–	10
Celinnaya 90	KZ		2	R-N	I	–	10
Kazakstanskaya 15	KZ		2	R-N	S	–	15
Pavlodarskaya 93	KZ		3	S-NC	I	–	15
Kargaly 9	KZ		2	R	I	–	0
Express	KZ		2	R-N	I	–	10
KP33	KZ	Winter	1	R-N	I	–	10
KP34	KZ		2	R	I	–	5
KP36	KZ		1	R	I	–	5
KSI6	KZ		1	R	I	–	5
KSI 9/374	KZ		2	R-N	I	–	10
KSI 16	KZ		1	R-N	I	–	10
KSI 17	KZ		2	R-N	I	–	5
Bogarnaya 56	KZ		4	S-NC	S	+	35
Mereke 70	KZ		3	S-N	I	+	50
Raminal	KZ		4	S-N	I	+	35
Nureke	KZ		4	S-C	S	+	50
Tungysh	KZ		2	R-N	S	+	50
Aray	KZ		4	S-N	S	+	35
Alem	KZ		4	S-N	S	+	35
Progress	KZ		3	S-N	S	+	50
Zhalyn	KZ		3	S-N	S	+	35
Karaspan	KZ		4	S-NC	S	+	30
Kazakstanskaya 4	KZ		4	S-NC	S	+	35
Anara	KZ		3	S-N	S	+	35
Akbidai	KZ		4	S-NC	S	+	50
Rausin	KZ		3	S-N	S	+	35
Zhadyra	KZ		3	S-N	S	+	35
KP 4	KZ		3	S-N	S	+	35
KP 18	KZ		3	S-NC	S	+	30
KP 35	KZ		3	S-N	S	+	50
Celinnaya 26	KZ	Spring	2	R-N	I	+	30
Akmola 2	KZ		4	S-NC	S	+	35
Bobwhite 04	CIMMYT		2	R-N	S	+	15

Table 2 (end)

Accession	Origin	Growth habit	Response to race 1		Response to Ptr ToxA	Allelic state of molecular marker	Field evaluation Ptr, %
			Lesion type	Reaction			
F133/SHA5//OPATA	CIMMYT	Winter	2	R-N	S	+	25
BR35/BR14	CIMMYT		1	R-N	I	–	20
F3.71/TRM/VORONA/3/OC14	CIMMYT		1	R-N	I	–	10
CEP80111/VEE	CIMMYT		1	R-N	I	–	15
TPAP#1/OPATA	CIMMYT		3	S-NC	I	–	10
P83-5112/V82274	CIMMYT		3	S-NC	S	+	0
JAC161/TEMU51.80	CIMMYT		1	R	I	–	0
CATBIRD	CIMMYT	Spring	1	R	I	–	20
GAN/AE.437SOVARROSA	CIMMYT	Winter	1	R-N	I	–	25
GAN/AE (408)	CIMMYT		1	R	I	–	15
EG, AUS/H 567.71//4* EG AVS/3/2	CIMMYT		4	S-NC	S	+	25
EFED/F5.83 7792 (BAJAS)	CIMMYT		3	S-NC	I	–	20
L.A.CJAT(SANTACARUS)	CIMMYT		3	S-NC	I	–	0
TALHUENJNJA	CIMMYT		1	R-N	I	–	15
EFED/22150	CIMMYT		1	R	I	–	15
TOO11/TOOOO7	CIMMYT		1	R	I	–	20
RECURRENT SELECTION 1	CIMMYT		1	R	I	–	25
Salomouni	Egypt	Spring	1	R	I	–	0
Glenlea	Canada		4	S-N	S	+	40

Note: *Xfcp623* is the SSR marker to the *Tsn1* locus; "+" indicates sensitive to Ptr ToxA samples, contain *Tsn1* allele, 380 bp DNA fragment; "–" indicates insensitive to Ptr ToxA samples, contain *tsn1*, null allele; Salamouni, the insensitive control for race 1 and toxin Ptr ToxA, carrier of the ressive gene *tsn1*; Glenlea, the susceptible control for race 1 and Ptr ToxA, carrier of the dominant *Tsn1*; 1–5 are the lesion type rating based on Lamari and Bernier (1989a) scale; 1, 2 – indicates resistance, and 3–5 – susceptibility; the reaction to Ptr ToxA: I, insensitivity, S, sensitivity to Ptr ToxA.

susceptible to isolate 29A-18 related to race 1. In general, a higher number of spring wheat cultivars exhibited suceptibility to race 1 as compared to winter wheat cultivars.

Field evaluation to tan spot. The results of the field evaluation (from naturally ocurring infections) to tan spot resistance showed that the severity level to *P. tritici-repentis* varied widely, from 0 to 50 % (see Table 2, Fig. 1). In the field, considerable disease (>30 %) developed on plants of 21 cultivars (32.8 %). High level of lesion development from naturally ocurring infections was observed in cultivars Mereke 70, Nureke, Progress, Akbidai and KP 35. Field resistance (<15 %) was observed in 37 (57.8 %) of the accessions tested. There was a tendency towards lower rating in the field, most cultivar had similar reactions when tested in the greenhouse and in the field.

Genotyping of wheat accessions with *Xfcp623* marker. The wheat cultivars were genotyped with *Xfcp623* marker to predict reaction to the Ptr ToxA. The marker of *Xfcp623* has two alleles: in the presence of *Tsn1* allele (Ptr ToxA sensitivity) in the genome, 380 bp fragment is amplified, the absence of a fragment of amplification (null-allele) suggests the presence of the recessive *tsn1* allele (Ptr ToxA insensitivity). The results of genotyping of wheat cultivars and lines with the *Xfcp623* marker are presented in Table 2. As an example, the results of the PCR amplification with *Xfcp623* marker on 16 wheat genotypes, are shown in the Figure 2. According to Figure 2, seven out of 16 entries (Bogarnaya 56, KP 18, Kazakstanskaya 4, Akmola 2, KP 4, P83-5112/V82274 and Anara) had

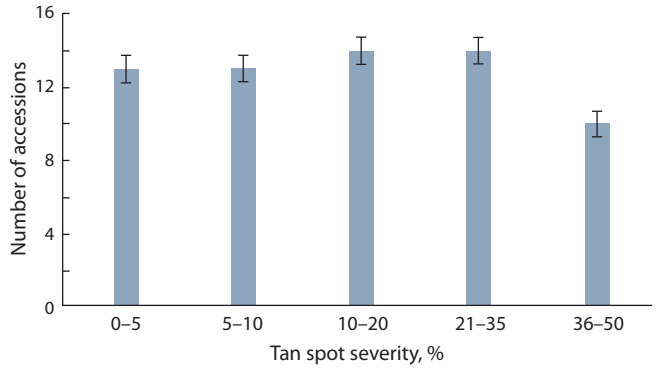

Fig. 1. Frequency distribution of wheat accession for severity to *P. tritici-repentis*, field evaluation, Almaty region.

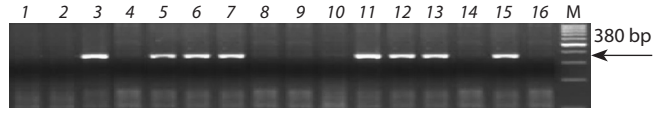

Fig. 2. DNA amplification profile for wheat cultivars and elite lines obtained with *Xfcp623* marker.

Lane: *1*, KSI 16; *2*, GAN/AE (408); *3*, Bogarnaya 56; *4*, KP 17; *5*, KP 18; *6*, Kazakstanskaya 4; *7*, Akmola 2; *8*, Taza; *9*, Kargaly 9; *10*, EFED/22150; *11*, KP 4; *12*, P83-5112/V82274; *13*, Anara; *14*, KP33; *15*, Glenlea (the sensitive control for race 1 and toxin Ptr ToxA, carrier of the dominant gene *Tsn1)*; *16*, Salamouni (the insensitive control for race 1 and toxin Ptr ToxA, carrier of the ressive gene *tsn1)*; M, DNA Ladder.

380 bp fragment, which allows us to postulate the presence of the dominant *Tsn1* allele conferring toxin Ptr ToxA sensitivity. Seven entries including KSI 16, GAN/AE (408), KP 17, Taza, Kargaly 9, EFED/22150 and KP33 showed a lack of amplification (null-allele) and suggests that these samples contain recessive *tsn1* allele conferring toxin Ptr ToxA insensitivity.

The results of genotyping showed that the frequency of *Tsn1* allele was 37.5 % (24 cultivars of 64 analyzed). Analysis of molecular and phytopathological data shows that carriers of the *tsn1* gene characterized by high field resistance with severity level ranging from 0 to 15 %. The carriers of the *Tsn1* gene, showed in the field susceptibility to the disease comprised at 30–45 %.

Twenty-seven wheat entries are of the greatest interest since they demonstrated resistance to the seedling inoculation by the race 1 of *P. tritici-repentis*, showed resistance to the pathogen in the field and were Ptr ToxA insensitive. This set of accessions includes 16 cultivars and lines from Kazakhstan (Reke, Taza, Zhenis, Ishimskaya 92, Karagandinskaya 22, Karabalykskaya 90, Celinnaya 90, Kargaly 9, Express, KP33, KP34, KP36, KSI6, KSI 9/374, KSI 16, KSI 17) and 11 CIMMYT lines (BR35/BR14, F3.71/TRM/VORONA/3/OC14, CEP80111/VEE, JAC161/TEMU51.80, CATBIRD, GAN/AE.437SOVARROSA, GAN/AE (408), TALHUENJNJA, EFED/22150, TOO11/TOOOO7, RECURRENT SELECTION 1).

Discussion

Ptr ToxA toxin is known to be one of the main factors associated with the development of *P. tritici-repentis* in susceptible wheat genotypes (Friesen et al., 2006). The new *P. tritici-repentis* races are emerging through natural selection, therefore development of new resistance sources is imperative (Ali et al., 2010). Several reports indicate the global prevalence of race 1 (Singh et al., 2010; Abdullah et al., 2017a). Since it was previously shown that the race 1 is the most prevalent race in Kazakhstan (Zhanarbekova et al., 2005; Maraite et al., 2006; Kokhmetova et al., 2016), in the present study, we searched for carriers of resistance to race 1 and Ptr ToxA toxin among wheat germplasm. The particular value of the experiment is the breeding material, which was developed and selected in Kazakhstan, as well as in CIMMYT. This material is representing different genetic background, including carriers of resistance to tan spot.

Molecular markers *Xfcp393*, *Xfcp394* and *Xfcp623* were developed as diagnostics for detection of insensitivity to Ptr ToxA and Sn ToxA (Zhang et al., 2009; Faris et al., 2010). On the basis of sequencing the marker *Xfcp623* was proposed as diagnostic for *Tsn1* gene. It was located in intron 5 of the locus in position 4901…5280 (Faris et al., 2010). The reliability of the diagnostic marker *Xfcp623* for identifying wheat genotypes with resistance to the fungus and insensitivity to Ptr ToxA was shown in some studies (Karelov et al., 2015; Kokhmetova et al., 2017, 2018; Mironenko et al., 2017). Taking into consideration the higher efficiency of the *Xfcp623* marker, wheat germplasm in our study were genotyped with this marker. In this study, the frequency of *tsn1* allele of the marker *Xfcp623* for Ptr ToxA insensitivity was 62.5 %. Sensitive reaction to the Ptr ToxA was predicted for the 20 remainder of the tested genotypes (37.5 %).

The majority of studied cultivars, 53.1 % were susceptible to isolate 29A-18 related to race 1. In general, the most part of spring wheat cultivars exhibited susceptibility to race 1 as compared to winter wheat cultivars. The same response to race 1 in a set of spring and winter wheat cultivars was observed by Abdullah et al. (2017b), who indicated that the high resistance of winter wheat to race 1 tan spot minimizes their role in establishing race 1 in the region.

Disease scores from seedling stage, assessed herein, positively correlated with field disease ratings. The most of the wheat entries with the *Tsn1* genotype (90.5 %) were sensitive to race 1 and showed susceptibility to the pathogen in the field. The exception was 2 varieties (Tungysh and Celinnaya 26), which showed an insensitive reaction to the race 1 of the fungus.

Among the studied wheat material 59 accessions (92.2 %) exhibited "sensitive" or "insensitive" alleles at marker loci *Xfcp623* in Ptr ToxA sensitive and insensitive accessions, respectively. In other words, no recombination was observed within the segment harboring the marker *Xfcp623* and *Tsn1* among these 59 accessions. Only 5 genotypes had recombination events between *Xfcp623* and *Tsn1*. In the cultivars Kazakstanskaya 3 and Kazakstanskaya 15 the sensitivity to Ptr ToxA and the presence of resistance gene *tsn1* was observed. The cultivars Mereke 70, Raminal and Celinnaya 26 showed the insensitivity to Ptr ToxA, but characterized by the presence of susceptible gene *Tsn1*. Apparently, recombinations are possible in the segment harboring the *Xfcp623* and *Tsn1* marker among these wheat cultivars.

The results of our study are in agreement with a previous research, suggesting that ToxA is not the major determinant in tan spot disease development in some host backgrounds and indicates the presence of additional effectors (Oliver et al., 2014; Rybak et al., 2017; See et al., 2018). The ToxA–*Tsn1* interaction alone is not a prerequisite for pathogenicity of race 1 Ptr isolates, and pathologists have started to recognize that race 1 Ptr isolates harbour additional uncharacterized effectors in addition to ToxA and ToxC (Manning, Ciuffetti, 2015). Ptr interacts with the host in a complex and intricate manner, leading to a variety of disease reactions that are dependent or independent of the ToxA–*Tsn1* interaction (See et al., 2018). ToxA is found ubiquitously in Australian Ptr isolates and the removal of ToxA sensitivity gene from wheat has been shown to have no effect on yield penalty (Oliver et al., 2014). Although the removal of the *ToxA* gene in Ptr does not severely impede the ability of the pathogen to infect in all varieties, the absence of the *Tsn1* gene in the wheat germplasm does generally improve resistance to tan spot disease (See et al., 2018).

Conclusion

In summary, the results presented in this study indicate that it is necessary to continue breeding for development of carriers of *tsn1* gene insensitive to Ptr ToxA toxin. The obtained results will contribute to wheat breeding by pyramiding Ptr ToxA insensitivity genes using the closely linked marker *Xfcp623* into the desired germplasm. The group of disease resistance germplasm identified in this study can be utilized to develop cultivars with broad-genetic base durable resistance to wheat tan spot.

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Acknowledgements. This study was supported by the Kazakhstan Ministry of Education and Sciences, project AP05132540.

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

Received August 22, 2019. Revised September 16, 2019. Accepted September 23, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):887-895
DOI 10.18699/VJ19.563

The Fifth International Scientific Conference PlantGen2019

Identification of QTLs for resistance to leaf and stem rusts in bread wheat (*Triticum aestivum* L.) using a mapping population of 'Pamyati Azieva × Paragon'

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Leaf rust (LR) and stem rust (SR) are harmful fungal diseases of bread wheat (*Triticum aestivum* L.). The purpose of this study was to identify QTLs for resistance to LR and SR that are effective in two wheat-growing regions of Kazakhstan. To accomplish this task, a population of recombinant inbred lines (RILs) of 'Pamyati Azieva × Paragon' was grown in the northern and southeastern parts of Kazakhstan, phenotyped for LR/SR severities, and analyzed for key yield components. The study revealed a negative correlation between disease severity and plant productivity in both areas. The mapping population was genotyped using a 20,000 Illumina SNP array. A total of 4595 polymorphic SNP markers were further selected for linkage analysis after filtering based on missing data percentage and segregation distortion. Windows QTL Cartographer was applied to identify QTLs associated with LR and SR resistances in the RIL mapping population studied. Two QTLs for LR resistance and eight for SR resistance were found in the north, and the genetic positions of eight of them have matched the positions of the known *Lr* and *Sr* genes, while two QTLs for SR were novel. In the southeast, eight QTLs for LR and one for SR were identified in total. The study is an initial step of the genetic mapping of LR and SR resistance loci of bread wheat in Kazakhstan. Field trials in two areas of the country and the genotyping of the selected mapping population have allowed identification of key QTLs that will be effective in regional breeding projects for better bread wheat productivity.
Key words: bread wheat; linkage mapping; recombinant inbred lines; qualitative trait loci; leaf rust; stem rust.

For citation: Genievskaya Y., Fedorenko Y., Sarbayev A., Amalova A., Abugalieva S., Griffiths S., Turuspekov Y. Identification of QTLs for resistance to leaf and stem rusts in bread wheat (*Triticum aestivum* L.) using a mapping population of 'Pamyati Azieva × Paragon'. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019; 23(7):887-895. DOI 10.18699/VJ19.563

Идентификация локусов количественных признаков устойчивости к листовой и стеблевой ржавчинам мягкой пшеницы (*Triticum aestivum* L.) с использованием картирующей популяции Памяти Азиева × Парагон

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Листовая и стеблевая ржавчины являются вредоносными грибными болезнями мягкой пшеницы (*Triticum aestivum* L.). Целью данного исследования была идентификация локусов количественных признаков (ЛКП), связанных с устойчивостью к болезням, в двух регионах возделывания пшеницы в Казахстане. Для этого картирующая популяция Памяти Азиева × Парагон, состоящая из 98 рекомбинантно-инбредных линий и выращиваемая на севере и юго-востоке Казахстана, была фенотипирована по степени поражения листовой и стеблевой ржавчиной и проанализирована по ключевым компонентам урожайности. Выявлена отрицательная корреляция между степенью поражения болезнью и продуктивностью растений в обоих регионах. Картирующая популяция была генотипирована с использованием ДНК микрочипа Illumina на 20000 маркеров ОНП (однонуклеотидный полиморфизм). Для дальнейшего анализа отобрано 4595 полиморфных маркеров ОНП. Для идентификации

ЛКП, связанных с устойчивостью картирующей популяции к листовой и стеблевой ржавчинам, использовалась программа Windows QTL Cartographer v2.5. В северном регионе были обнаружены два ЛКП устойчивости к листовой ржавчине и восемь ЛКП – к стеблевой. Локализации восьми из них совпали с позициями известных генов *Lr* и *Sr*. Два ЛКП для стеблевой ржавчины были обозначены как новые. Для юго-восточного региона обнаружено восемь ЛКП, ассоциированных с устойчивостью к листовой ржавчине, и один – к стеблевой. Настоящее исследование является первым шагом в генетическом картировании локусов устойчивости к листовой и стеблевой ржавчинам мягкой пшеницы в Казахстане. Полевые исследования в двух регионах страны и генотипирование выбранной картирующей популяции позволили выявить ключевые ЛКП, которые будут эффективны в региональных селекционных проектах, направленных на улучшение продуктивности мягкой пшеницы.
Ключевые слова: мягкая пшеница; картирование; рекомбинантно-инбредные линии; локусы количественных признаков; листовая ржавчина; стеблевая ржавчина.

Introduction

Wheat is one of the most important cereal crops in the World and Kazakhstan (http://www.fao.org). In Kazakhstan wheat is grown on about 13 million hectares annually. The country produces up to 20–25 million tons of bread wheat per year, and exports up to 5–7 million tons of the grain (http://stat.gov.kz). However, an annual infection of bread wheat by fungal diseases is causing a serious yield reduction (Koyshybaev et al., 2017).

The three most common wheat fungal pathogens in the world are *Puccinia triticina* Erikss. (leaf rust), *Puccinia graminis* Pers. f. sp. *tritici* Erikss. & Henn. (stem rust), and *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (stripe or yellow rust) (Bushnell, Roelfs, 1984). *P. recondita* is now recognized as one of the most dangerous pathogens in wheat production worldwide, causing significant yield losses over the large geographical areas (Bolton et al., 2008). The infection with any rust fungus results in decreased numbers of kernels per spike and lower kernel weights due to the parasitic consumption of host nutrients, which leads to apparent yield losses and poor quality of the grains (Afzal et al., 2008).

In Kazakhstan, leaf rust (LR) and stem rust (SR) together cause the most severe yield losses in bread wheat (Rsaliev et al., 2005). When the epidemic develops at the early stage, and the infection persists until wheat is fully ripe, the yield loss increases up to 40–60 % (Koyshybaev, 2010). It happens because of the favorable climate conditions for the spreading of *P. recondita* in the fields, especially in south and south-east of Kazakhstan, where the high temperature and water deficiency stimulate the expansion of spores (Koyshybaev, 2010). As for the SR, the constantly widening areal of aggressive stem rust race Ug99 creates a threat to the food security of the entire planet (Singh et al., 2011; Bhardwaj et al., 2014), including Kazakhstan (Shamanin et al., 2010; Rsaliev, 2011). With epiphytotic SR development, the yield losses of spring wheat can potentially reach 40–50 % (Koyshybaev, 2010; Soko et al., 2018).

One of the most effective ways to protect wheat from LR and SR is the development of resistant cultivars with high yield potential (Ellis et al., 2014). In the last 100 years, approximately 80 LR resistance genes designated from *Lr1* to *Lr78*, *Lrac104*, and *Lrac124*, have been identified and described in common wheat, durum wheat and diploid wheat species (McIntosh et al., 1998, 2007, 2017). In the last 10 years in Kazakhstan, there were active research works on the identification of genes, which are effective against LR, screening of wheat cultivars for the presence of resistance gene (Kokhmetova et al., 2009; Akhmetova et al., 2015) and investigation on population of *P. recondita* in the country and neighboring territories (Agabaeva, Rsaliev, 2013; Gultyaeva et al., 2018).

As for the SR, to date, nearly 60 *Sr* genes have been identified in wheat and its wild relatives (McIntosh et al., 2017). Almost all of the wheat cultivars approved for use on the territory of Kazakhstan demonstrate poor resistance to SR pathogens (Koyshybaev et al., 2017). For this reason, the analysis of SR and methods of its prevention in Kazakhstan are an important issue and require comprehensive genetic and breeding studies. Several experiments were conducted to search SR resistance sources in wheat germplasm of Kazakhstan (Rsaliev, 2011; Kokhmetova, Atishova, 2012). However, no efforts were done to identify effective genes and quantitative trait loci (QTL) based on genetic mapping approach. Genetic mapping is an effective tool for the identification of QTLs that are responsible for natural phenotypic variations in complex traits, such as resistance to rust diseases (Goutam et al., 2015; Xu et al., 2017). During the past two decades, linkage mapping has been commonly used in various plant species, numerous wheat dense genetic maps were developed (Yang et al., 2017), and a large number of QTL have been cloned or tagged (Price, 2006).

The purpose of this study was the identification of QTL for LR and SR resistance by using 98 recombinant inbred lines (RILs) of 'Pamyati Azieva × Paragon' mapping population (MP). As these lines were tested in environmental conditions of North and South-East Kazakhstan, it was expected that important insights of the genetic control for two types of rust disease resistance in bread wheat will be revealed. This work is a continuation of our recent studies of bread wheat undertaken in our research organization (Turuspekov et al., 2017a, b).

Materials and methods

'Pamyati Azieva × Paragon' mapping population. The MP comprising of 98 F_8 RILs was assembled via crossing between two spring wheat cultivars – 'Pamyati Azieva' (PA) and 'Paragon' (P). These two cultivars were chosen because of their different genetic background and differences in morphological traits. The first parental cultivar is Russian medium-early spring wheat cultivar 'Pamyati Azieva' recommended for the Western Siberian region (https://reestr.gossort.com), approved for commercial cultivation in the North Kazakhstan (http://www.goscomsort.kz/index.php/ru), and susceptible to LR and SR. The second parental cultivar was a modern UK elite spring wheat cultivar 'Paragon' that was used as a key parent for Wheat Genetic Improvement Programme (http://www.wgin.org.uk) resources but poorly studied for the resistance to LR and SR. The MP, as well as the genetic

map, was developed within ADAPTAWHEAT project in greenhouse conditions by using facilities of the John Innes Centre (Norwich, UK) during 2011–2015 (https://www.jic.ac.uk/adaptawheat).

Evaluation of the MP for variation in agronomic traits, and LR/SR severity in South-East and North Kazakhstan. Field evaluations of the MP were conducted in North Kazakhstan agricultural experimental station (North Kazakhstan region) and Kazakh Research Institute of Agriculture and Plant Industry (South-East Kazakhstan, Almaty region). Ninety-eight RILs, the parental cultivars ('Pamyati Azieva' and 'Paragon'), and standard check cultivars ('Astana' and 'Omskaya 35' in the North, and 'Kazakhstanskaya 4' and 'Kazakhstanskaya rannespelaya' in the South-East) were evaluated in 2018 under field conditions for resistance to LR and SR, as well as for key adaptation traits and yield components. The population was planted at each site in randomized triplicated experiments. Plants were grown in 15 cm distance between rows and 5 cm distance between plants within a row. Each row contained 25 plants. In the field conditions the MP was tested using 11 traits, including HT (heading time), MT (seed maturation time), PH (plant height), PL (peduncle length), SL (spike length), NPS (number of productive spikes per plant), NKS (number of kernels per spike), WKS (weight of kernels per spike), TKW (thousand kernels weight), WKP (weight of kernels per plant), YSM (yield per square meter).

Evaluation of rusts resistance in both locations was conducted in two randomized replicates with a natural source of infection. LR and SR resistance was evaluated on two growth stages – phase of grain formation on 75 of Zadoks scale and at the beginning of grain ripening on 83 of Zadoks scale (Zadoks et al., 1974). Averaged values for both diseases in two regions were calculated. Field infection response of the test materials was assessed visually. In both regions assessment of resistance/susceptibility levels was performed using the scale of Stakman (Stakman et al., 1962) for SR, the scale of Mains and Jackson (Mains, Jackson, 1926) for LR. The severity of rust infection on leaf and stem surfaces was assessed using the modified Cobb scale (Peterson et al., 1948; Roelfs et al., 1992). To meet the data format required for association analysis, the conventional scale was converted to the 0–9 linear disease scale described by Zhang and co-authors (Zhang et al., 2011). Pearson correlation analysis between agronomic traits and SR/LR severity was performed using the R statistical platform (https://www.r-project.org).

Linkage mapping and QTL analysis. Genomic DNA for 98 RILs and their parental cultivars was extracted from seedlings using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle J.J., Doyle J.L., 1990) and genotyped with the Illumina's iSelect 20K SNP array at the TraitGenetics Company (TraitGenetics GmbH, Gatersleben, Germany). MapChart v2.32 software was used to draw the genetic map (Voorrips, 2002). Each repetition experiments and their average results for studied traits in each environment were used for QTL analysis. For analysis of QTL, the Windows QTL Cartographer v2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) with composite interval mapping (CIM) method at a logarithm of the odds (LOD) for the threshold of 3.0 was used.

Results

Phenotypic variations of resistance to stem and leaf rusts in two environmental conditions. Generally, mean values of SR and LR severity of two parental cultivars and 98 RILs in two regions demonstrated non-equal distribution with deviations towards resistance in the North and susceptibility in the South-East for both diseases (Fig. 1). Out of 98 RILs, fourteen lines were recognized as fully resistant to SR (1 point), 67 lines as moderate resistant on the level of 2–3 points, and only one line was determined as susceptible with 8 points of infection severity (see Fig. 1, *a*). In the South-East area, the severity of SR infection at the stage of grain ripening is higher and less diverse than in the North. SR scores of RILs at the adult plant stage were not normally distributed and were strongly skewed towards susceptibility. Here, 89 lines were affected by stem rust on the level of 8 points, with no lines identified as resistant (see Fig. 1, *a*).

As per the LR resistance, parents and lines of the MP grown in North Kazakhstan had demonstrated clear evidence of infection at the phase of grain ripening. The majority of RILs (81 lines) was identified as moderately resistant with the severity level on 2–3 points. The remaining six lines were resistant, and 11 lines had shown intermediate (4–5 points) level of infection (see Fig. 1, *b*). In the region of South-East Kazakhstan, as in the case of stem rust, the severity of leaf rust infection was significantly higher than in the northern part of the country.

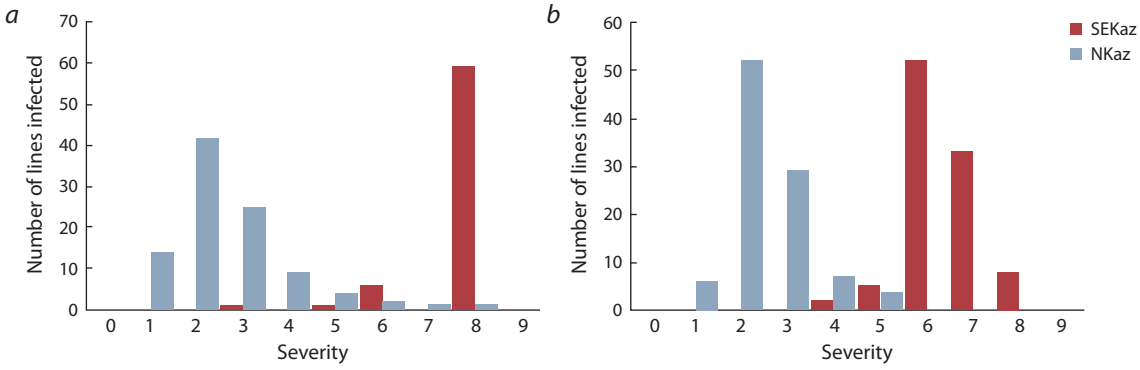

Fig. 1. Phenotypic variations of recombinant inbred lines for stem (*a*) and leaf rust (*b*) severity in two environments.
The severity of infections was determined based on the 9-point scale. SEKaz – South-East Kazakhstan, NKaz – North Kazakhstan.

Coefficients of pairwise Pearson correlations (*r*) between the leaf and stem rusts infections severities and adaptation/yield-related traits in RILs population grown in North Kazakhstan

Parameter	SR (grain formation)	SR (grain ripening)	SR (average)	LR (average)
HT	–0.254*	–0.072	–0.059	0.129
MT	–0.295**	–0.191	–0.211*	–0.054
PH	–0.061	–0.219*	–0.214*	–0.123
PL	–0.047	–0.037	–0.042	–0.085
NPS	0.159	–0.113	–0.060	–0.215*
SL	0.208*	0.098	0.124	0.019
NFS	–0.180	–0.113	–0.155	–0.094
NKS	–0.148	–0.117	–0.151	–0.237*
WKS	0.053	–0.144	–0.141	–0.295**
WKP	0.010	–0.214*	–0.230*	–0.168
TKW	0.031	–0.168	–0.198	–0.059

Notes: SR, stem rust severity; LR, leaf rust severity; HT, heading time; MT, maturation time; PH, plant height; PL, peduncle length; NPS, number of productive spikes per plant; SL, spike length; NFS, number of fertile spikelets per spike; NKS, number of kernels per spike; WKS, weight of kernels per spike; WKP, weight of kernels per plant; TKW, thousand kernels weight.
* Significance level at $p < 0.05$; ** significance level at $p < 0.01$.

Correlation analysis for resistance to LR/SR and agronomic traits. North Kazakhstan is the biggest wheat-growing area in Kazakhstan that gives around 85 % of bread wheat grain annually (http://stat.gov.kz). Therefore, a separate evaluation of the relationship between yield components and rust indexes was performed (see the Table).

The severity of LR and SR infections measured on two growth stages and averaged values revealed generally negative influence on all key adaptation and yield-related traits. In North Kazakhstan, the averaged level of SR infections was negatively correlated with three important traits – MT, PH, and WKP. At the same time, the level of SR infections measured during the phase of grain formation demonstrated a negative correlation with HT, MT, while measures at the beginning of grain ripening were negatively correlated with PH and WKP. LR severity made a significant negative impact on NPS, NKS, and WKS.

Genetic linkage map of the studied RILs population. A total of 4595 polymorphic SNP markers from 21 chromosomes were used in the current study. All SNPs showed a good fit to 1:1 segregation in the RILs mapping population ($p > 0.001$ in Chi-squared test). The distribution of markers among genomes was the following: A genome – 1939 SNPs, B genome – 2099 SNPs, and D genome – 557 SNPs. The lengths of genetic maps for individual chromosomes ranged from 218.9 cM (chromosome 3B) to 16.9 cM (chromosome 4D). Chromosome 2B was identified as the densest with 563 SNPs per 150.6 cM (average spacing 0.27 cM), while chromosome 5D demonstrated the least markers density with the average 2.65 cM between neighboring SNPs.

QTL analysis of resistance to LR and SR. Information about QTL identified in this research work is summarized in Supplementary 1[1]. Ten putative QTL for LR resistance were identified in seven different chromosomes (Fig. 2, Supplementary 1). The majority of QTL was revealed in South-East Kazakhstan, where the severity of LR was on a maximum level. Four of QTL for LR resistance were located on 3B chromosome on short distances from each other. One QTL is observed on 3A chromosome while remaining five QTL were on chromosomes 1B, 1D, 2A, 2B and 4B.

Among all identified QTL for LR, the *QLR.IPBB-3B.1* located on the 3B chromosome was detected in the South-East region during the peak of infection. It had demonstrated the highest 7.8 LOD score among the others and explained 27 % of the phenotypic variances. Other QTL demonstrated LOD score in the range from 3.3 up to 6.0 and phenotypic variances from 11 to 20 %.

Nine tentative QTL for SR were detected in this study (Fig. 3, see Supplementary 1). All of them are distributed among six chromosomes, where 3B chromosome contained three QTL, 6B chromosome – two QTL while remaining QTL were spread in chromosomes 1A, 2B, 2D, and 4A. The majority of QTL for SR resistance was identified in the North region, while there was only one QTL identified in the South-East. The highest LOD score was observed for two QTL – *QSR.IPBB-2D* and *QSR.IPBB-6B.1* – on chromosomes 2D and 6B, and explained 22 and 20 % of the stem rust resistance variances, respectively.

Discussion

Identified QTL for LR resistance and their comparison to previously LR mapping studies. The literature survey suggests that LR pathotypes of infections, as well as the sources of infection and wheat genes that are effective against them, are different in two regions. For example, in South-East Kazakhstan, seven *Lr* genes were reported to be highly effective (0R, 1–5 MR) – *Lr9*, *Lr12*, *Lr13*, *Lr18*, *Lr19*, *Lr24*, and *Lr37* (Koyshybaev, 2018). In the North, the difference in *Lr* genes effectiveness was observed even between two sites. For Akmola site, there were nine genes with good effective-

[1] Supplementary Materials 1 and 2 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx20.pdf

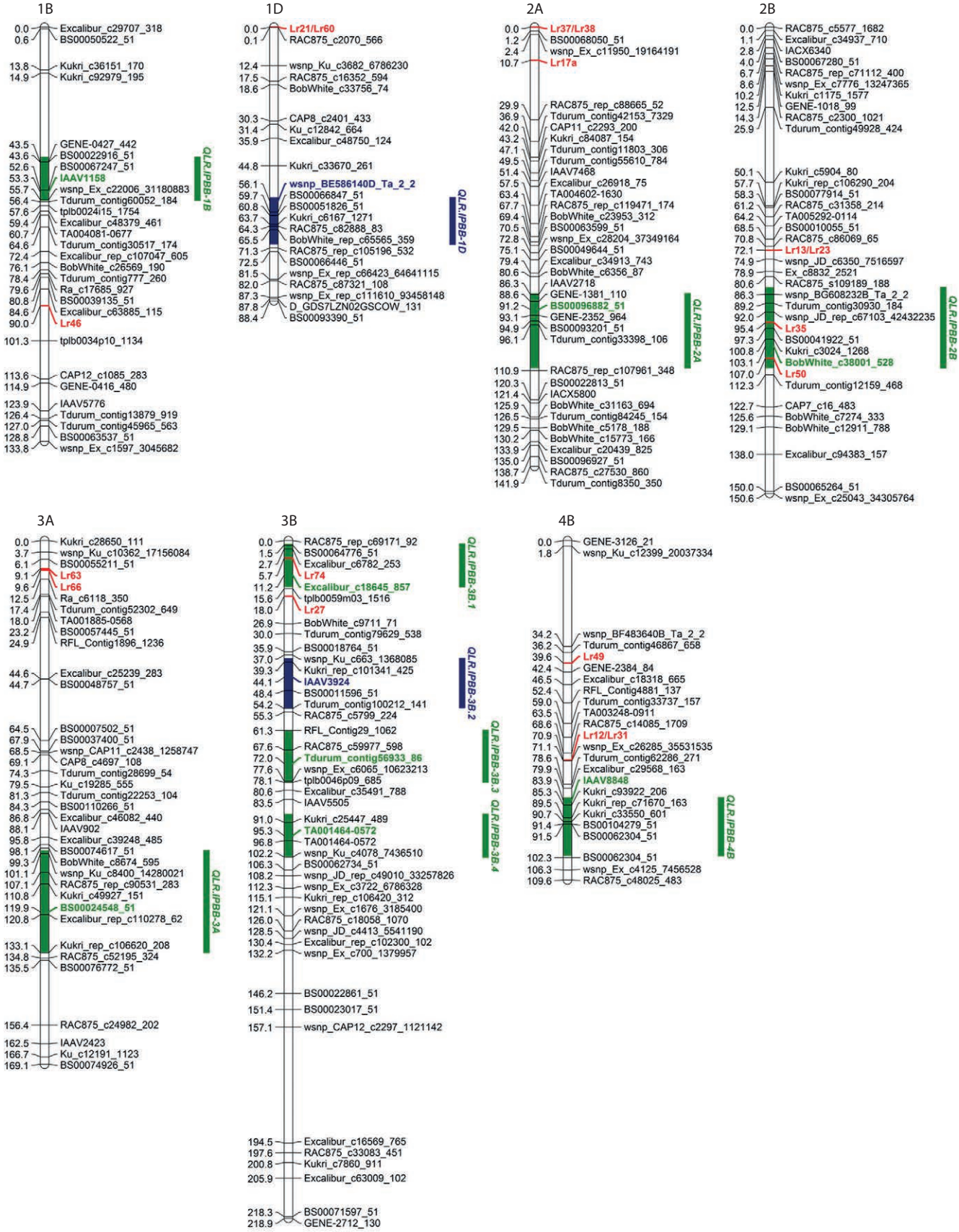

Fig. 2. Genetic map with QTL for leaf rust (LR) resistance detected using mapping population 'Pamyati Azieva × Paragon' and previously mapped *Lr* genes.

In each case, the genomic region containing the QTL is indicated by the vertical bar on the right and followed by the name of the QTL. SNP markers are indicated on the right, and their genetic positions (cM) are shown on the left. Peak marker for each QTL is highlighted in bold.

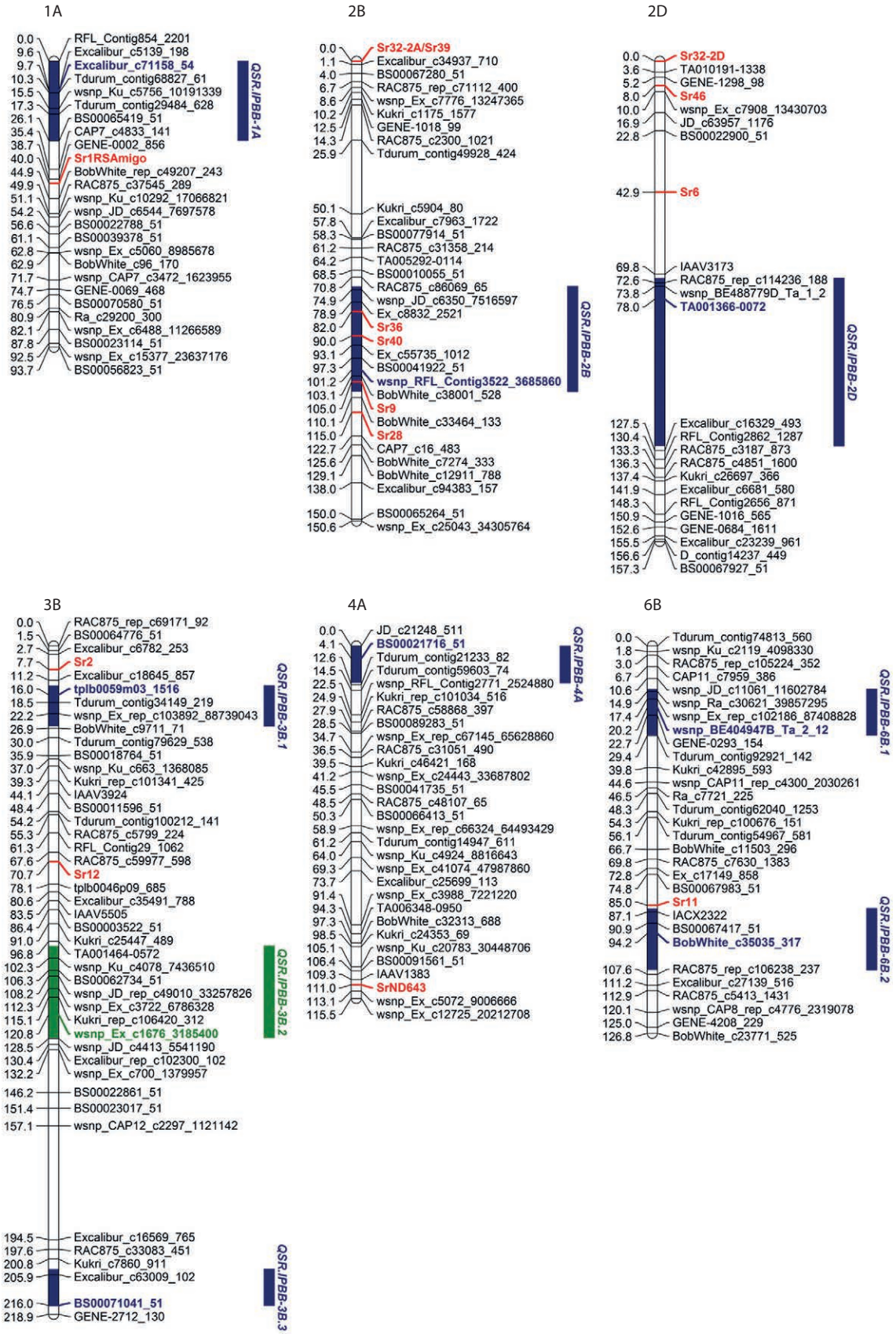

Fig. 3. QTL for stem rust (SR) resistance identified in mapping population 'Pamyati Azieva × Paragon' and previously mapped *Sr* genes.

In each case, the genomic region containing the QTL is indicated by the vertical bar on the right and followed by the name of the QTL. SNPs are indicated on the right, and their genetic positions (cM) are shown on the left. Peak marker for each QTL is highlighted in bold.

ness – *Lr9*, *Lr12*, *Lr13*, *Lr19*, *Lr23*, *Lr24*, *Lr28*, *Lr33*, and *Lr35*, while for North Kazakhstan site only three genes were highly effective – *Lr9*, *Lr28*, and *Lr36* (Koyshybaev, 2018). Here, ten QTL for LR were identified for two studied regions, and there were no matchings between them. Differences in QTL identification in North and South-East regions agreed with the data on differences in the composition of the pathogen populations between these regions (Koyshybaev, 2018). All comparison information concerning candidate genes and previously mentioned resistance QTL in the literature is presented in Supplementary 2.

One of two QTL identified in the North (*QLR.IPBB-1D*) is located in the long arm of 1D chromosome. The 1D chromosome has four *Lr* genes (see Supplementary 2) positioned on the far distances from the *QLR.IPBB-1D*. The second association found in the North region was *QLR.IPBB-3B.2*. The locus was within the interval of 38.0–54.0 cM on the 3B chromosome, near the locus *QLR.IPBB-3B.3*, which was identified in the South-East study. The *QLR.IPBB-3B.2* was distantly located from both *Lr27* and *Lr74* genes (see Fig. 2, Supplementary 2), but in close proximity to QTL described earlier (Gao et al., 2016; Zhang et al., 2017). Interestingly, none of *Lr* genes or QTL on chromosomes 1D and 3B had been described as effective in Kazakhstan before.

Field assessment of LR resistance in South-East allowed revealing eight QTL in six different chromosomes (see Supplementary 2). These QTL can be formally separated into two groups: the first group has QTL overlapping with previously identified and well described *Lr* genes, and the second group has QTL identified in this study. The first group is presented by two QTL on 2B and 3B chromosomes. On 2B chromosome, the *QLR.IPBB-2B* has similar positions with *Lr35* and *Lr50* (see Supplementary 2). Also, Gao and colleagues (Gao et al., 2016) and Zhang with co-authors (Zhang et al., 2017) identified similar QTL for LR in this part of the genome. The *Lr35* was previously described as highly effective in East, West, and North Kazakhstan regions (Koyshybaev, 2018). The second QTL *QLR.IPBB-3B.1* is positioned in the interval 1.1–15.0 cM of 3B chromosome, where it possibly overlaps with *Lr74* located approximately 4.9 cM away from *xgwm533* at 10.6 cM (Quarrie et al., 2005). Also, *Lr27* is another previously reported gene located in this region (see Supplementary 2). Notably, *QLR.IPBB-3B.1* was the most significant QTL for LR identified in this study with the highest R2 and additive effect.

The remaining six QTL for LR belong to the second group of putatively new genetic factors for studied environments. The first QTL from this group is *QLR.IPBB-1B* that located on the 1B chromosome. There are two QTL for LR described by Kumar and colleagues (Kumar et al., 2013) and Gao with co-authors (Gao et al., 2016) that were positioned in the same vicinity as the *QLR.IPBB-1B*. The *QLR.IPBB-2A* was the only identified association on 2A chromosomes in this study, and it was mapped in the interval 86.0–110.1 cM. The interval of the *QLR.IPBB-2A* is near to genetic positions of QTL for LR resistance that were described in previous studies (Kumar et al., 2013; Gao et al., 2016). The *QLR.IPBB-3A* was located in the interval 100.0–133.1 cM, and it is coinciding with the position of QTL for LR resistance described by Chu with colleagues (Chu et al., 2009). On the 3B chromosome, two QTL for LR were identified in this group of study in South-East region. These are *QLR.IPBB-3B.3* and *QLR.IPBB-3B.4* positioned in 61.2–78.1 and 88.2–102.3 cM intervals, respectively. It appears that QTL for LR in these regions were previously identified (Kumar et al., 2013; Muhammad et al., 2018). Finally, the *QLR.IPBB-4B* was located in the interval 82.9–101.8 cM, which is overlapping with the position of QTL for LR resistance described by Gao and co-authors (Gao et al., 2016).

As all identified genetic factors associated with the resistance to LR in this study were genetically positioned with associations identified in recent GWAS for LR resistance (Gao et al., 2016; Muhammad et al., 2018), it is strong indications that QTL identified in this study may play an important role in local breeding projects.

Identified QTL for SR resistance and their comparison to previously SR mapping studies. Unlike in LR study, where the majority of QTL for SR were found based on the data from South-East, in SR study almost all QTL (8 out of 9) were identified in the North region. The only SR resistant locus form the South-East was *QSR.IPBB-3B.2* in the interval 98.3–128.3 cM on the 3B chromosome, and it was significantly far from *Sr* genes mapped in this linkage group (see Supplementary 2). Other QTL for SR resistance can also be formally divided into two groups, likewise in LR study. The first group of marker-trait associations includes four QTL. The *QSR.IPBB-1A* was located in the interval 0–26.0 cM at a relatively short distance from the *Sr1RS^{Amigo}* mapped at 40.0 cM (Yu et al., 2014), and two QTL described in other studies (Yu et al., 2012; Bajgain et al., 2016). The next QTL *QSR.IPBB-2B* lies in the interval 73.8–108.2 cM and overlaps with three mapped *Sr* genes (*Sr9*, *Sr 36*, and *Sr40*) and adjoins *Sr28* (see Fig. 3), as well as several QTL for SR from literature (Yu et al., 2012; Bajgain et al., 2015; Edae et al., 2018). Two of these genes – *Sr9* and *Sr36* – were distinguished as effective against the Western Siberian population of SR (Shamanin et al., 2011). On the 3B chromosome, there are three identified QTL for SR, but only *QSR.IPBB-3B.1* was positioned in the vicinity of previously mapped gene *Sr2*, and QTL for SR resistance described by Elbasyoni with co-authors (Elbasyoni et al., 2017). Notably, the *Sr2* is the most important disease resistance gene to be deployed in modern plant breeding and provided partial resistance for many years over large areas and under high and prolonged disease pressure in the field (Ellis et al., 2014). Finally, the *QSR.IPBB-6B.2* was positioned just in 2.1 cM from *Sr11* (see Supplementary 2).

The second group of QTL for SR resistance included associations that previously were not mentioned in Kazakhstan. This group was comprised of five QTL located on chromosomes 2D, 3B, 4A, and 6B. The region 71.1–126.0 cM of chromosome 2D, which is associated with the *QSR.IPBB-2D*, has not been mentioned in connection with previous QTL for SR mapping studies. The *QSR.IPBB-2D* demonstrated the highest impact on the SR resistance in this study, explaining 22 % of the variation. Also, on the 3B chromosome, there is *QSR.IPBB-3B.3*, which is another presumably novel QTL for SR resistance in Kazakhstan. The *QSR.IPBB-4A* on the 4A chromosome resembles two SR-associated loci described in previous studies (see Supplementary 2) (Basnet et al., 2015), but it has no candidate *Sr* genes nearby. The remaining QTL

QSR.IPBB-6B.1 was located in the short arm of the 6B chromosome. It seems that the position of the *QSR.IPBB-6B.1* is matching the position of QTL for SR resistance that was previously described by Yu with co-authors (Yu et al., 2012).

Conclusion

The constructed RILs MP 'Pamyati Azieva × Paragon' was very efficient in the identification of QTL for LR and SR resistance in bread wheat. The MP consisted of 98 RILs and analyzed by using 4595 polymorphic SNP markers densely populated all 21 wheat chromosomes. Field trials output suggested a negative correlation between LR and SR severity and key yield components in the North and South-East regions of Kazakhstan. The CIM method allowed the identification of ten QTL for LR and nine QTL SR resistance associated with resistance to these two rust diseases. The comparative analysis of the findings in this study and reports from previously published data suggested that the majority of identified QTL were well described in existed literature, confirming the robustness of obtained results. Nevertheless, two QTL for SR identified in the North region of the country were never described before, and they are presumably novel genetic factors. Overall, identified QTL both for LR and SR resistances in newly developed MP 'Pamyati Azieva × Paragon' can be efficiently used in local breeding projects for higher yield in bread wheat.

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Acknowledgements. This work was done in the framework of the project "Development of new DNA markers associated with the resistance of bread wheat to the most dangerous fungal diseases in Kazakhstan" in Program "Development of the innovative systems for increasing the resistance of wheat varieties to especially dangerous diseases in the Republic of Kazakhstan" BR06249329 supported by the Ministry of Agriculture of the Republic of Kazakhstan.

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

Received August 21, 2019. Revised September 23, 2019. Accepted September 23, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):896-901
DOI 10.18699/VJ19.564

The Fifth International Scientific Conference PlantGen2019

The cinnamyl alcohol dehydrogenase gene family is involved in the response to *Fusarium oxysporum* in resistant and susceptible flax genotypes

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Flax (*Linum usitatissimum* L.) is used for the production of textile, oils, pharmaceuticals, and composite materials. Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *lini*, is a very harmful disease that reduces flax production. Flax cultivars that are resistant to Fusarium wilt have been developed, and the genes that are involved in the host response to *F. oxysporum* have been identified. However, the mechanisms underlying resistance to this pathogen remain unclear. In the present study, we used transcriptome sequencing data obtained from susceptible and resistant flax genotypes grown under control conditions or *F. oxysporum* infection. Approximately 250 million reads, generated with an Illumina NextSeq instrument, were analyzed. After filtering to exclude the *F. oxysporum* transcriptome, the remaining reads were mapped to the *L. usitatissimum* genome and quantified. Then, the expression levels of cinnamyl alcohol dehydrogenase (*CAD*) family genes, which are known to be involved in the response to *F. oxysporum*, were evaluated in resistant and susceptible flax genotypes. Expression alterations in response to the pathogen were detected for all 13 examined *CAD* genes. The most significant differences in expression between control and infected plants were observed for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B*, with strong upregulation of *CAD1B*, *CAD5A*, and *CAD5B* and strong downregulation of *CAD4A*. When plants were grown under the same conditions, the expression levels were similar in all studied flax genotypes for most *CAD* genes, and statistically significant differences in expression between resistant and susceptible genotypes were only observed for *CAD1A*. Our study indicates the strong involvement of *CAD* genes in flax response to *F. oxysporum* but brings no evidence of their role as resistance gene candidates. These findings contribute to the understanding of the mechanisms underlying the response of flax to *F. oxysporum* infection and the role of *CAD* genes in stress resistance.
Key words: flax; *Linum usitatissimum*; resistant cultivars; *Fusarium oxysporum*; RNA-Seq; transcriptome; *CAD*.

For citation: Novakovskiy R.O., Povkhova L.V., Krasnov G.S., Rozhmina T.A., Zhuchenko A.A., Kudryavtseva L.P., Pushkova E.N., Kezimana P., Kudryavtseva A.V., Dmitriev A.A., Melnikova N.V. The cinnamyl alcohol dehydrogenase gene family is involved in the response to *Fusarium oxysporum* in resistant and susceptible flax genotypes. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):896-901. DOI 10.18699/VJ19.564

Семейство генов дегидрогеназ коричного спирта вовлечено в ответ устойчивых и восприимчивых генотипов льна на заражение *Fusarium oxysporum*

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Лен (*Linum usitatissimum* L.) используется для производства текстиля, масел, фармацевтических препаратов и композитных материалов. Крайне вредоносным заболеванием, снижающим урожайность льна, является фузариозное увядание, вызываемое грибом *Fusarium oxysporum* f. sp. *lini*. Созданы устойчивые к фузариозному увяданию сорта льна и определены гены, вовлеченные в ответ на *F. oxysporum*, однако механизмы устойчивости *L. usitatissimum* к этому патогену до сих пор неясны. В настоящем исследовании мы использо-

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вали данные секвенирования транскриптомов восприимчивых и устойчивых генотипов льна, выращенных в контрольных условиях или зараженных *F. oxysporum*. Проанализировано около 250 миллионов прочтений, полученных на секвенаторе NextSeq Illumina. После фильтрации прочтений для исключения транскриптома *F. oxysporum* оставшиеся прочтения картировали на геном *L. usitatissimum* и провели их количественный анализ. Оценили экспрессию генов семейства *CAD*, которые, как известно, участвуют в ответе на заражение *F. oxysporum*, у устойчивых и восприимчивых к фузариозному увяданию генотипов. Изменение экспрессии в ответ на возбудителя выявили для всех 13 исследованных генов *CAD*. Наиболее значительные различия в экспрессии между контрольными и инфицированными растениями наблюдались для генов *CAD1B*, *CAD4A*, *CAD5A* и *CAD5B*: сильное повышение экспрессии выявлено для *CAD1B*, *CAD5A* и *CAD5B*, а сильное снижение – для *CAD4A*. Для большинства генов *CAD* уровни экспрессии были близкими при одинаковых условиях выращивания для всех изученных генотипов льна. Статистически значимое различие в изменении экспрессии между группами устойчивых и восприимчивых генотипов выявлено только для гена *CAD1A*. Наше исследование указывает на активное участие генов *CAD* в ответе растений льна на *F. oxysporum*, но не приводит свидетельств их роли в качестве кандидатов в гены устойчивости. Полученные результаты вносят вклад в понимание механизмов ответа льна на заражение *F. oxysporum* и роли генов *CAD* в устойчивости к стрессовым воздействиям.

Ключевые слова: лен; *Linum usitatissimum*; устойчивые сорта; *Fusarium oxysporum*; RNA-Seq; транскриптом; *CAD*.

Introduction

Flax (*Linum usitatissimum* L.) is an agricultural crop with numerous uses. High-quality fiber can be obtained from flax stems and is used for the production of textile and fiber-based materials for the healthcare, military, aerospace, and electronics industries (Costa et al., 2018). Flaxseed is also used in the production of pharmaceuticals, functional foods, and other products for human consumption, while linseed is used in paints, varnishes, and animal feed (Singh et al., 2011; Goyal et al., 2014). *Fusarium oxysporum* f. sp. *lini* is a harmful pathogen that reduces flax production and quality (Rashid, 2003). Flax genotypes showing resistance to Fusarium wilt have been identified, and cultivars with improved resistance have been bred (Diederichsen et al., 2008; Rozhmina et al., 2017). However, the molecular mechanisms underlying resistance to Fusarium wilt remain unclear, and the search for genes involved in the response to *F. oxysporum* is an area of active research. The involvement of pathogenesis-related proteins in the response to *F. oxysporum* infection was demonstrated (Wrobel-Kwiatkowska et al., 2004; Wojtasik et al., 2014; Galindo-Gonzalez, Deyholos, 2016), and the roles of antioxidants, polyamines, and phenolic compounds in response to the pathogen were shown (Lorenc-Kukula et al., 2007, 2009; Boba et al., 2011, 2016; Zeitoun et al., 2014; Wojtasik et al., 2015). Moreover, *F. oxysporum*-infected flax plants show cell wall rearrangements (Wojtasik et al., 2015, 2016; Boba et al., 2016).

Cinnamyl-alcohol dehydrogenases (CADs) are involved in the biosynthesis of lignin, which can function as a barrier against pathogens, and the role of *CAD* genes in the response to *F. oxysporum* was previously shown (Wrobel-Kwiatkowska et al., 2007; Preisner et al., 2014, 2018). Plants with downregulated *CAD* showed reduced (Wrobel-Kwiatkowska et al., 2007) or slightly decreased (Preisner et al., 2014) resistance to *F. oxysporum*. Sixteen *CAD* genes were identified in *L. usitatissimum* and their roles in plant growth and stress responses were examined in the Nike cultivar, which is relatively resistant to *Fusarium* infection (Preisner et al., 2018). In the present study, we evaluated the expression of *CAD* genes in resistant and susceptible flax genotypes under control conditions and *F. oxysporum* infection to determine the general trends in response to the pathogen and genotype-specific alterations in expression.

Materials and methods

Two *F. oxysporum*-susceptible flax cultivars (TOST and AP5), two resistant cultivars (3896 and Dakota), and two resistant BC_2F_5 populations (3896 × AP5, recurrent parent AP5, and Dakota × AP5, recurrent parent AP5) were used in the present study. Seeds were obtained from the Institute for Flax (Torzhok, Russia) and sterilized, first in 70 % ethanol for 1 min and then in 1 % sodium hypochlorite for 20 min. The plants were grown in 15 ml glass tubes on Murashige–Skoog medium in a growth chamber at 22 °C under a 16/8 h day/night cycle for seven days. Then, half of the plants were inoculated with *F. oxysporum* (pathogenic isolate #39 from the phytopathogen collection of the Institute for Flax); the remaining uninoculated plants were used as controls. Forty-eight hours later, the root tips were collected and frozen in liquid nitrogen. In total, the material was obtained from 240 plants.

Total RNA was extracted from the pooled plants (10–12 plants each) using the RNeasy Plant Mini Kit (Qiagen, USA). We obtained 24 RNA samples from the TOST, AP5, 3896, Dakota, 3896 × AP5, and Dakota × AP5 plants under control and infection in duplicate. The quality and concentration of the isolated RNA were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and Qubit 2.0 fluorometer (Life Technologies, USA). The TruSeq Stranded Total RNA Sample Prep Kit (Illumina, USA) was used to prepare the cDNA library. The libraries were sequenced on a NextSeq500 sequencer (Illumina) with 80-nucleotide paired-end reads (SRP119227, Sequence Read Archive).

Reads were trimmed using Trimmomatic (Bolger et al., 2014) and filtered against the *F. oxysporum* reference genome as described in our previous study (Dmitriev et al., 2017). Then, the remaining reads were mapped to the *L. usitatissimum* genome (GenBank assembly: GCA_000224295.2) using STAR (Dobin et al., 2013) and quantified using BEDTools (Quinlan, Hall, 2010). The genome sites where the *CAD* family genes (*CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, *CAD7*, and *CAD8*) are localized were identified in the latest *L. usitatissimum* genome assembly (GenBank assembly: GCA_000224295.2) using the data of Preisner et al. (2018). Namely, the sequences of the scaffold regions of the old *L. usitatissimum* genome assembly (GenBank: AFSQ00000000.1) that encode *CAD* transcripts were mapped to the latest *L. usitatissimum* ge-

nome assembly (GCA_000224295.2) and new coordinates were identified for the *CAD* transcripts. The counts per million (CPM) values were determined for 13 *CAD* genes in each cultivar and population under both control conditions and *Fusarium* infection, and then the log(*CPM Fusarium*/*CPM control*) values were calculated for each cultivar and BC_2F_5 population. This was performed using the equipment of the Genome Center of Engelhardt Institute of Molecular Biology (http://www.eimb.ru/rus/ckp/ccu_genome_c.php).

Results

In our previous study on the response of *L. usitatissimum* to *F. oxysporum*, we used RNA-Seq data for *de novo* transcriptome assembly and annotation and then quantified the expression levels of the identified transcripts (Dmitriev et al., 2017). Unfortunately, we had failed to identify a significant number of *CAD* family genes in our *de novo* transcriptome assembly. In the present study, we used an improved assembly of the *L. usitatissimum* genome (GCA_000224295.2), in which the scaffolds were mapped to specific chromosomes, as a reference for RNA-Seq read mapping, and *CAD* gene sequence data (Preisner et al., 2018) were used to identify the genome sites in which the *CAD* genes are located. This approach enabled us to evaluate the expression levels of all presently identified *CAD* genes in flax. Unique chromosome locations were determined for *CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, and *CAD7*, while *CAD8A*, *CAD8B*, *CAD8C*, and *CAD8D* were mapped to the same region of chromosome Lu7. Therefore, we performed an expression analysis of *CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, *CAD7*, and *CAD8* genes.

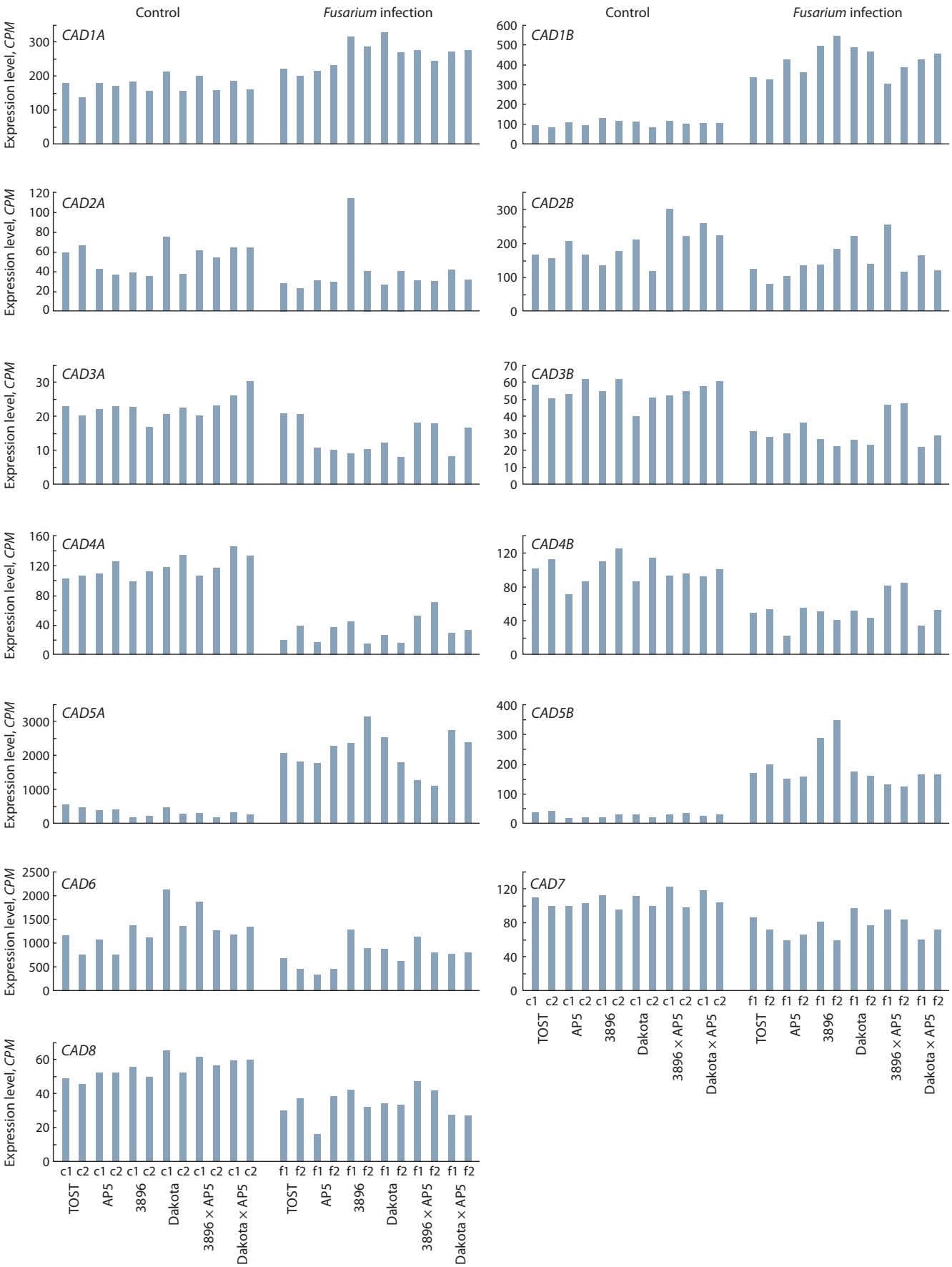
The expression levels of the 13 *CAD* family genes in six flax cultivars and populations grown under control conditions or inoculated with a pathogenic isolate of *F. oxysporum* were evaluated based on RNA-Seq data. The results are shown in the Figure, which presents the CPM values for each studied gene in resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and susceptible (TOST and AP5) genotypes under control conditions and 48 h after inoculation with *F. oxysporum* in biological replicates. Under both conditions, the expression levels of *CAD5A* and *CAD6* were the highest, and the expression levels of *CAD2A*, *CAD3A*, *CAD3B*, and *CAD8* were the lowest. Compared to control conditions, statistically significant alterations in expression under *Fusarium* infection were observed for all 13 genes ($p < 0.01$ for all genes except *CAD2B*, which was $p < 0.05$, Mann–Whitney test). The most significant differences in expression between the control and infected plants were observed for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B*; *CAD1B*, *CAD5A*, and *CAD5B* showed strong upregulation under *F. oxysporum* infection, and *CAD4A* showed strong downregulation. Under the same conditions, the expression levels were similar in all studied flax genotypes for most *CAD* genes; however, some exceptions were observed. For example, the expression levels of *CAD3A*, *CAD3B*, *CAD4A*, and *CAD4B* in the infected plants of the 3896 × AP5 population were higher than those in other infected genotypes.

To identify genotype-specific expression alterations in response to *F. oxysporum* infection, the log(*CPM Fusarium*/*CPM control*) values were calculated (see the Table). In all studied genotypes, the most significant (more than 3-fold in average) increases in expression were observed for *CAD1B*, *CAD5A*, and *CAD5B*, while the most significant decreases in expression were observed for *CAD4A*. For *CAD2A*, *CAD3A*, *CAD3B*, *CAD4B*, and *CAD6*, downregulation was observed in the majority of genotypes after *F. oxysporum* infection. For *CAD2B*, *CAD7*, and *CAD8*, the decrease in expression was only slight, and for some genotypes, no decrease was observed. Statistically significant differences in expression between resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and susceptible (TOST and AP5) genotypes were only observed for the *CAD1A* gene ($p < 0.05$, Mann–Whitney test).

Expression alterations in the *CAD* genes of *F. oxysporum* resistant and susceptible flax cultivars and populations

Gene	log(*CPM Fusarium/CPM control*)					
	Susceptible genotypes		Resistant genotypes			
	TOST	AP5	3896	Dakota	3896 × AP5	Dakota × AP5
CAD1A	0.42	0.36	0.82	0.70	0.54	0.65
CAD1B	1.86	1.91	2.08	2.24	1.67	2.04
CAD2A	–1.28	–0.38	1.04*	–0.73	–0.89	–0.80
CAD2B	–0.65	–0.64	0.04	0.14	–0.49	–0.75
CAD3A	–0.07	–1.10	–1.03	–1.09	–0.26	–1.17
CAD3B	–0.88	–0.80	–1.26	–0.87	–0.18	–1.23
CAD4A	–1.80	–2.08	–1.80	–2.56	–0.86	–2.14
CAD4B	–1.05	–1.03	–1.34	–1.07	–0.19	–1.14
CAD5A	1.91	2.32	3.66	2.51	2.22	3.09
CAD5B	2.14	2.85	3.56	2.66	1.98	2.55
CAD6	–0.73	–1.18	–0.19	–1.21	–0.69	–0.70
CAD7	–0.40	–0.70	–0.57	–0.28	–0.30	–0.75
CAD8	–0.47	–0.93	–0.50	–0.77	–0.37	–1.13

* A 3-fold difference between biological replicates was observed for the *CAD2A* gene in cultivar 3896 under *Fusarium* infection (see the Figure).



Expression of *CAD* genes in resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and susceptible (TOST and AP5) flax genotypes under control conditions (c1 and c2) and 48 h after inoculation with *F. oxysporum* (f1 and f2) in biological replicates.

Discussion

Recent progress in molecular analysis has provided novel opportunities for plant studies (Kage et al., 2015; Poland, 2015). Using high-throughput sequencing, genome and transcriptome sequences for many plant species can be obtained in a short time period and used for further research (Varshney et al., 2009; He et al., 2014). The draft genome sequence for flax was obtained in 2012 (Wang et al., 2012), and in 2018, the genome assembly was improved and chromosome-scale pseudomolecules were obtained using BioNano genome optical mapping (You et al., 2018). Flax transcriptomes and small RNAs obtained from different tissues, development stages, and under biotic and abiotic stresses have been sequenced. Such data has enabled the identification of genes and miRNAs that are expressed in particular organs and in the definite time and could play key roles in plant development, as well as the discovery of genes and miRNAs with altered expression under unfavorable conditions that are likely involved in the response to stress (Yu et al., 2014, 2016; Melnikova et al., 2015, 2016; Dmitriev et al., 2016, 2017, 2019; Galindo-Gonzalez, Deyholos, 2016; Dash et al., 2017; Gorshkova et al., 2018; Zyablitsin et al., 2018; Gorshkov et al., 2019; Krasnov et al., 2019; Wu et al., 2019).

In the present study, we used RNA-Seq data from *F. oxysporum*-resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and -susceptible (TOST and AP5) flax genotypes grown under control conditions or 48 h after inoculation with *F. oxysporum* to evaluate the expression of *CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, *CAD7*, and *CAD8* and identified the *CAD* genes that were involved in the response to the pathogen. The genes showed different expression changes after *F. oxysporum* infection: in most genotypes, *CAD1A*, *CAD1B*, *CAD5A*, and *CAD5B* were upregulated, while *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD6*, *CAD7*, and *CAD8* were downregulated. In the study by Preisner et al. (2018), decreased expression was observed for most *CAD* genes at 24 and 96 h after *F. oxysporum* infection in the flax cultivar Nike. In the present study, the greatest expression changes in infected plants were observed for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B*; three of these genes were upregulated, while *CAD4A* was downregulated. Our data for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B* are consistent with the results of the previous study at 24 h after infection (Preisner et al., 2018). However, the changes observed by us were more significant, with a 3–5-fold change for *CAD1B*, a 2–5-fold change for *CAD4A*, a 4–13-fold change for *CAD5A*, and a 4–12-fold change for *CAD5B*. Based on our results, we suggest that *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B* are the most involved in the response of flax to *F. oxysporum*.

Searching for genes with diverse expression alterations in resistant and susceptible genotypes under stress conditions is important for the identification of resistance genes. In our study, statistically significant differences in expression between resistant and susceptible genotypes in response to the pathogen were observed only for *CAD1A*. Therefore, this gene could be involved in resistance to *F. oxysporum*. However, the changes were not pronounced. Thus, further investigations are necessary.

Conclusion

We performed expression analysis of *CAD* family genes after *F. oxysporum* inoculation based on RNA-Seq data and identified genes with significant up- and down-regulation after pathogen infection. The results of the present study indicate the involvement of *CAD* genes in response to *Fusarium* infection, but their role as resistance genes in the studied cultivars and populations is questionable. Our data also contribute to the understanding of the role of *CAD* genes in stress response and resistance.

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Acknowledgements. This work was financially supported by the Russian Science Foundation (Grant No. 16-16-00114).

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

Received June 02, 2019. Revised August 16, 2019. Accepted August 19, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):902-909
DOI 10.18699/VJ19.565

The Fifth International Scientific Conference PlantGen2019

Сравнительная оценка вариабельности ядерного и хлоропластного генома лука-порея (*Allium porrum* L.)

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К роду *Allium* L. (сем. Amaryllidaceae), самому многочисленному среди однодольных растений, относятся такие экономически значимые овощные культуры, как лук репчатый (*A. cepa*), чеснок (*A. sativum*) и лук-порей (*A. porrum*). Лук-порей обладает высокими вкусовыми качествами и доказанными ценными диетическими свойствами и является одной из самых популярных овощных культур в Западной Европе. Несмотря на высокую значимость лука-порея как овощной культуры, этот вид редко бывает объектом молекулярно-генетических исследований. Генетическое разнообразие лука-порея практически не изучали ранее. Поэтому в настоящей работе на широкой выборке образцов изучена вариабельность ядерного (метод AFLP) и хлоропластного (анализ нуклеотидных последовательностей) геномов. Для проведения работы было отобрано 65 образцов лука-порея из коллекции Федерального научного центра овощеводства, которая включала сорта отечественной и зарубежной селекции. В результате проведения AFLP-анализа и обработки полученных ДНК-спектров идентифицировано 760 фрагментов, из которых 716 были полиморфны для анализируемых образцов лука-порея. Рассчитанные генетические расстояния между образцами лука-порея варьировали от 0.4 до 0.76, что сопоставимо с внутривидовым полиморфизмом родственных видов *Allium* (лук репчатый, чеснок). Анализ геномной структуры в программе STRUCTURE 2.3.4 разделил исследуемые образцы лука-порея на семь групп, что в целом совпадает с кластеризацией этих образцов по результатам кластерного анализа. Для оценки вариабельности хлоропластного генома у анализируемых образцов лука-порея были секвенированы девять участков хлоропластного генома, как некодирующие: межгенные спейсеры *rpl32-trnL*, *ndhJ-trnL* и интрон гена *rps16*, так и белок-кодирующие: гены *psaA*, *psaB*, *psbA*, *psbB*, *psbE*, *petB*. Проведенный анализ участков хлоропластного генома лука-порея выявил крайне низкий уровень их полиморфизма, было обнаружено всего шесть SNP в изученных последовательностях суммарной длиной около 10500 п. н. Таким образом, в результате работы был установлен высокий уровень полиморфизма ядерного генома лука-порея, при этом полиморфизм хлоропластного генома оказался крайне низким.
Ключевые слова: лук-порей; *Allium porrum*; AFLP; генетическое разнообразие; анализ генома; хлоропластный геном.

Для цитирования: Дьяченко Е.А., Середин Т.М., Филюшин М.А. Сравнительная оценка вариабельности ядерного и хлоропластного генома лука-порея (*Allium porrum* L.). Вавиловский журнал генетики и селекции. 2019; 23(7):902-909. DOI 10.18699/VJ19.565

Nuclear and chloroplast genome variability in leek (*Allium porrum* L.)

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The genus *Allium* L. (Amaryllidaceae), the most numerous among monocotyledonous plants, includes such economically important vegetable crops as onion (*A. cepa*), garlic (*A. sativum*) and leek (*A. porrum*). Leek has a high taste and proven valuable dietary properties and is one of the most popular vegetable crops in Western Europe. Despite a high importance of leek as a vegetable, this species is rarely the subject of molecular genetic studies. The genetic diversity of leeks has never been studied before. Therefore, in this work, we studied the nuclear variability (AFLP) and the chloroplast (nucleotide sequence analysis) genomes using a broad sample. For this work, 65 leek accessions were selected from the collection of the Scientific Center of Vegetable Crops, which included varieties of domestic and foreign breeding. As a result of an AFLP analysis and processing of the DNA spectra obtained, 760 fragments were identified, of which 716 were polymorphic for the leek accessions being analyzed. The calculated genetic distances between the leek samples varied from 0.4 to 0.76, which is comparable to the intraspecific polymorphism of related *Allium* species (onions, garlic). Analysis of the genomic structure with STRUCTURE 2.3.4 divided the leek samples into seven groups, which generally coincides with the clustering of these samples. To

assess the variability of the chloroplast genome, nine sites of the chloroplast genome were sequenced in the leek samples, both non-coding (intergenic spacers *rpl32-trnL*, *ndhJ-trnL*, and intron *rps16* gene), and protein coding genes (*psaA*, *psaB*, *psbA*, *psbB*, *psbE*, *petB*). The analysis of the sites of the leek chloroplast genome revealed an extremely low level of their polymorphism, only six SNPs were detected in the studied sequences with a total length of about 10,500 bp. Thus, as a result of this work, a high level of polymorphism of the leek nuclear genome was revealed, while the polymorphism of the chloroplast genome was extremely low.
Key words: leek; *Allium porrum*; AFLP; genetic diversity; genome analysis; chloroplast genome.

For citation: Dyachenko E.A., Seredin T.M., Filyushin M.A. Nuclear and chloroplast genome variability in leek (*Allium porrum* L.). Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):902-909. DOI 10.18699/VJ19.565 (in Russian)

Введение

Род *Allium* L. (сем. Amaryllidaceae) – самый многочисленный среди однодольных растений, объединяет около 1200 видов, каждый год описываются новые виды луков (Govaerts et al., 2018; Brullo et al., 2019). Представители рода распространены в Северном полушарии, многие виды являются эндемиками. К роду *Allium* относятся такие важные сельскохозяйственные культуры, как лук репчатый (*A. cepa*), чеснок (*A. sativum*), лук-порей (*A. porrum*), возделываемые в настоящее время во всем мире.

Лук-порей – ценная овощная культура с двухлетним циклом выращивания. В пищу пригодно практически все растение – утолщенный ложный стебель и широкие зеленые линейно-ланцетные листья. Обе части содержат важные для диеты человека вторичные метаболиты: сераорганические, полифенольные соединения, витамин С (Bernaert et al., 2014; Агафонов, Дубов, 2018). Высокие вкусовые качества и доказанные ценные диетические свойства сделали лук-порей популярным в Европе, Северной Америке и Азии. Ежегодное производство этой культуры составляет более 2 млн т в год (по данным FAO, 2017 г.), крупнейшим производителем и потребителем лука-порея является Западная Европа (Soininen et al., 2014; Агафонов, Дубов, 2018).

Культуру лук-порей, столь популярную в Западной Европе, в России начали выращивать сравнительно недавно. Первый из отечественных сортов – Карантанский – внесен в Государственный реестр селекционных достижений, допущенных к использованию (Госсортреестр) в 1961 г., и только с 1993 г. этот список начал расширяться, включая к настоящему времени 27 сортов, преимущественно западноевропейской селекции. В Российской Федерации активная селекция лука-порея ведется в Федеральном научном центре овощеводства (ФНЦО), на сегодняшний день четыре сорта лука-порея включены в Госсортреестр.

Лук-порей – популярный объект для биохимических исследований (Bernaert et al., 2013, 2014; Soininen et al., 2014), однако генные и геномные исследования этой культуры ранее практически не проводили. Так, был определен транскриптом листа лука-порея, который использовали для сравнительного анализа экспрессии генов у видов *Allium* с различной морфологией листа (Zhu et al., 2017). Методами AFLP и ISSR изучены геномный полиморфизм 16 образцов лука-порея и внутрисортовая вариабельность (Филюшин и др., 2011; Филюшин, Агафонов, 2015).

Комплексная оценка биоразнообразия, включающая, помимо классического морфофизиологического описания образцов, геномный анализ, становится в настоящее время обязательной для характеристики сельскохозяйственных видов растений. Поэтому целью нашей работы стали оценка и сравнительный анализ вариабельности ядерного и хлоропластного геномов у широкой выборки образцов лука-порея.

Материалы и методы

Для изучения были взяты 65 образцов лука-порея из коллекции Федерального научного центра овощеводства (пос. ВНИИССОК, Московская область) (табл. 1). В качестве внешней группы использованы родственные виды из коллекции Ботанического сада Университета Оснабрюк (Германия), предоставленные профессором Н. Фризеном: *A. ampeloprasum* (кат. № 1023 и 1744, Грузия), *A. commutatum* (кат. № 1662, Франция) и *A. pyrenaicum* (кат. № 3018, Испания). ДНК выделяли из пятидневных проростков CTAB-методом с двойной депротеинизацией хлороформом, для выделения использовали по 10 проростков каждого сорта для охвата возможного внутрисортового полиморфизма.

Полиморфизм ядерного генома определяли методом AFLP, согласно стандартному протоколу (Vos et al., 1995). В связи с тем, что лук-порей имеет большой размер генома, для AFLP-анализа применяли праймерные комбинации с девятью селективными нуклеотидами: E-ACT/M-CAAGCG и E-AGG/M-CAAGCG. Из 12 протестированных комбинаций только эти пары позволили выявить внутривидовой полиморфизм и получить оптимальное количество фрагментов на геле. ДНК гидролизовали с помощью рестриктаз *EcoRI* и *MseI* (Thermo Fisher Scientific, США). Продукты амплификации разделяли в 6.5 % денатурирующем полиакриламидном геле с помощью системы фрагментного анализа LI-COR 4300 DNA Analyzer (LI-COR Biosciences, США).

Определение генетических расстояний (1 – коэф. Жаккара) и построение дендрограммы (метод UPGMA) проводили в программе PAST3 (https://folk.uio.no/ohammer/past/). Геномную структуру выявляли с помощью программы STRUCTURE 2.3.4 (https://web.stanford.edu/group/pritchardlab/structure.html) со следующими параметрами: ADMIXTURE model с allele frequencies correlated, burn-in 500.000 и длиной прогона 500.000 MCMC, числом кластеров (set K) от 2 до 19. Наиболее вероятное число кластеров определяли по методике (Evanno et al., 2005).

Для анализа полиморфизма участков хлоропластного генома были амплифицированы и секвенированы межгенные спейсеры *rpl32-trnL* и *ndhJ-trnL*, интрон гена *rps16* (последовательности праймеров взяты из работы (Shaw et al., 2007) и белок-кодирующие гены *psaA* (5′-CTTGACTGTTGGCGGGTCT-3′; 5′-GTAC

Table 1. Leek accessions from the FSCVC collection used in the work

No.	Cultivars*	Country of origin (if available)*	Acc. no. in FSCVC (if available)*	Acc. no. in VIR (if available)*
1	Agria	Netherlands	K-16	2398
2	Amarillo	France	K-13	2307
3	American flag	Denmark	K-3	2191
4	Arcona	Netherlands	K-24	5162
5	Bentons Monorel	–	K-59	2084
6	Blauwgroene Winter	Germany	K-17	2403
7	Carentan	Germany	K-60	2092
8	Colonna	Netherlands	K-54	2243
9	Empire	Denmark	K-14	2350
10	Florena	Netherlands	K-25	5989
11	Geant d'Hiver Tezier	France	K-64	2203
12	Giant anelioro	Netherlands	K-7	2238
13	Giant Musselburgh	India	K-69	2140
14	Italion gent	Denmark	K-15	2353
15	Lincoln	Yugoslavia	K-21	2544
16	Long d'hiver	France	K-5	2212
17	Lyon	United Kingdom	K-2	2159
18	Lyon	United Kingdom	K-55	2252
19	Merlin	Netherlands	K-45	2545
20	Monstruoso	Argentina	K-1	2114
21	Monstrouso	United Kingdom	K-10	2248
22	Monstruena de Carentan	France	K-65	2204
23	Monstrueux	France	K-67	2198
24	Monstrueux d'Elbeuf	France	–	2236
25	Musselburgh	Netherlands	K-11	2253
26	Olifant-Exelsior	Netherlands	K-4	2196
27	Poireau	France	K-6	2231
28	Porree dicker	Germany	K-41	2017
29	Praza 2	Italy	K-9	2245
30	Siegfried	Denmark	K-70	2187
31	Suttons Prizetaker	India	K-68	2120
32	The Legon	Netherlands	K-50	5998
33	Timperley Light	United Kingdom	–	2239
34	Tres long Hiver	Botswana	K-19	2518
35	Tynis	Tunisia	K-57	2276
36	Wastlandia Winterreusen	Netherlands	K-62	2197
37	Winter lauch Rijen fon Carentan	Germany	K-71	2005
38	Zocal Type	–	K-53	2580
39	Zocalen	Iran	K-22	2574
40	Alligator	–	–	–
41	Vesta	–	–	–
42	Goliath	–	K-28	–
43	Gulliver	–	K-119	–
44	Dobryy molodec	–	K-39	–
45	Giraffe	–	K-29	–
46	Zimniy gigant	–	K-118	–
47	Kazachok (kyavar)	Russia	K-113	–
48	Kazimir	Germany	K-31	–

Table 1 (end)

No.	Cultivars*	Country of origin (if available)*	Acc. no. in FSCVC (if available)*	Acc. no. in VIR (if available)*
49	Karantanskiy	Russia	K-40	2001
50	Kilima	–	K-111	–
51	Columbus	Netherlands	–	–
52	Kyavar	Azerbaijan	K-61	2094
53	Letnii briz	–	K-33	–
54	Osenniy gigant	–	–	–
55	Pras	Russia	K-42	2038
56	Premier	Russia	–	–
57	Slon (Elephant)	–	K-36	–
58	Hobot slona (Elephant trunk)	–	K-37	–
59	Elefant	Czech Republic	–	–
60	–	Syria	K-43	2054
61	–	United Kingdom	K-44	2026
62	–	United Kingdom	K-63	2200
63	–	Canada	K-23	4895
64	–	–	K-51	5954
65	–	–	K-52	2563

* If available.

CATAGTCAGTAGCTAGA-3′), *psaB* (5′-TTCTGGG TGGAATTGCCAC-3′; 5′-ATAGCTCCATGAGCAAAG GC-3′), *psbA* (5′-AGTACGTGTGCTTGGGAGT-3′; 5′-TATAGCCCCTTCGTTCGAC-3′), *psbB* (5′-GTC TATTGCAATGCGATAAAG-3′; 5′-ACCATAGAA CTCAACAGTTAC-3′), *psbE* (5′-TCCCATCTTTCAC CGAATC-3′; 5′-AGTCCGTGAATAGCTAACC-3′), *petB* (5′-TGTGTTTCTGTTTGAGCCGT-3′; 5′-TATT GTTCCTTCCCGATAGG-3′). Праймеры для белок-кодирующих генов были разработаны на основе секвенированных пластомов видов *Allium* (Filyushin et al., 2016, 2018). Нуклеотидные последовательности выравнивали и анализировали в программе MEGA7.0 (https://www.megasoftware.net/).

Результаты и обсуждение

AFLP-анализ ядерного генома лука-порея. С использованием отобранных комбинаций праймеров E-ACT/M-CAAGCG и E-AGG/M-CAAGCG проведен AFLP-анализ ядерного генома 65 образцов лука-порея, включающих сорта отечественной и зарубежной селекции. В результате анализа и обработки полученных ДНК-спектров идентифицировано 760 фрагментов, 716 из которых были полиморфны для анализируемых образцов. Рассчитанные генетические расстояния между образцами лука-порея варьировали от 0.4 до 0.76, а между образцами лука-порея и видами, взятыми в качестве внешней группы, – от 0.65 до 0.82.

На дендрограмме (рис. 1) все образцы *A. porrum* формировали единый субкластер, а виды, взятые в качестве внешней группы, – сестринский субкластер. Выбор видов *A. ampeloprasum*, *A. commutatum* и *A. pyrenaicum* в качестве внешней группы обусловлен тем, что ряд исследователей объединяет их вместе с *A. porrum* и еще несколькими родственными видами в *Allium ampeloprasum* complex (Jones, Mann, 1963; Hirschegger et al., 2010; Guenaoui et al., 2013). Согласно системе APG IV, *A. porrum* – самостоятельный вид, в то время как ряд исследователей считает лук-порей подвидом *A. ampeloprasum* (Hirschegger et al., 2010; Guenaoui et al., 2013). Однако на дендрограмме образцы *A. ampeloprasum* не образуют единой группы с образцами *A. porrum*, а кластеризуются с образцами *A. commutatum* и *A. pyrenaicum* (см. рис. 1).

Анализ геномной структуры изучаемых образцов лука-порея в программе STRUCTURE 2.3.4 в сочетании с методикой (Evanno et al., 2005) показал их вероятное разделение на семь групп. Полученная диаграмма была наложена на AFLP-дендрограмму (см. рис. 1). Как видно на диаграмме, наборы ДНК-фрагментов образцов лука-порея отличаются друг от друга менее чем 10 % (примеси других цветов на красном фоне), при этом образцы *A. porrum*, формирующие на дендрограмме отдельные группы, имеют схожую геномную структуру (см. рис. 1).

Выявленный высокий уровень полиморфизма ядерного генома *A. porrum* логично было бы объяснить типом размножения (перекрестное опыление). Аналогично высокий уровень внутривидовой вариабельности был показан для перекрестно-опыляемого лука репчатого *A. cepa* (Karić et al., 2018). При этом интересно отметить, что геном чеснока *A. sativum*, который размножается только вегетативно, также является крайне вариабельным (Volk et al., 2004; Zhao et al., 2011; Egea et al., 2017). По всей видимости, высокий уровень полиморфизма ядерного генома характерен для видов *Allium* и может быть связан с насыщенностью мобильными элементами и повторяющимися последовательностями, о чем свидетельствуют проведенные ранее цитологические и геномные исследования некоторых видов *Allium* (Suzuki et al., 2001; Jakse et al., 2008; Vitte et al., 2013; Peška et al., 2019). Помимо этого, для некоторых сельскохозяйственных культур было по-

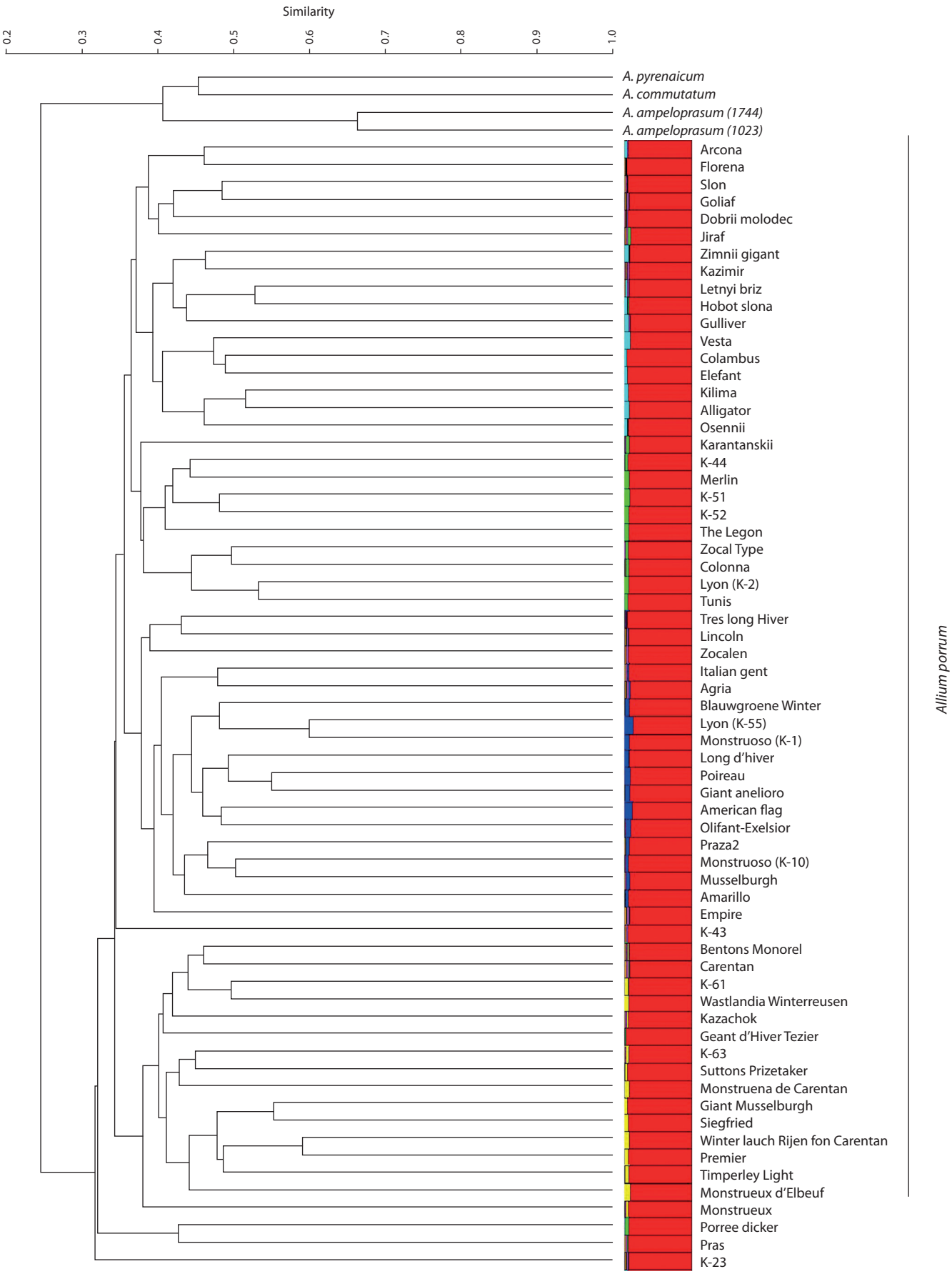

Fig. 1. UPGMA consensus tree of genetic differences in 65 *Allium porrum* accessions and 4 related species; diagram of the genomic structure of 65 analyzed leek accessions.

Table 2. Characteristics of the chloroplast genome regions analyzed in *A. porrum* accessions

Parameter	*rpl32-trnL*	*ndhJ-trnL*	*rps16* (intron)	*psaA*	*psaB*	*psbA*	*psbB*	*psbE*
Length, bp	793	837	844, 891, 913	2253	2205	1062	1527	252
SNP	2	3	0	0	1	0	0	0
Indels	0	0	2	0	0	0	0	0

казано, что уровень генетического разнообразия может быть связан с доместикацией и селекцией (Sun et al., 2018; Fu et al., 2019).

Анализ вариабельности участков хлоропластного генома. У изучаемых образцов лука-порея были амплифицированы и секвенированы девять участков хлоропластного генома, как некодирующие: межгенные спейсеры *rpl32-trnL* и *ndhJ-trnL*, интрон гена *rps16*, так и белок-кодирующие: гены *psaA*, *psaB*, *psbA*, *psbB*, *psbE*, *petB* (табл. 2).

Размеры межгенных спейсеров *rpl32-trnL* и *ndhJ-trnL* у всех исследуемых образцов лука-порея оказались инвариантны, в нуклеотидных последовательностях было выявлено два и три вариабельных сайта соответственно (см. табл. 2). Размеры этих участков у видов луков, взятых в качестве внешней группы, варьировали за счет наличия коротких инделей. Так, длина последовательности *rpl32-trnL* у образцов *A. ampeloprasum* составила 839 п. н., *A. commutatum* – 822 п. н., *A. pyrenaicum* – 785 п. н. Интересно, что в последовательностях *rpl32-trnL* и *ndhJ-trnL* у *A. ampeloprasum* обнаружены короткие индели и нуклеотидные замены, не встречающиеся у образцов *A. porrum*, но присутствующие у *A. commutatum*.

Длина последовательности интрона гена *rps16* у образцов лука-порея была различна. У большинства образцов интрон имел размер 913 п. н. У трех образцов *A. porrum* – Monstruoso (К-1), Wastlandia Winterreusen и Премьер – длина последовательности составила 891 п. н. Разница в длинах обусловлена тандемно повторяющимся 22-нуклеотидным фрагментом. У образцов видов *A. ampeloprasum*, *A. commutatum* и *A. pyrenaicum* этот повтор представлен одной копией. У единственного образца лука-порея – сорта Merlin – выявлена специфичная 69-нуклеотидная делеция, за счет чего длина интрона составила 844 п. н.

Последовательности шести генов фотосинтетического аппарата, кодирующих субъединицы фотосистем 1 и 2 (*psaA*, *psaB*, *psbA*, *psbB*, *psbE*), а также субъединицу цитохрома b (*petB*), были инвариантны по размеру у анализируемых образцов лука-порея и мономорфны (см. табл. 2). Единственная замена T327A в гене *psaB* была обнаружена у сортов Monstruoso (К-1), Wastlandia Winterreusen (К-62) и Казачок, однако она не приводила к замещению аминокислотного остатка. У образцов видов, взятых в качестве внешней группы, размеры изучаемых шести генов также были инвариантны.

Полученные для каждого образца последовательности были объединены и использованы для построения дендрограммы (рис. 2). На дендрограмме анализируемые образцы лука-порея образуют единый субкластер, базальную ветвь к которому формирует образец *A. pyrenaicum*. Образцы *A. ampeloprasum* и *A. commutatum* образуют

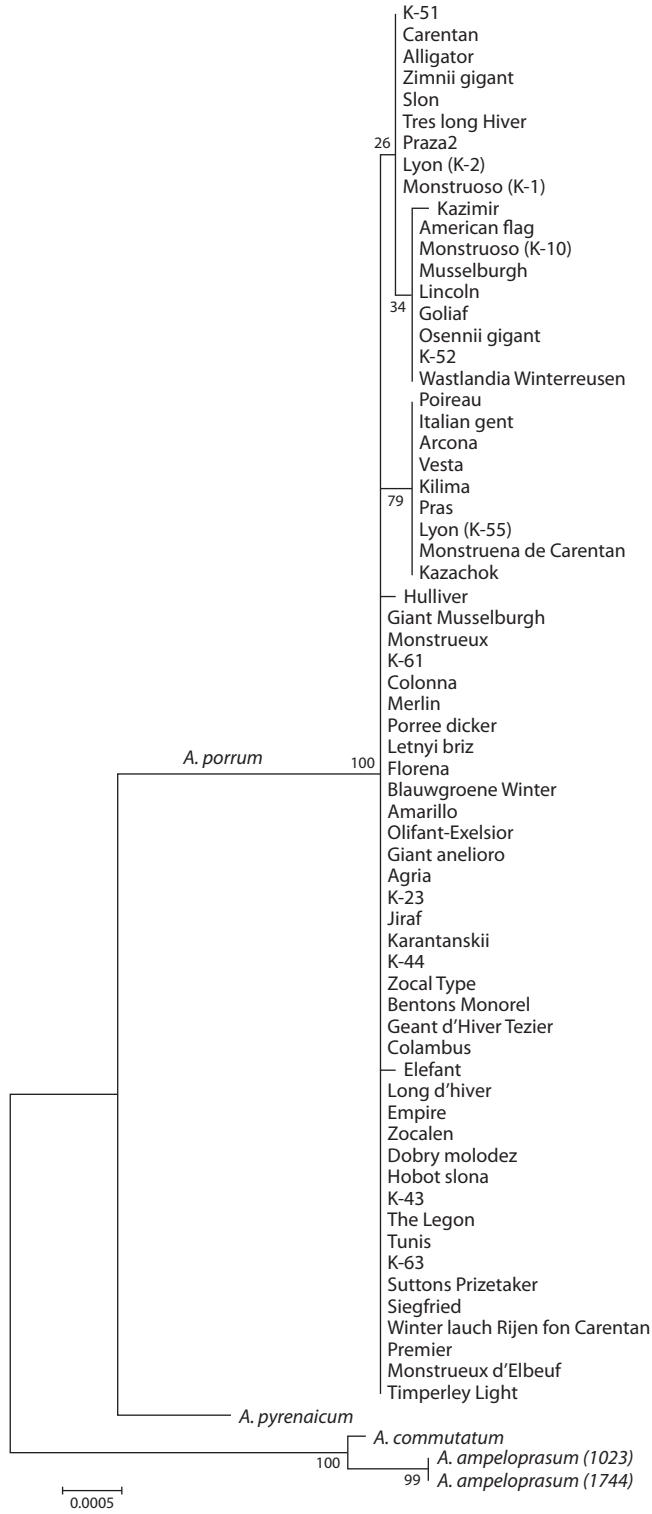

Fig. 2. Consensus tree based on nine regions of the chloroplast genome (MEGA7.0, Maximum Likelihood method, HKY model).

обособленный субкластер, в изученных последовательностях хлоропластного генома этих видов был обнаружен ряд общих инделей и SNP, отсутствующих у *A. porrum* и *A. pyrenaicum*, последовательности которых были высокогомологичны (99.62 %).

Анализ кодирующих и некодирующих участков хлоропластного генома суммарной длиной около 10 500 п. н. для каждого из 65 образцов лука-порея показал крайне низкий уровень их полиморфизма – всего 6 SNP.

Данные о внутривидовом полиморфизме участков пластома известны лишь для нескольких видов *Allium*. Так, анализ трех межгенных спейсеров у 24 образцов дикорастущих популяций *A. roseum* также выявил низкую вариабельность хпДНК (Guetat et al., 2010). Отсутствие внутривидовой вариабельности пластома обнаружено при исследовании небольших выборок (до 10 образцов) видов секции Rhizirideum (Sinitsyna et al., 2016). Низкий уровень вариабельности участков хлоропластного генома, по-видимому, – характерный признак для представителей *Allium*, что, как предполагается, может быть следствием гибридизации или интрогрессии хлоропластного генома (Hanelt, 1996; Li et al., 2010, 2016).

Заключение

В настоящей работе впервые на широкой выборке образцов изучен внутривидовой полиморфизм генома *A. porrum*. В результате выявлен высокий уровень полиморфизма ядерного генома лука-порея, сопоставимый с таковым у других сельскохозяйственно значимых видов *Allium*. Несмотря на то что лук-порей – популярная овощная культура, молекулярно-генетические исследования как всего генома в целом, так и отдельных локусов/генов ранее практически не проводились. Наши результаты по вариабельности ядерного генома *A. porrum* будут использованы для научно обоснованного подбора родительских пар для скрещивания с целью получения возможного гетерозисного эффекта в F_1 при селекции лука-порея в Федеральном научном центре овощеводства.

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Acknowledgements. The AFLP analysis was supported by the Russian Foundation for Basic Research, project 19-016-00054. The sequencing of chloroplast genome regions was supported by the program of the President of the Russian Federation for governmental support of young Russian Candidates of Sciences, project MK 3350.2019.11.

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

Received May 23, 2019. Revised July 1, 2019. Accepted July 15, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):910-915
DOI 10.18699/VJ19.566

The Fifth International Scientific Conference PlantGen2019

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

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

For citation: Davoyan E.R., Bespalova L.A., Davoyan R.O., Agaeva E.V., Bukreeva G.I., Zubanova Yu.S., Mikov D.S., Boldakov D.M. Allelic variants for *Waxy* genes in common wheat lines bred at the Lukyanenko National Grain Center. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7): 910-915. DOI 10.18699/VJ19.566

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

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

отобранных нами молекулярных маркеров для идентификации аллельного состояния генов *Wx-A1*, *Wx-B1*, *Wx-D1* может стать эффективной основой для селекции с помощью молекулярных маркеров (MAS) по данному признаку. Отобранные линии с идентифицированными в них нуль-аллелями генов *Wx* представляют интерес для селекционных программ, направленных на улучшение технологических качеств зерна и получение сортов мягкой пшеницы с новыми свойствами крахмала.
Ключевые слова: мягкая пшеница; молекулярные маркеры; гены *Wx*.

Introduction

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

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

Starch quality is closely related to the ratio between amylose and amylopectin, the two major constituents of starch. Granule-bound starch synthase (GBSSI) is the enzyme responsible for amylose synthesis in wheat grain. As some important technological properties of starch, such as gelatinization, bonding, and gelation, depend on the amylose/amylopectin ratio (Zeng et al., 1997). The GBSSI enzyme, or Waxy protein, has been the subject of many studies in recent years. In common wheat, this enzyme is encoded by three homologous *Waxy* (*Wx*) genes located on chromosomes 7A (locus *Wx-A1*), 7D (*Wx-D1*), and 4A (*Wx-B1*), (Shure et al., 1983; Chao et al., 1989; Yamamori et al., 1994). Each of the *Wx* genes has several allelic variants. The most common wild-type alleles are called *Wx-A1a*, *Wx-B1a*, and *Wx-D1a* (Yamamori et al., 1994; Nakamura et al., 1995). These alleles carry no mutations, and they intensely express GBSSI protein. Another type of allele (null allele) is nonfunctional and less common. It leads to a decrease in amylose content in starch. It is known that the *Wx-B1* gene has the greatest effect on amylose content in common wheat starch, followed by *Wx-D1* and *Wx-A1* (Yamamori et al., 1994). Functional alleles different from those of the wild type were also isolated, but their effects remain poorly understood. The presence of three null alleles for the *Wx-A1*, *Wx-B1*, and *Wx-D1* genes leads to complete elimination of GBSS1, absence of amylose synthesis, and the formation of amylopectin-type starch (Nakamura et al., 2002).

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

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

This article presents the results of molecular marker-assisted analysis of F_6 lines of common wheat for allelic variants of *Wx* genes. The purpose of the work was to select valuable genotypes carrying both individual null alleles and their combinations for their subsequent involvement in the breeding process aimed at obtaining varieties with improved technological qualities of grain.

Materials and methods

The study was conducted with 502 lines of common wheat generation F_6. The lines were obtained at NGC by crossing carriers of inactive (null) alleles *Wx-A1*, *Wx-B1*, *Wx-D1* to commercial varieties of common wheat. The mutant forms PI619381, PI619384, PI619376, PI619386, PI619377, and PI619378, in which the functional Waxy protein was not synthesized, were used as null alleles donors. These wheat forms were obtained from CIMMYT Turkey as part of a collaboration on the exchange of breeding material. The mutant forms had been created at the National Center for the Study of Small-Grain Germplasm, USDA-ARS, United States, by crossbreeding of Bai Huo common wheat varieties from China, a null allele carrier of the *Wx-D1* gene, to Kanto 107 and Ike varieties from the USA, carrying null alleles of the *Wx-A1* and *Wx-B1* genes. Varieties Starshina, Vassa, Utrish, Tabor, Esaul, Kuma, Grom, and Sila, raised at NGC, and variety Korotyshka, bred at the Belgorod Research Institute of Agriculture, were used as recipients. The crossing combinations of the lines under study are shown in Table 1.

DNA was isolated from 5 to 7-day-old etiolated wheat seedlings according to the method (Plaschke et al., 1995). The lines were genotyped for the allelic state of *Wx* genes by PCR. Primers were selected on the basis of literature data; their names and amplification conditions are presented in Table 2. The reaction mixture of the volume 25 µl contained 1× buffer for TaqDNA polymerase (50 mM KCl, 20 mM TrisHCl pH 8.4, 2–5 mM $MgCl_2$, and 0.01 % tween-20), 2 mM $MgCl_2$, 0.2 mM each dNTP, 12.5 pM each primer, 50 ng of DNA and 1 U of Taq polymerase.

Amplification was carried out according to the conditions recommended by the authors with minor modifications (see Table 2). PCR products were resolved by agarose gel elec-

Table 1. Crossing combinations of the lines under study (year of crossing 2012)

Cross combination No.	Crossbreeding combination
54	(PI 619381/Korotyshka) × Starshina
55	(PI 619384/Korotyshka) × Starshina
56	(PI 619376/Vassa) × Vassa
58	(PI 619384/Utrish) × Utrish
59	(PI 619384/Korotyshka) × Sila
60	(Esaul/PI 619386//Vassa) × Vassa
61	Tabor × (PI619381/Korotyshka/Starshina)
62	Grom × (PI619378/Korotyshka/Starshina)
70	(Esaul/PI 619381) × Esaul
71	(Esaul/PI 619377) × Kuma

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

Results

A total of 502 soft wheat generation F_6 lines were analyzed for the allelic state of the *Wx-A1*, *Wx-B1*, and *Wx-D1* genes with the use of molecular markers. The numbers of samples studied for each crossing combination, as well as the numbers of identified alleles, are presented in Table 3.

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

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

The null allele *Wx-B1b* was detected in one line obtained from cross combination No. 56, in four lines of combination No. 62, and in five lines of combination No. 71. It was also found that out of six mutant forms used as donors, the *Wx-B1b* allele was present in three: PI619376, PI619377, and PI619378. A functional allele of *Wx-B1e*, other than *Wx-B1a*, was detected in 108 lines. It was absent from combinations No. 56, 59, 62, and 71.

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

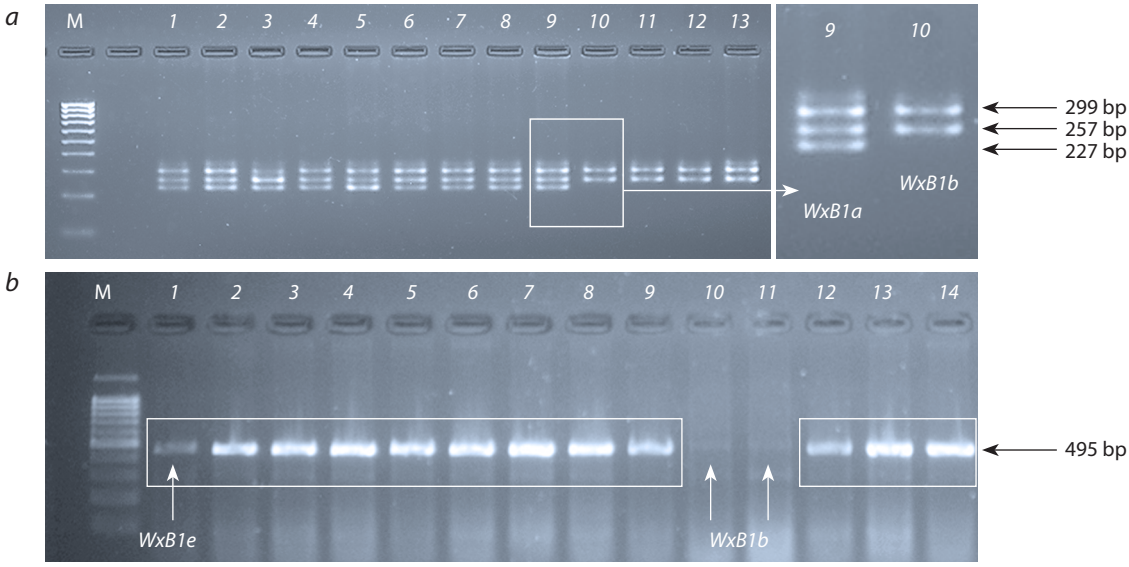
The combination of two null alleles (*Wx-A1b* + *Wx-D1b*) was detected in 19 lines from cross combination No. 58, in three lines in combinations No. 61 and 62, in two lines in combination No. 70, and in one line in combination No. 71. The presence of three null alleles (*Wx-A1b* + *Wx-B1b* + *Wx-D1b*),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

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

Gene	Primers	Annealing temperature, °C	Allele	Amplicon size, bp
WxA1	AFC and AR2 (Nakamura et al., 2002)	65	*Wx-A1a*	389
			Wx-A1b	370
WxB1	4F and 4R (McLauchlan et al., 2001)	65	*Wx-B1a*	3 fragments, 299, 257, 227
			Wx-B1b	2 fragments, 299, 257
	Wx-B1L and Wx-B1R (Vanzetti et al., 2009)	65	*Wx-B1a*	461
			Wx-B1b	Lack of fragment
			Wx-B1e	495
WxD1	Wx-D1-2-F and Wx-D1-2-R (Shariflou et al., 2001)	55	*Wx-D1a*	900
			Wx-D1b	279

Table 3. Numbers of crossing combinations and lines with identified null alleles *Wx-A1b*, *Wx-B1b*, and *Wx-D1b* and the functional allele *Wx-B1e*

Cross combination No.	Number of lines	Numbers of lines with identified alleles			
		Wx-A1b	*Wx-B1b*	*Wx-B1e*	*Wx-D1b*
54	26	26		22	17
55	45	27		20	4
56	27	11	1		
58	43	22		1	17
59	33				
60	62			5	1
61	47	17		13	4
62	60	9	4		3
70	100	3		47	42
71	59	7	5		12
	502	122	10	108	100



Electrophoretic image of PCR products with primers:

a – 4F and 4R (McLauchlan et al., 2001). *1–12* – line crossing combination No. 54; *13* – K+ (mutant form PI619381); *b* – Wx-B1L and Wx-B1R (Vanzetti et al., 2009). *1* – K+ (mutant form PI619381); *2–9*, *12–14* – lines of crossing combination 54; *10* – mutant form PI619377; *11* – mutant form PI619378. M – molecular weight ladder.

Discussion

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

Table 4. The ordinal numbers of the combination of crosses and the numbers of lines with the identified combination of alleles of *Wx* genes

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

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

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

Conclusion

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

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

Received May 31, 2019. Revised July 19, 2019. Accepted July 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):916-925
DOI 10.18699/VJ19.567

The Fifth International Scientific Conference PlantGen2019

Фенотипическое проявление аллеля низкостебельности *Rht-B1p* (*Rht-17*) у яровой твердой пшеницы в двух климатических условиях

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Аллели генов, определяющие низкостебельность, играют большую роль в селекции твердой пшеницы, так как не только снижают высоту растений, обеспечивая их устойчивость к полеганию, но и обладают рядом плейотропных эффектов. Твердая пшеница несет два субгенома, A и B, что ограничивает использование аллелей генов субгенома D и требует расширения арсенала аллелей низкостебельности и изучения их влияния на высоту и агрономически важные признаки. В настоящей работе изучали фенотипическое проявление аллеля *Rht-B1p* (*Rht-17*) в семьях $B_2F_{2:3}$, полученных в результате скрещивания Chris Mutant/#517//LD222 в полевом опыте в Москве и Краснодаре. Показано, что растения, гомозиготные по аллелю *Rht-B1p*, по сравнению с растениями, несущими аллель дикого типа *Rht-B1a*, были ниже на 36.3 см (40 %) в Москве и на 49.5 см (48 %) в Краснодаре. В полевом опыте в Краснодаре у растений с *Rht-B1p* было на одно междоузлие меньше, чем у растений дикого типа, что также внесло вклад в снижение высоты растений. Масса зерна в главном колосе у растений с аллелем *Rht-B1p* была ниже, чем у растений с *Rht-B1a*, на 12 % в Москве и на 23 % в Краснодаре из-за снижения массы 1000 зерен в обоих регионах проведения полевого опыта. Число зерен в главном колосе у растений с *Rht-B1p* было выше по сравнению с растениями с *Rht-B1a* на 6.5 % в Москве благодаря увеличению числа колосков в главном колосе и на 11 % в Краснодаре вследствие большей озерненности колоска. Колошение у растений с аллелем низкостельности *Rht-B1p* по сравнению с растениями с аллелем дикого типа *Rht-B1a* в Краснодаре наступило позже в среднем на семь дней. Обсуждаются возможность и перспективы использования *Rht-B1p* в селекции твердой пшеницы.
Ключевые слова: твердая пшеница; молекулярные маркеры; плеойтропное действие гена; полевой опыт; гены низкостебельности; агрономически ценные признаки; высота растения.

Для цитирования: Черноок А.Г., Крупин П.Ю., Беспалова Л.А., Панченко В.В., Ковтуненко В.Я., Баженов М.С., Назарова Л.А., Карлов Г.И., Крупина А.Ю., Дивашук М.Г. Фенотипическое проявление аллеля низкостебельности *Rht-B1p* (*Rht-17*) у яровой твердой пшеницы в двух климатических условиях. Вавиловский журнал генетики и селекции. 2019;23(7):916-925. DOI 10.18699/VJ19.567

Phenotypic effects of the dwarfing gene *Rht-17* in spring durum wheat under two climatic conditions

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Alleles of the genes, conferring a dwarfing phenotype, play a crucial role in wheat breeding, as they not only reduce plant height, ensuring their resistance to lodging, but also have a number of positive and negative pleiotropic effects on plant productivity. Durum wheat carries only two subgenomes (A and B), which limits the use of the D-subgenome genes and requires the expansion of the arsenal of dwarfing alleles and the study of their effects on height and agronomically important traits. We studied the effect of the gibberellin-insensitive allele *Rht-B1p* in the $B_2F_{2:3}$ families, developed by crossing Chris Mutant /#517//LD222 in a field experiment in Moscow and Krasnodar. In our experiments, plants homozygous for *Rht-B1p* were shorter than those homozygous for the wild-type allele *Rht-B1a* by 36.3 cm (40 %) in Moscow and 49.5 cm (48 %) in Krasnodar. In the field experiment in Krasnodar, each plant with *Rht-B1p* had one less internode than any plant with *Rht-B1a*, which additionally contributed to the decrease in plant height. Grain weight per main spike was lower in plants with *Rht-B1p* than in plants with *Rht-B1a* by 12 % in Moscow and by 23 % in Krasnodar due to a decrease in 1000 grain weight in both regions of the field experiment. The number of grains per main spike in plants with *Rht-B1p* was higher in comparison to that with *Rht-B1a* by 6.5 % in Moscow due to an increase in spikelet

number per main spike and by 11 % in Krasnodar due to an increase in grain number per spikelet. The onset of heading in plants with *Rht-B1p* in comparison with the plants with the wild-type allele *Rht-B1a* was 7 days later in Krasnodar. The possibility and prospects for the use of *Rht-B1p* in the breeding of durum wheat are discussed.
Key words: durum wheat; molecular markers; pleotropic gene effect; plot experiment; dwarfing genes; valuable agronomic traits; plant height.

For citation: Chernook A.G., Kroupin P.Yu., Bespalova L.A., Panchenko V.V., Kovtunenko V.Ya., Bazhenov M.S., Nazarova L.A., Karlov G.I., Kroupina A.Yu., Divashuk M.G. Phenotypic effects of the dwarfing gene *Rht-17* in spring durum wheat under two climatic conditions. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7): 916-925. DOI 10.18699/VJ19.567 (in Russian)

Введение

Высота пшеницы – сложный признак, который определяется различными группами генов и связан с морфологией и фертильностью колоса, временем цветения, продуктивностью растения и качеством зерна (Chen et al., 2016; Würschum et al., 2018a, b). Одним из путей повышения урожайности пшеницы, особенно в условиях интенсивного земледелия, является оптимизация высоты растений за счет создания низкостебельных сортов (Лукьяненко, 1970; Беспалова, 2001; Divashuk et al., 2013; Grover et al., 2018). Наибольшее значение в создании форм пшеницы с оптимальной высотой растений в настоящее время имеют аллельные варианты генов, определяющих высоту растения, обладающие фенотипическим эффектом снижения высоты растений. Среди многообразия генетических факторов, отвечающих за высоту растения пшеницы, особое место занимают аллельные варианты гена *Rht* (Reduced height), представленного у мягкой пшеницы тремя гомеологами: *Rht-A1*, *Rht-B1* и *Rht-D1*. Эти гены кодируют белок DELLA, который подавляет рост клеток и репрессирует гиббереллиновый сигнал (Peng et al., 1999). Гиббереллин активирует рост клеток, запуская деградацию DELLA и снимая репрессию с генов роста. Дикий тип аллелей генов *Rht-A1a*, *Rht-B1a* и *Rht-D1a* не имеет собственного фенотипического проявления. Мутации в этих генах могут приводить к образованию белков DELLA с повышенной стабильностью, которые не подвергаются гиббереллин-опосредованному протеолизу. Фенотипический эффект таких мутантных аллелей – растения со сниженной высотой, которую нельзя восстановить внешней обработкой гиббереллинами (гиббереллин-нечувствительный фенотип) (Chebotar et al., 2012; Билова и др., 2016; Van De Velde et al., 2017).

Умеренное снижение высоты растений как результат экспрессии таких аллелей позволяет повысить урожайность благодаря следующим факторам: низкорослые растения более устойчивы к полеганию, вследствие чего уменьшаются потери при уборке; у сортов, устойчивых к полеганию, можно увеличить норму высева по сравнению с неустойчивыми, а, следовательно, увеличить стеблестой на единицу площади; у низкорослых растений происходит перераспределение ассимилятов в пользу развивающегося колоса, а не вегетативных органов (Лукьяненко, 1970; Беспалова, 2001; Hedden, 2003). Аллели генов, определяющие низкостебельность, *Rht-B1b* (*Rht1*) и *Rht-D1b* (*Rht2*), сыграли большую роль в Зеленой революции. Благодаря их использованию в селекции в Мексике, США и Европе были созданы продуктивные сорта, устойчивые к полеганию при высоких дозах удобрений и орошении, что позволило отказаться от применения ретардантов (Кулаева, 2000; Hedden 2003; Borojevic K., Borojevic K., 2005; Knopf et al., 2008). Аллели *Rht-B1b* и *Rht-D1b* наряду с другими аллелями низкостебельности получили большое распространение среди отечественных сортов мягкой пшеницы (Беспалова и др., 2012; Divashuk et al., 2013; Миков и др., 2018). Что касается твердой пшеницы, то в отечественной селекции в последние 20 лет отмечен значительный рост продуктивности сортов благодаря интрогрессии *Rht-B1b* и *Rht-B1e* (Самофалова и др., 2014; Мудрова, Яновский, 2016), а в современных итальянских и испанских сортах наибольшее распространение получил аллель *Rht-B1b*, который увеличил урожайность за счет озерненности колоска (Alvaro et al., 2008). В современных сортах представлены преимущественно эти два аллеля, несмотря на разнообразие мутантных аллелей низкостебельности (Alvaro et al., 2008; Мальчиков, 2009).

Помимо преимуществ, которые дает *Rht-B1b*, он обладает и рядом недостатков. Так, растения твердой пшеницы, несущие этот аллель, имеют более короткий колеоптиль по сравнению с растениями с аллелем *Rht-B1a*, что ограничивает глубокую заделку семян в регионах с недостатком почвенной влаги в период посева (Trethowan et al., 2001). В условиях засухи высокорослые растения твердой пшеницы с аллелем *Rht-B1a* могут показать более высокую продуктивность, чем растения с *Rht-B1b* (Mathews et al., 2006). Вместе с тем растения твердой пшеницы с *Rht-B1b* давали зерно с меньшим весом и более низким содержанием белка, что может быть серьезной проблемой (McClung et al., 1986; Zaccai et al., 1987). Разнообразие аллелей гена *Rht-B1*, которые можно использовать для снижения высоты растений пшеницы твердой, задействовано в настоящее время не полностью. Продолжается активная работа по расширению разнообразия генов низкостебельности, вовлеченных в создание новых сортов твердой пшеницы, и изучению их влияния на хозяйственно ценные признаки (Watanabe, 2008; Мальчиков и др., 2017; Vikhe et al., 2017, 2019). Сравнительно недавно с молекулярной точки зрения был описан аллель низкостебельности *Rht-B1p*, ранее обозначаемый как *Rht-17* (Bazhenov et al., 2015).

Московская область (Центральный район Нечерноземной зоны) и Краснодарский край являются важными для экономики с точки зрения производства зерна для нужд населения, животноводства и перерабатывающей промышленности. Они отличаются по почвенно-климатическим условиям и позволяют оценить фенотипическое проявление аллеля в различных условиях. Настоящее исследование направлено на изучение фенотипического эффекта аллеля *Rht-B1p* на высоту растений и другие хозяйственно ценные признаки у пшеницы твердой в полевом опыте, проведенном параллельно в Москве и Краснодаре.

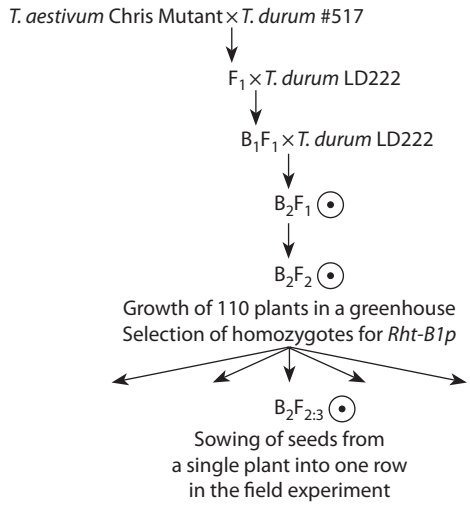

Fig. 1. Cultivation of plant material for the field experiment.
The dot-centered circle indicates self-pollination.

Материалы и методы

Растительный материал. В качестве исходного материала нами использовалась популяция B_2F_2 Chris Mutant/#517//LD222, полученная от профессора N. Watanabe из Университета Ибараки (факультет агрономии, Япония) (Bazhenov et al., 2015) (рис. 1). Низкостебельная линия мягкой пшеницы Chris Mutant служит донором аллеля *Rht-B1p*, она получена путем химического мутагенеза из сорта Chris (Heiner, Elsayed, 1974).

Растения популяции B_2F_2 выращивали в количестве 110 шт. в теплице Центра молекулярной биотехнологии (Российский государственный аграрный университет – МСХА им. К.А. Тимирязева) при дозированном поливе и внесении равных доз удобрений. У каждого индивидуального растения B_2F_2 с помощью молекулярного маркера (см. раздел Молекулярный анализ) определяли аллельное состояние гена *Rht-B1*, после чего отбирали растения-гомозиготы по аллелям *Rht-B1p* и *Rht-B1a*. Гомозиготные растения по достижении ими фазы полной спелости обмолачивали вручную, семена, собранные с отдельного растения B_2F_2, принимали за одну семью. В результате для посева в полевом опыте были отобраны семена от растений, гомозиготных по аллелям *Rht-B1a* (25 семей) и *Rht-B1p* (18 семей). Семена от каждой семьи были разделены на две части для посева в полевом опыте в двух регионах (Москва и Краснодар).

Полевой опыт с растениями $B_2F_{2:3}$ проводили в Москве на Полевой опытной станции РГАУ–МСХА им. К.А. Тимирязева (55°50′ с. ш., 37°33′ в. д.) и Краснодаре на земельном участке Национального центра зерна им. П.П. Лукьяненко (45°.41′ с. ш., 38°.55′ в. д.) в 2018 г. (далее Москва и Краснодар соответственно). Москва (Центральный район Нечерноземья, умеренно континентальный климат) отличается большим количеством осадков, умеренными температурами и дерново-подзолистыми почвами, а для Краснодарского края (Северо-Кавказский район, мягкий континентальный климат) типичны высокие температуры при обильных осадках и черноземные почвы, при этом для него характерны резкие погодные изменения. Погодные условия (температура и осадки) во время проведения полевого опыта от посева до окончательной уборки показаны в табл. 1. Длина светового дня в период выращивания растений в условиях Москвы составляла 15:35 на момент посева (5 мая), увеличивалась до 17:33 (24 июня), далее снижаясь до 14:49 ко дню окончательной уборки (18 августа); средняя длина дня составила 16:40 (длинный световой день). Длина светового дня в период выращивания растений в Краснодаре составляла 12:10 на момент посева (21 марта) и увеличивалась, достигнув 15:34, ко дню окончательной уборки (30 июня); средняя длина дня составила 14:23 (короткий световой день). Посев осуществляли в Москве 5 мая, в Краснодаре – 21 марта 2018 г. Посев в двух регионах производили кассетным способом селекционной сеялкой СКС-6-10 при следующих параметрах: длина делянки 1 м, в 4 рядка с расстоянием между рядками 30 см (Москва) или 40 см (Краснодар), расстояние между делянками 50 см. Сорняки выпалывали вручную, проводили необходимую обработку пестицидами для защиты растений от вредителей. Каждое растение убирали вручную отдельно, по достижению им фазы полной спелости; окончательный день уборки в Москве пришелся на 19 августа, в Краснодаре – на 30 июня. Обмолот проводили на колосовой молотилке МКС-1М (ВИМ-МЗОК, Москва, Россия).

Фенотипирование. Выполняли структурный анализ каждого индивидуального растения по следующим фенотипическим признакам: высота растений (см), длина

Table 1. Agrometeorological conditions during the field experiment conducted in Moscow and Krasnodar in 2018

Month	Moscow May 5 – August 19, 2018			Krasnodar March 21 – June 30, 2018		
	Sum of active temperatures	Mean temperature	Sum of precipitation	Sum of active temperatures	Mean temperature	Sum of precipitation
March	–	–	–	142	6.4	38
April	–	–	–	2845	13.8	26
May	3288	16.1	105	4731	19.4	43
June	3914	17.3	107	5407	24.1	11
July	5097	20.5	190	–	–	–
August	2926	20.3	39	–	–	–
Total	15225	–	441	13125	–	118

каждого междоузлия (см), количество междоузлий, длина главного колоса (см), число колосков в главном колосе, масса зерна с главного колоса (г), число зерен с главного колоса (г), число зерен в колосе (отношение числа зерен в главном колосе к числу колосков в главном колосе); вегетативная масса надземной части главного побега до обмолота. На основании измеренных величин рассчитывали следующие показатели: плотность колоса (число колосков в главном колосе на 10 см длины колосового стрежня), масса 1000 зерен (тысячекратная масса зерен с главного колоса по отношению к числу зерен с главного колоса), озерненность колоска (число зерен в главном колосе по отношению к числу колосков в главном колосе), уборочный индекс (Кхоз, отношение массы зерен с главного колоса к вегетативной массе надземной части главного побега до обмолота). Дату наступления колошения определяли в целом для отдельной семьи визуально. Из каждой семьи анализировали по 15 растений. Подсчет семян осуществляли с помощью приложения SeedCounter (Komyshev et al., 2017).

Молекулярный анализ. Из каждого индивидуального вегетирующего растения из листа была выделена ДНК и с помощью молекулярных маркеров установлено аллельное состояние гена *Rht-B1*. Геномную ДНК экстрагировали из индивидуальных вегетирующих растений CTAB методом (Bernatzky, 1986). Аллельное состояние гена *Rht-B1* определяли методом ПЦР с применением двух пар праймеров для каждого из изучаемых аллелей: для выявления аллеля *Rht-B1a* использовали пару праймеров Rht-B1-R1a и BF; а аллеля *Rht-B1p* – пару праймеров Rht-B1p-R и BF (табл. 2). Праймеры синтезированы ООО «Синтол» (Москва, Россия). Таким образом, каждое растение проверяли дважды: на наличие *Rht-B1a* и на присутствие *Rht-B1p*.

Состав реакционной смеси и условия ПЦР соответствовали протоколу, описанному в (Bazhenov et al., 2015). Реакцию ПЦР проводили в приборе GeneAmp PCR System 9700 (Applied Biosystems, Фостер Сити, Калифорния, США). Размер ампликонов устанавливали методом электрофореза в 1.5 % агарозном геле с добавлением бромистого этидия в Трис-борат-ЭДТА буфере и последующей визуализацией геля в системе Gel Doc XR+ (Bio-Rad Laboratories, Inc., Геркулес, Калифорния, США). В качестве маркера размеров применяли маркер GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Уолтем, Массачусетс, США). Размер целевого продукта амплификации при использовании обеих пар праймеров составлял 226 п. н. (Bazhenov et al., 2015).

Статистический анализ. Для каждого фенотипического признака определяли среднее значение и стандартное отклонение. Полученные данные обрабатывали с помощью двухфакторного дисперсионного анализа: фактор 1 – аллельное состояние гена *Rht-B1*, фактор 2 – регион проведения полевого опыта. Значимость разности между средними значениями по всем изучаемым признакам у растений, гомозиготных по *Rht-B1a* и *Rht-B1p*, в двух регионах проведения полевого опыта выявляли посредством критерия наименьшей существенной разности на 95 % уровне доверительной вероятности ($НСР_{0.05}$). Для визуализации влияния *Rht-B1p* на высоту, массу зерен с главного колоса, числа зерен с главного колоса и массу

Table 2. Primers for identification of the *Rht-B1a* and *Rht-B1p* alleles

Primer	Nucleotide sequence
BF	5′-GGT AGG GAG GCG AGA GGC GAG-3′
Rht-B1a-R	5′-CCA TCT CCA GCT GCT CCA GCT TAT G-3′
Rht-B1p-R	5′-CCA TCT CCA GCT GCT CCA GCT TAT A-3′

1000 зерен нами был проведен анализ методом главных компонент. Результаты анализа интерпретировали на основании учебных пособий по статистике (Доспехов, 1985; StatSoft, Inc., 2012). Все анализы осуществляли с помощью программы Statistica 12.0 (StatSoft, Inc., Талса, Оклахома, США).

Фенотипический эффект *Rht-B1p* относительно *Rht-B1a* определяли как разницу между средними (Ср) для каждого фенотипического признака у групп растений, гомозиготных по аллелям *Rht-B1p* (генотип *Rht-B1p Rht-B1p*) и *Rht-B1a* (генотип *Rht-B1a Rht-B1a*). Значение фенотипического эффекта *Rht-B1p*, нормированное относительно *Rht-B1a* (НФЭ), определяли как указанную разницу относительно среднего значения изучаемого фенотипического признака у группы растений, гомозиготных по аллелю *Rht-B1a* (генотип *Rht-B1a Rht-B1a*), выраженную в процентах:

$$НФЭ\ Rht\text{-}B1p\ (\%) = \frac{Ср(RhtB1p\,RhtB1p) - Ср(RhtB1a\,RhtB1a)}{Ср(RhtB1a\,RhtB1a)} \cdot 100.$$

Результаты

Молекулярный анализ. В результате молекулярного анализа нами идентифицировано аллельное состояние гена *Rht-B1* у отдельных растений популяции B_2F_2 (Приложение 1)[1]. На основании полученных данных были отобраны растения, гомозиготные по аллелям *Rht-B1a* (25 растений) и *Rht-B1p* (18 растений). Семена с этих растений были разделены на две части и высеяны в Москве и Краснодаре в полевом опыте. Проведено изучение фенотипического проявления *Rht-B1p* на основные агрономически ценные признаки у твердой пшеницы в условиях Москвы и Краснодара.

Высота и междоузлия. Дисперсионный анализ результатов полевого опыта в Москве и Краснодаре показывает значимость на 5 % уровне влияния аллельного состояния *Rht-B1*, региона проведения полевого опыта, а также их взаимодействия на высоту растений (Приложение 2). Оценка значимости разностей между средними значениями изучаемых признаков у растений, гомозиготных по *Rht-B1a* и *Rht-B1p*, с помощью критерия $НСР_{0.05}$ дала следующие результаты. Растения с аллелем дикого типа *Rht-B1a* значимо выше растений с аллелем дикого типа *Rht-B1a* в Москве – на 13.7 см. В то же время растения с аллелем низкостебельности *Rht-B1p* в Краснодаре и Москве существенно не отличаются друг от друга по высоте (табл. 3, Приложение 3). Растения с аллелем *Rht-B1p* по сравнению с растениями, несущими аллель дикого типа *Rht-B1a*, были ниже на 36.3 см (40 %) в Москве и на 49.5 см (48 %) в Краснодаре (см. табл. 3, Приложение 2). При

[1] Приложения 1–4 см. по адресу:
http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx21.pdf

Table 3. The effect of *Rht-B1b* on the main agronomic traits in plants in the $B_2F_{2:3}$ (Chris M1/#517 × LD222) families of durum wheat

Agronomic traits	Moscow		Krasnodar	
	Rht-B1a Rht-B1a	*Rht-B1p Rht-B1p*	*Rht-B1a Rht-B1a*	*Rht-B1p Rht-B1p*
Plant height, cm	89.8 ± 14.6a*	53.5 ± 8.9c	103.5 ± 5.6b	53.9 ± 3.3c
Length of peduncle, cm	48.4 ± 11.3a	26.0 ± 7.3b	51.6 ± 3.9c	24.6 ± 1.9b
Length of the second upper internode, cm	17.0 ± 3.1a	10.1 ± 2.2b	17.7 ± 1.2a	9.2 ± 1.3b
Length of the third upper internode, cm	9.5 ± 1.6a	5.5 ± 1.3b	12.7 ± 1.2c	6.4 ± 0.7d
Length of the fourth upper internode, cm	–	–	9.0 ± 1.5a	5.0 ± 0.7b
Length of the second lower internode, cm	6.82 ± 1.37a	4.00 ± 1.07b	5.11 ± 2.35c	3.96 ± 0.92b
Length of the first lower internode, cm	3.75 ± 1.48a	2.81 ± 2.09b	1.36 ± 1.03c	1.42 ± 0.83c
Number of internodes	4.6 ± 0.5a	4.4 ± 0.7b	5.7 ± 0.5c	5.0 ± 0.3d
Main spike length, cm	6.8 ± 1.0d	7.0 ± 0.8c	9.8 ± 0.7a	9.5 ± 0.6b
Number of spikelets per spike	15.9 ± 2.0a	16.5 ± 1.7b	18.9 ± 1.4c	18.2 ± 1.6d
Spike compactness	23.5 ± 3.7a	23.6 ± 2.5a	19.41 ± 1.5b	19.15 ± 1.5b
Grain weight per spike, g	1.32 ± 0.45a	1.17 ± 0.42b	1.62 ± 0.25c	1.25 ± 0.30d
Grain number per spike	30.4 ± 8.0a	32.4 ± 9.5b	36.7 ± 5.1c	40.9 ± 7.7d
1000 grain weight, g	42.9 ± 7.0a	35.9 ± 6.7b	44.5 ± 5.5c	30.8 ± 5.4d
Spike fertility (grain number per spikelet)	1.91 ± 0.02a	1.96 ± 0.04a	1.94 ± 0.02a	2.25 ± 0.03b
Harvest index	0.50 ± 0.11b	0.55 ± 0.07a	0.37 ± 0.04d	0.41 ± 0.07c
Plant biomass, g	2.7 ± 0.9a	2.1 ± 0.6a	4.4 ± 0.5a	3.0 ± 0.5a
Heading date (days after sowing)	57.2 ± 0.8a	58.9 ± 1.8b	66.5 ± 0.8c	73.7 ± 1.2d

Data are presented in the tables as mean ± standard error.
* The means labeled with different letters are different at $HCP_{0.05}$.

сравнении длины междоузлий наибольшая абсолютная разность между растениями, гомозиготными по *Rht-B1p* и по *Rht-B1a*, была выявлена по первому верхнему междоузлию. Первое верхнее междоузлие у растений, гомозиготных по *Rht-B1p*, короче, чем у растений, гомозиготных по *Rht-B1a*, в Москве на 22.4 см (46 %), а в Краснодаре – на 27.0 см (52 %) (см. табл. 3, Приложение 3).

По результатам дисперсионного анализа показана значимость на 5 % уровне влияния аллельного состояния *Rht-B1*, региона проведения полевого опыта и их взаимодействия на количество междоузлий (см. Приложение 2). По сравнению с растениями, выращенными в Краснодаре, у растений с аллелем дикого типа *Rht-B1a* в Москве было в среднем на одно междоузлие меньше, а у низкорослых с аллелем *Rht-B1p* – в среднем на 0.6 междоузлий меньше (см. табл. 3, Приложение 3). У растений с *Rht-B1p* количество междоузлий в условиях Москвы было на 0.2 шт. меньше, чем у растений с *Rht-B1a*, а в Краснодаре – на 0.7 шт.; разница наблюдалась по четвертому верхнему междоузлию (см. табл. 3, Приложение 3). В опыте в Москве четвертое верхнее междоузлие было только у 1 % растений обоих генотипов (рис. 2). В опыте в Краснодаре четвертое верхнее междоузлие имели 69 % растений-гомозигот по *Rht-B1a*, и только 4 % гомозигот – по *Rht-B1p*.

Строение и продуктивность колоса. Результаты дисперсионного анализа данных полевого опыта показывают значимость на 5 % уровне влияния региона проведения полевого опыта и взаимодействия региона и аллельного

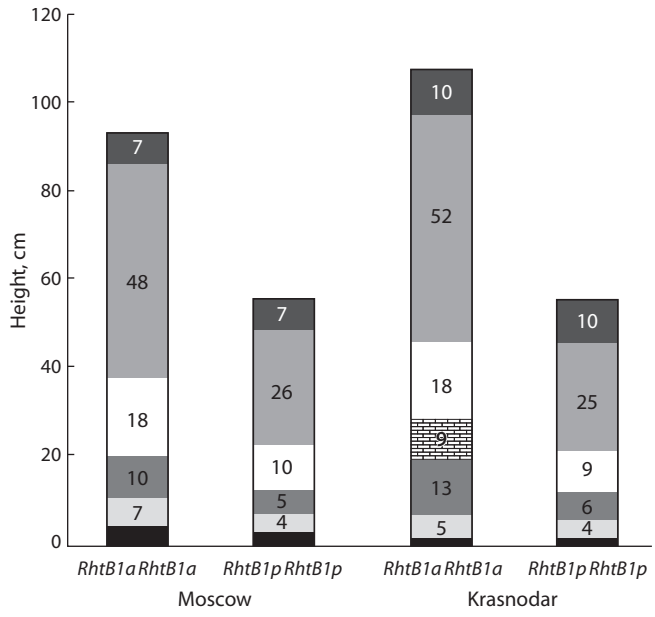

Fig. 2. The lengths of internodes of the main culm and main spike of durum wheat plants in $B_2F_{2:3}$ (Chris M1/#517 × LD222) families in the field plot experiment in Moscow and Krasnodar.

Y axis, plant height (cm); *X* axis, genotype (*Rht-B1a Rht-B1a* and *Rht-B1p Rht-B1p*). From top to bottom: main spike peduncle, 2nd upper internode, 3rd upper internode, 4th upper internode (in *Rht-B1a Rht-B1a* in Krasnodar only, shown with texture), 2nd lower internode, 1st lower internode.

состояния *Rht-B1* на длину колоса и число колосков, а также региона – на плотность колоса (см. Приложение 2). Колос у растений как с аллелем *Rht-B1a*, так и с аллелем *Rht-B1p*, в Краснодаре имеет значимо большую длину и число колосков и значимо меньшую плотность. Однако разница по числу колосков у низкостебельных растений с аллелем *Rht-B1p* между Москвой и Краснодаром заметно меньше (см. табл. 3, Приложение 3).

Хотя длина главного колоса у растений, гомозиготных по *Rht-B1p*, в Москве и Краснодаре значимо меньше длины главного колоса у растений, на 2 и 3 мм соответственно, однако это различие в условиях полевого опыта не играет существенной роли (см. табл. 3, Приложение 3). Число колосков у растений, гомозиготных по *Rht-B1p*, по сравнению с растениями, гомозиготными по *Rht-B1a*, было больше на 0.6 шт. (4 %) в Москве и меньше на 0.7 шт. (4 %) в Краснодаре (см. табл. 3, Приложение 3).

По результатам дисперсионного анализа показана значимость на 5 % уровне влияния аллельного состояния *Rht-B1*, региона проведения полевого опыта и их взаимодействия на массу 1000 зерен, озерненность колоска, массу и число зерен в колосе (см. Приложение 2). Растения с аллелем *Rht-B1a* в условиях опыта в Москве не отличаются от таковых в условиях опыта в Краснодаре по озерненности колоска, но существенно уступают им по числу зерен в колосе (за счет меньшего числа колосков в колосе) и массе 1000 зерен. Таким образом, масса зерен в главном колосе у растений с аллелем *Rht-B1a* на 18.4 % ниже в Москве, чем в Краснодаре. Анализ растений с аллелем *Rht-B1p* показывает, что в условиях опыта в Москве растения имеют значимо меньшие озерненность колоска и число зерен в колосе по сравнению с Краснодаром, но благодаря тому, что масса 1000 зерен у низкостебельных растений в Москве выше, чем в Краснодаре, разница по массе зерен в главном колосе между Москвой и Краснодаром почти в два раза меньше, чем между растениями с аллелем дикого типа *Rht-B1a*, и составляет 7 %. Таким образом, продуктивность колоса у растений в Краснодаре выше, чем в Москве, как у растений с аллелем *Rht-B1a*, так и с аллелем *Rht-B1p*, при этом различие между низкорослыми растениями меньше, чем между высокорослыми. Это объясняется тем, что снижение массы 1000 зерен под влиянием *Rht-B1p* более сильное в опыте в Краснодаре (до 30.8 г), чем в Москве (до 35.9 г) (см. табл. 3, Приложение 3).

Масса зерен главного колоса у растений, гомозиготных по *Rht-B1p*, была статистически значимо ниже, чем у растений, гомозиготных по *Rht-B1a*, в обоих регионах проведения полевого опыта: в Москве на 0.2 г (12 %), в Краснодаре на 0.4 г (23 %). Масса 1000 зерен у растений, гомозиготных по *Rht-B1p*, была статистически значимо ниже, чем у растений, гомозиготных по *Rht-B1a*, в обоих регионах проведения полевого опыта: в Москве на 7.0 г (16 %), в Краснодаре на 13.7 г (31 %). Число зерен в колоске (озерненность) в Москве не различалось между генотипами *Rht-B1p* и *Rht-B1a*; в Краснодаре у растений с *Rht-B1p* число зерен в колоске было на 0.3 больше, чем у растений с *Rht-B1a*. Число зерен главного колоса у растений, гомозиготных по *Rht-B1p*, было статистически значимо выше, чем у растений, гомозиготных по *Rht-B1a*, в обоих регионах проведения полевого опыта: в Москве на 2.0 шт. (6.5 %), в Краснодаре на 4.2 шт. (11 %) (см. табл. 3, Приложение 3).

Таким образом, в результате влияния *Rht-B1p* в Москве наблюдалось увеличение числа зерен в колосе за счет увеличения числа колосков, а в Краснодаре – за счет увеличения озерненности колоска относительно растений с аллелем *Rht-B1a*. Позитивный фенотипический эффект *Rht-B1p* на число зерен с главного колоса и отрицательное его воздействие на массу зерна с колоса и массу 1000 зерен в Краснодаре примерно в два раза больше, чем в Москве (см. табл. 3, Приложение 3).

Дисперсионный анализ показал значимость на 5 % уровне влияния аллельного состояния *Rht-B1* и региона проведения полевого опыта на уборочный индекс (см. Приложение 2). Сравнение средних значений показало, что уборочный индекс растений в Москве значимо выше, чем в Краснодаре, у растений как с аллелями *Rht-B1a* (на 33 %), так и с *Rht-B1p* (на 34 %), хотя и масса зерен в колосе, и вегетативная масса растений в Москве были статистически меньше по обеим группам генотипов (см. табл. 3, Приложение 3). Таким образом, растения в полевом опыте в Москве оказались более эффективными по использованию накопленных ассимилятов.

Уборочный индекс растений, гомозиготных по *Rht-B1p*, был значимо выше, чем у растений, гомозиготных по *Rht-B1a*, в Москве на 11.3 %, в Краснодаре – на 10.1 %. При этом вегетативная масса была меньше у растений, гомозиготных по *Rht-B1p*, по сравнению с растениями, гомозиготными по *Rht-B1a*, на 22 и 30 % в Москве и Краснодаре соответственно. Таким образом, увеличение уборочного индекса произошло не за счет увеличения массы зерна (у низкостебельных растений она была меньше, чем у высокостебельных), а вследствие уменьшения непродуктивной биомассы (см. табл. 3, Приложение 3).

Сроки колошения. Дисперсионный анализ показал достоверное влияние (на 5 % уровне значимости) на сроки колошения как аллельного состояния *Rht-B1*, так и региона проведения полевого опыта, а также и их взаимодействия (см. Приложение 2). В полевом опыте в Москве растения, гомозиготные по *Rht-B1a* и *Rht-B1p*, выколашивались соответственно на 9 и 15 дней значимо раньше, чем в опыте в Краснодаре (см. табл. 3, Приложение 3).

В среднем период от посева до колошения в опыте в Москве был короче, чем в Краснодаре, что может быть обусловлено разницей в продолжительности и динамике изменения светового дня. Колошение у растений с *Rht-B1p* в опыте в Москве наступило в среднем на 2 дня позже, чем у растений с *Rht-B1a*; в опыте в Краснодаре колошение наступило в среднем на 7.2 дня позже по сравнению с растениями с *Rht-B1a* (см. табл. 3, Приложение 3).

Оценка плейотропных эффектов аллеля *Rht-B1p* методом главных компонент. В результате анализа методом главных компонент нами было выделено два фактора: фактор 1 имеет наиболее высокую корреляцию с высотой, аллельным состоянием *Rht-B1p*, массой зерен в колосе и массой 1000 зерен, сроками колошения, фактор 2 – с массой зерен в главном колосе, числом зерен в главном колосе и уборочным индексом (Кхоз) в обоих регионах проведения полевого опыта (см. Приложение 4).

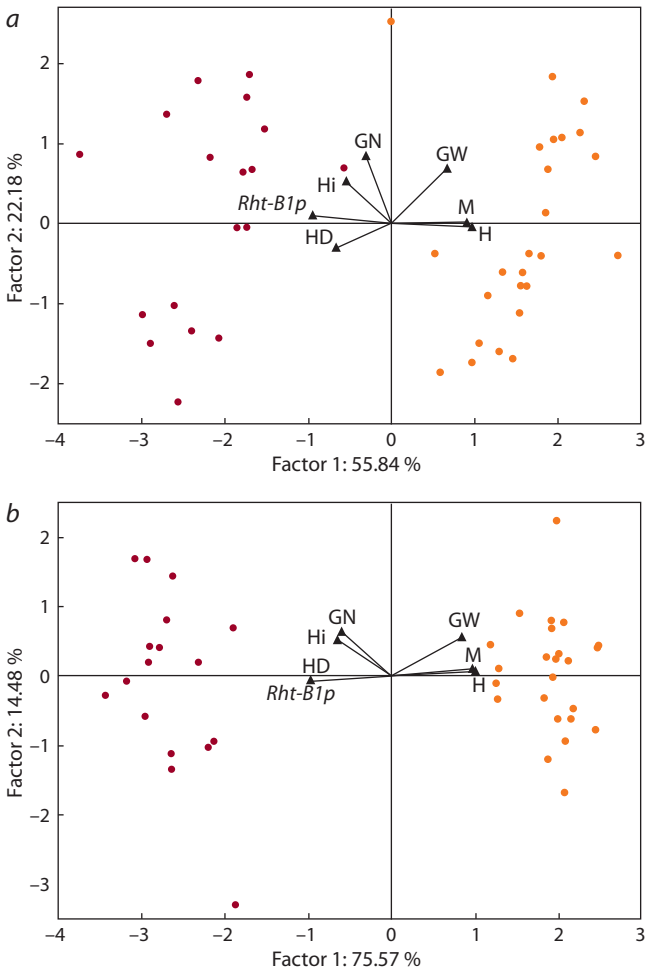

Fig. 3. Analysis of principal components of the effect of *Rht-B1b* on the main agronomic traits in the $B_2F_{2:3}$ (Chris M1/#517×LD222) families in the field plot experiment in Moscow (*a*) and Krasnodar (*b*).

H, plant height; GW, grain weight per spike; GN, grain number per spike; M, 1000 grain weight; HD, heading date (days after sowing). Red dots indicate families with the *Rht-B1p* allele and orange dots indicate families with the *Rht-B1a* allele.

Фактор 1 объясняет 56 и 76 % дисперсии, в то время как фактор 2 – 22 и 15 % дисперсии в Москве и Краснодаре соответственно. Семьи сгруппировались по горизонтальной оси по высоте растений, а по вертикальной оси наблюдается тенденция группировки по элементам продуктивности. На диаграмме на рис. 3 показано, что в обоих регионах вектор *Rht-B1p* имеет направление, противоположное высоте (В) и массе 1000 зерен (М), более слабое отрицательное влияние *Rht-B1p* оказывает на массу зерен в колосе; имеет одно направление с вектором сроков колошения (К) и уборочного индекса (Кхоз) и более слабое положительное влияние *Rht-B1p* на число зерен в колосе.

Обсуждение

Аллели генов, определяющие низкостебельность, не только снижают высоту растения, но и обладают плейотропным эффектом на агрономически ценные признаки (Rebetzke et al., 2011; Liu et al., 2017; Kroupin et al., 2019). Нами было изучено фенотипическое проявление *Rht-B1p* на одних и тех же семьях $B_2F_{2:3}$ твердой пшеницы в Москве и Краснодаре. При этом показано, что в Краснодаре влияние *Rht-B1p* на высоту, параметры зерна и период до колошения более выражено, чем в Москве. Общие тенденции воздействия аллеля *Rht-B1p* на изучаемые признаки, определенные методом дисперсионного анализа и методом главных компонент, совпадали между регионами проведения полевого опыта.

Влияние одного из наиболее распространенных аллелей, определяющих нечувствительность к гиббереллину, *Rht-B1b*, на высоту может варьировать от 10 до 25 % у мягкой пшеницы и от 30 до 40 % у твердой пшеницы по сравнению с аллелем дикого типа *Rht-B1a* (Mathews et al., 2006; Rebetzke et al., 2012; Subira et al., 2016; Liu et al., 2017). Снижение высоты растений в полевых опытах, вызванное наличием в геноме *Rht-B1p*, в наших исследованиях в опыте в Москве составило 41 %, в Краснодаре – 55 %, что сопоставимо с данными других исследований, полученных в вегетационных опытах (Ellis et al., 2004; Bazhenov et al., 2015). Сравнение данных полевого опыта с помощью дисперсионного анализа между регионами позволило выявить следующие тенденции. Растения с *Rht-B1a* в условиях полевого опыта в Краснодаре были значимо выше таковых в Москве, а с аллелем *Rht-B1p* значимо не различались. У растений с аллелем *Rht-B1a* можно предполагать разную реакцию клеток и тканей на факторы роста (в частности, гиббереллиновой кислоты) в различных условиях среды (освещение, температура, влажность). В то же время у растений с аллелем *Rht-B1p* эти факторы роста блокируются из-за мутации в гене, при этом нами отмечено, что растения значимо по высоте не различаются. Таким образом, в этом эксперименте различия в условиях среды не повлияли на фенотипический эффект *Rht-B1p* на высоту растений.

Снижение высоты в двух регионах происходит за счет уменьшения длины междоузлий (прежде всего, подколосового). Но при этом в Краснодаре длина междоузлий меняется непропорционально, а высота растений снижается дополнительно за счет уменьшения количества междоузлий. Явление непропорционального уменьшения подколосового междоузлия было описано у растений с аллелем *Rht-13* (Rebetzke et al., 2011), а снижение числа междоузлий в результате влияния аллелей низкостебельности в литературе практически не описано. Интересно, что этот фенотипический эффект был отмечен только в одном из регионов проведения полевого опыта. Возможно, что это связано со взаимодействием аллеля низкостебельности *Rht-B1p* с другими генами, например генов чувствительности к фотопериоду.

Влияние *Rht-B1p* на число колосков сопоставимо между регионами проведения полевого опыта по силе, но противоположно по направлению: в Москве число колосков возрастает, в Краснодаре уменьшается; общее число зерен в колосе в двух регионах возросло, что может быть обусловлено увеличением числа цветков, фертильности и завязываемостью. Меньшая разница между низкорослыми и высокорослыми растениями, выращенными в опыте в Москве и Краснодаре, по длине колоса и числу колосков в главном колосе может быть обусловлена тем, что при отсутствии внутренних факторов роста (гормоны, белки),

вызванного мутацией в аллеле *Rht-B1p*, снижается восприимчивость клеток и тканей колоса к внешним условиям (температура, освещенность, влажность).

Число зерен в колосе определяется числом колосков и озерненностью каждого колоска. В нашем полевом опыте в Краснодаре у растений с *Rht-B1p* число колосков было на 4 % меньше, чем у растений с аллелем дикого типа, а в Москве – на 4 % больше. Похожая разнонаправленность в проявлении аллеля *Rht-B1b* относительно числа колосков наблюдалась в работе (Álvaro et al., 2008): у итальянских сортов твердой пшеницы с *Rht-B1b* число колосков в колосе на 7 % больше, чем у сортов с аллелем дикого типа, а у испанских наоборот – на 2 % меньше. Число зерен в колосе в наших опытах у растений с *Rht-B1b* оказалось выше, чем у растений с аллелем дикого типа в Москве за счет увеличения числа колосков, а в Краснодаре – за счет более высокой озерненности колоска по сравнению с растениями с аллелем *Rht-B1a*. В исследовании (Álvaro et al., 2008) у низкорослых итальянских и испанских сортов число зерен также было выше, чем у высокорослых (на 20 и 13 % соответственно), что объяснялось их более высокой озерненностью (на 11 и 16 % соответственно).

Гиббереллин-нечувствительные фенотипы с аллелями низкостебельности гена *Rht*, как правило, имеют большее число зерен в колосе и меньшую массу 1000 зерен (или эквивалент этого показателя – массу одного зерна) по сравнению с растениями, несущими аллель дикого типа, что связано с меньшим количеством клеток в перикарпе (Mirrales et al., 1998; Zhang et al., 2013). Как было показано (Álvaro et al., 2008), в целом у современных итальянских и испанских сортов твердой пшеницы масса одного зерна ниже, чем у стародавних, что также может быть обусловлено внедрением аллелей низкостебельности. По литературным данным, разница в массе одного зерна между низкостебельными растениями с аллелем *Rht-B1b* и растениями с аллелем дикого типа *Rht-B1a* составляет 5–10 % (Liu et al., 2017). По сравнению с *Rht-B1b*, в наших экспериментах наблюдалась бóльшая разница: разность между растениями с *Rht-B1p* и растениями с аллелем дикого типа *Rht-B1a* составила 17 и 32 % в Москве и Краснодаре соответственно. При этом в Краснодаре отмечается более сильное снижение массы 1000 зерен, чем в Москве. В ряде исследований было продемонстрировано, что высокостебельные формы в засушливых условиях имеют преимущество перед низкостебельными в отношении массы и натуры зерна (Richards, 1992a, b; Butler et al., 2005). Таким образом, более выраженный негативный плейотропный эффект *Rht-B1p* в отношении массы 1000 зерен в полевом опыте в Краснодаре по сравнению с опытом в Москве также может быть связан с разницей в количестве осадков между регионами проведения полевого опыта (см. табл. 1).

Нами продемонстрировано, что растения с аллелем дикого типа *Rht-B1a* и с аллелем низкостебельности *Rht-B1p* в условиях опыта в Краснодаре более продуктивны, чем в Москве. Однако при этом масса 1000 зерен у растений с *Rht-B1p* в Москве была выше, чем в Краснодаре. Полученные нами данные говорят о том, что условия опыта в Москве были более благоприятны, чем в Краснодаре, для формирования отдельной зерновки и ее налива для растений, не чувствительных к гиббереллиновой кислоте. Это может говорить о возможности получения в условиях, близких к условиям проведения опыта в Москве, меньшего количества зерна, но более крупного.

Яровая пшеница – растение длинного дня, и в условиях длинного светового дня в Москве она переходит к колошению быстрее, чем в условиях короткого дня в Краснодаре. В Краснодаре низкостебельные растения отставали от высокостебельных на неделю, что может быть критичным в условиях наступления летней засухи. Как отмечают R. Motzo и F. Giunta (2007), *Rht-B1b* в целом никак не влияет на темпы развития у пшеницы, хотя были отдельные сообщения о том, что *Rht-B1b* ускоряет заложение соцветий. В нашем исследовании в двух регионах проведения полевого опыта результатом фенотипического проявления *Rht-B1p* стало более позднее колошение по сравнению с растениями, гомозиготными по *Rht-B1a*. Этот плейотропный эффект аллелей низкостебльности *Rht-B1* ранее не был отмечен.

Негативное влияние *Rht-B1p* на массу зерна компенсируется увеличением числа зерен в колосе и уборочного индекса. Низкостебельные сорта более устойчивы к полеганию, их можно высевать с большей нормой высева. Это позволит увеличить стеблестой на единицу площади и сбор зерна, как показывает опыт использования сортов с наиболее распространенными аллелями низкостебельности *Rht-B1b* и *Rht-B1e* (Беспалова, 2001). Кроме того, возможно комбинирование *Rht-B1p* с аллелями низкостебельности, приводящими к формированию гиббереллин-чувствительного фенотипа (Rebetzke et al., 2012; Liu et al., 2017), и аллелями генов яровизации и чувствительности к фотопериоду, в том числе и вновь выявленными (Shcherban et al., 2012; Kiseleva et al., 2016; Chen et al., 2018; Okada et al., 2019).

Таким образом, сравнение наших данных и опубликованных научных исследований показывает, что, несмотря на ряд отличий, *Rht-B1p* по своему фенотипическому эффекту сопоставим с *Rht-B1b* и может быть использован в селекции твердой пшеницы наравне с ним. *Rht-B1p* также может оказаться эффективен при создании гетерозисных гибридов твердой пшеницы. В южных регионах нашей страны внедрение *Rht-B1p* в геном твердой пшеницы может быть более перспективным, так как этот аллель не только снижает высоту растений, обеспечивая их устойчивость к полеганию, но и обладает рядом плейотропных эффектов, а именно – под его влиянием увеличивается число зерен в колосе, что потенциально может дать больший урожай, несмотря на более мелкое зерно.

Заключение

Нами изучено фенотипическое проявление аллеля низкостебельности *Rht-B1p* в семьях твердой пшеницы $B_2F_{2:3}$ в условиях полевого опыта в Москве и Краснодаре. Показано, что растения, несущие этот аллель, обладают низкостебельным фенотипом, масса зерна в колосе была меньше по сравнению с растениями с *Rht-B1a*, количество зерен было больше за счет увеличения колосков в полевом опыте в Москве и озерненности колоска в полевом опыте в Краснодаре. Растения с аллелем низкостебельности *Rht-B1p* по сравнению с высокорослыми растениями

характеризовались более высоким уборочным индексом и более поздним сроком колошения. Более перспективным использование аллеля *Rht-B1p* может быть в южных регионах, так как этот аллель не только снижает высоту растений, обеспечивая устойчивость к полеганию, но и обладает рядом плейотропных эффектов, потенциально способных дать больший урожай.

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Acknowledgements. This work was supported by the Russian Science Foundation, project 17-76-20023.

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

Received June 1, 2019. Revised September 24, 2019. Accepted September 25, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):926-932
DOI 10.18699/VJ19.568

The Fifth International Scientific Conference PlantGen2019

The formation and the study of a collection of the *Miscanthus* resource species gene pool in the conditions of the West Siberian forest steppe

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Several species of the genus *Miscanthus* Anderss. (elephant grass) characterized by a high rate of growth of the aboveground vegetative mass are currently in the focus of attention due to their high practical application as a source of bioethanol and cellulose. The main goals of this study were: (1) molecular genetic identification and (2) histochemical analysis of the genus *Miscanthus* Anderss. species in the collection of Central Siberian Botanical Garden SB RAS in order to identify the most perspective and technically valuable individuals. To study the collection of *Miscanthus* samples, a multi-disciplinary approach was applied. To collect the samples of different species from native habitats, traditional systematic and geobotanical methods (comparative morphological and phytocenological) were used. According to the results of the ISSR-analysis, 16 samples of three *Miscanthus* species were divided into two clades: Sinensis and Sacchariflorus, the former including two subclades. For the samples of *M. purpurascens*_I and II, a hybrid origin of this species was confirmed by ISSR data. The molecular data obtained from the study allowed us to hypothesize that the samples involved in the subclade I of the Sinensis clade could be used as donors of resistance to adverse environments, and the samples of the subclade II, as donors of high biomass productivity. Based on histochemical analysis, sclerenchyma cells were characterized by the most lignin-rich thickened membranes, so the most appropriate direction in *Miscanthus* selection should be based on identification and using less lignin-containing samples.
Key words: *Miscanthus*; ISSR analysis; histochemical analysis; biomorphology; microecology; cluster dendrogram; bioethanol.

For citation: Dorogina O.V., Vasilyeva O.Yu., Nuzhdina N.S., Buglova L.V., Zhmud E.V., Zueva G.A., Komina O.V., Kuban I.S., Gusar A.S., Dudkin R.V. The formation and the study of a collection of the *Miscanthus* resource species gene pool in the conditions of the West Siberian forest steppe. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):926-932. DOI 10.18699/VJ19.568

Формирование и изучение коллекционного генофонда ресурсных видов рода *Miscanthus* Anderss. в условиях лесостепи Западной Сибири

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В настоящее время особый интерес представляют растения, характеризующиеся высокой скоростью нарастания надземной вегетативной массы и имеющие практическое применение в качестве источника биоэтанола и целлюлозы, например, виды рода *Miscanthus* Anderss. (веерник). Цель данного исследования – молекулярно-генетическая идентификация и гистохимический анализ видов рода *Miscanthus*: *M. sacchariflorus* (Maxim.) Benth., *M. sinensis* Anderss. и *M. purpurascens* Anderss. коллекционного генофонда Центрального сибирского ботанического сада СО РАН для выявления перспективных технических сырьевых растений. Для формирования, изучения и идентификации коллекционного генофонда рода *Miscanthus* применялся мультидисциплинарный подход, заключавшийся в следующем: при сборе образцов в природе использовались традиционные методы классической систематики и геоботаники (сравнительно-морфологический и фитоценологический); при выделении форм под конкретные ресурсные (биоэнергетические), технологические задачи применялись биоморфологические подходы и проводилось описание микроэкологических условий с акцентом на наименее благоприятные для произрастания факторы. Молекулярно-генетические исследования были направлены на уточнение таксономической принадлежности и анализ генетических дистанций между представителями трех видов *Miscanthus*. По результатам ISSR-анализа изученные образцы трех видов мискантуса разделились на две клады: Sinensis и Sacchariflorus, что хорошо согласуется с видовой при-

надлежностью большинства образцов. Исключение составили растения *M. purpurascens*_I и II, которые по данным молекулярно-генетического анализа отнесены к кладе Sacchariflorus, что может служить подтверждением гибридного происхождения данного вида. Клада Sinensis разделилась на две субклады. В субкладу I вошли образцы, произраставшие в наименее благоприятных микроэкологических условиях – на более засоленных почвах, а в субкладе II сосредоточены наиболее габитуально мощные образцы. Эти данные позволяют предположить, что среди образцов субклады I могут оказаться доноры устойчивости, а среди образцов субклады II – доноры продуктивности. Известно, что из-за повышенного содержания лигнина снижается технологическая ценность сырья. Согласно проведенному нами гистохимическому анализу, мощность механической лигнин-содержащей ткани различается у разных растений мискантуса, поэтому наиболее целесообразен селективный отбор особей, накапливающих меньше лигнина (с наименее развитой склеренхимой). Выявленные молекулярные признаки разных видов мискантуса могут быть использованы для идентификации и паспортизации перспективных форм и линий в качестве альтернативного источника биотоплива и целлюлозы.
Ключевые слова: род *Miscanthus*; ISSR-анализ; гистохимический анализ; биоморфология; микроэкология; кластерная дендрограмма; биоэтанол.

Introduction

Miscanthus is considered to be one of the most efficient solar energy accumulators among representatives of the Earth vegetative kingdom (Dohleman, Long, 2009). High cellulose content and significant plant biomass make it possible to treat *Miscanthus* as a promising alternative energy source (Lewandowski et al., 2000; McCalmont et al., 2017; Van Der Weijde et al., 2017). Physiologists and biochemists deem *Miscanthus* species to be unique highly productive sources of renewable raw materials for producing ethylene and cellulose (Slynko et al., 2013).

Miscanthus is a valuable ameliorative culture as well. High plant growth rates, unpretentiousness to soil conditions, pronounced drought resistance have contributed to *Miscanthus* wide use to stabilize and reduce the intensity of soil erosion processes (Kahle et al., 1999, 2001).

In the late 20 century, many domestic and foreign botanical gardens started introducing *M. sacchariflorus* and *M. sinensis* into the culture as decorative cereals. The criteria for *ex situ* selection – rhythmological and ontogenetic ones – have been developed in conformity with the prospects for their use in landscape architecture. In the 21st century, the above-mentioned species, as well as *M.* × *giganteus*, are recognized by the world scientific community as the main resource species for elaborating the alternative energetics sphere.

The Central Siberian Botanical Garden of the Siberian Branch of the Russian Academy of Sciences (CSBG SB RAS, Novosibirsk) have been investigating *Miscanthus* species as a part of the lawn and decorative cereals collection since the late 1990s. Forming and studying of the collection gene pool of the genus *Miscanthus* complex have recently carried out in accordance with the world trends – decorative and bioenergetic directions. Searching perspective forms in nature and further studying their bioenergetic potential outside the natural range have required the additional selection criteria development, and economic and biological traits and properties evaluation.

The Russian Far East is the main region of field works to study the intraspecific polymorphism of *Miscanthus* species and search promising samples, where *M. sacchariflorus* occupies northern habitats, but *M. sinensis* spreads southward. In 2013, the collection gene pool was replenished with *M. sinensis* samples from the Gamov Peninsula (Khasan District, Primorsky Krai). In 2017, *M. sacchariflorus* was collected in the Chuguev District, and *M. sinensis* and, presumably, *M. purpurascens*, in the Khasan District of Primorsky Krai. In addition, living material was collected on the Kuril islands – Shikotan, Kunashir, Iturup.

The biomorphological and microecological criteria have been allotted as main ones to gather samples in nature. During the expeditions, the vegetative parts (rhizome) were selected from plants with the most powerful aboveground vegetative mass. At the same time, it was marked microecological conditions of growing, among which the following considered as unfavorable: saline splash zone, sites with arid compacted soil, open windy hilltops. Plants with normal shoot formation in such habitats should obtain high adaptive potential under more severe climatic conditions of cultivation.

It became necessary to study genetic polymorphism and DNA identification as a result of cumulating the intraspecific and form diversity in the collection gene pool. The study objective is molecular-genetic identification and histochemical analysis of the CSBG SB RAS' collection gene pool of the genus *Miscanthus* species to reveal promising forms as technical raw plants.

Materials and methods

An integrated multi-disciplinary approach to the collection gene pool formation and certification was used in the study. The traditional techniques of classical systematics and geobotany were applied to choose field sites, and collect samples of various species in nature. While isolating forms in natural habitats, biomorphological approaches were employed to solve specific resource problems, microecological conditions are described. The geographical coordinates of collecting sites were recorded when selecting material for molecular genetic and histochemical research.

16 samples of three species (*M. sinensis*, *M. sacchariflorus* and *M. purpurascens*) were studied (Table 1). *M. sacchariflorus* samples were gathered in the Chuguev District of Sikhote-Alin, as well as the northern part of the National Park "Zov Tigra (Call of the Tiger)" vicinities situated in the Chuguev, Olgin, and Lazov Districts. This species grows here in open flat areas and non-arable lands, which are secondary successions.

M. sinensis and *M. purpurascens* were collected in the Khasan District (Primorsky Krai). These species grow as a part of shrub-grass groupings, some specimens are taken in

Table 1. The origin of the samples of three *Miscanthus* species analyzed in the paper

Species	Place of collection, geographical coordinates
	M. sinensis
M. sinensis_I	Gamow Peninsula, Khasansky region. Observation deck on the road, overlooks Vityaz Bay. 42°36′24″ N, 131°09′56″ E
M. sinensis _II	Gamow Peninsula, Khasansky region. On the way to the lighthouse
M. sinensis_III	Gamow Peninsula, Khasansky region. On the way to the lighthouse
M. sinensis_IV	Gamow Peninsula, Khasansky region. The Bay Vityaz. 42°60′65″ N, 131°16′59″ E
M. sinensis_V	Gamow Peninsula, Khasansky region. Plateau above Vityaz Bay. 42°60′88″ N, 131°19′48″ E
M. sinensis_VI	Gamow Peninsula, Khasansky region. Plateau above Vityaz Bay. 42°61′51″ N, 131°17′89″ E
M. sinensis_VII	Gamow Peninsula, Khasansky region. Supralittoral in Telyakovsky Bay. 42°35′25″ N, 131°17′74″ E
M. sinensis_VIII	Gamow Peninsula, Khasansky region
M. sinensis_IX	Gamow Peninsula, Khasansky region
M. sinensis_X	Gamow Peninsula, Khasansky region. Gas Stop at the turn into the village Andreevka. 42°40′33″ N, 131°06′17″ E
	M. sacchariflorus
M. sacchariflorus_I	Sykhote-Alin. One of the most Northern Checkpoints of the area. Chuguevsky region, not reaching the river Pokrovka. Route A181. 44°51′21″ N, 134°52′95″ E
M. sacchariflorus_II	Sykhote-Alin. In the same population
M. sacchariflorus_III	Sykhote-Alin. In the area surrounding the National Park "Call of the Tiger". 43°33′3″ N, 134°66′7″ E
M. sacchariflorus_IV	Sykhote-Alin. 43°53′24″ N, 134°12′57″ E
	M. purpurascens
M. purpurascens_I	Far East of the Russian Federation. Collection of living plants CSBG (No. USU 440534)
M. purpurascens_II	Gamow Peninsula, Khasansky region. Mountain Tumannaya (foggy). 42°33′54″ N, 131°12′34″ E

a light shrub-forb oakery (*Quercus dentata* Thunb.) on the Telyakovsky Bay slope. The main works were carried out at the Gamov Peninsula, 14–112 m altitudes, with a wide range of microecological conditions in *M. sinensis* and *M. purpurascens* habitats.

The molecular genetic analysis to clarify samples taxonomic position and identification was carried out using plant dried leaves of three *Miscanthus* species: *M. sinensis*, *M. sacchariflorus*, and *M. purpurascens* collected of 15 plants in natural populations (see Table 1). Besides, *M. purpurascens* sample from the Living Plant Collection of the CSBG SB RAS (see Table 1, *M. purpurascens_I*, UNU No. USU 440534) was analyzed.

DNA extraction was performed by the NucleoSpin Plant II kit (Macherey and Nagel, USA). The purity and concentration of DNA extracts were determined with spectrophotometry (Spectrophotometer kinetic and μ-cuvette, Eppendorf, Germany). DNA purity was calculated as the ratio of the solution optical power at 260 and 280 nm wavelength.

The 25 μl reaction PCR mixture consisted of: 2.7 mM $MgCl_2$, 1.25 mM primer, 0.4 mM mononucleotides, 1x PCR buffer, 1.5 units Taq DNA polymerase (Medigen, Russia) and 20–30 ng matrix. The amplification program included the following steps: DNA denaturation: 90 s at 94 °C; 35 amplification cycles: 40 s at 94 °C, 45 s at 41–58 °C (primer annealing), and 90 s at 72 °C; 5 min at 72 °C. The amplification was made in C 1000 Thermal Cycler (BioRad Laboratories, USA), its products were separated by electrophoresis in a 0.8 % agarose gel in 1x TBE buffer. The obtained ISSR fragments were stained with SYBR-Green (Medigen, Russia), visualized using the Gel Doc XR + gel documentation system, analyzed by Image Lab Software (Bio-Rad Laboratories, USA).

The size of the identified ISSR fragments was determined with a molecular mass marker (Medigen, Russia). Each amplified fragment was considered as a dominant marker, and its presence (1) or absence (0) was noted for every compared samples.

Statistical data processing, cluster dendrogram construction and principal component analysis (Principal Components, PCO) were carried on applied the PAST software (Hammer et al., 2001).

As for the histochemical analysis, dry vegetative shoots were selected at the final vegetation stage from 3 samples (*IV*, *V* and *VI*) of *M. sinensis* and 1 sample (*II*) of *M. purpurascens*, taken as live rhizomes from 4 natural populations in the Khasan District, Primorsky Krai, Russia. Their subsequent vegetation took place in similar conditions as a part of CSBG SB RAS' Bioresource Scientific Collection USU No. 440534. A stem part was taken from a minimal industrial height – 10 cm from the ground level (10 cm length).

Further studies were carried out at CSBG SB RAS' Center for Collective Use. The bottom side shoots were longitudinally cut with a scalpel into bars of about 3 mm, placed on a freezing microtome, and 60–90 μm longitudinal sections were made. Staining was practiced in two variants: phloroglucinol in hydrochloric acid, alcian blue in acetic acid according to standard techniques (Barykina et al., 2004). Besides, the possibility of differentiated staining with these dyes was checked.

Microscopying with photography was performed by Carl Zeiss Axio Scope A1 light microscope.

Results

Molecular genetic analysis

The study had previously tested 16 ISSR primers, nine of which were used for the analysis (Table 2). They are characterized by the greatest amount and polymorphism of the amplified fragments, and are suitable to investigate the genetic variability of the genus *Miscanthus* plants at the intra- and interspecific levels.

The extracts of *Miscanthus* DNA obtained from dried leaves had 3–24 ng/μl concentration, 1.25–1.83 purity expressed with A_{260}/A_{280} ratio. The analysis of intermicro-satellite DNA sections of the studied samples using nine ISSR primers allowed identifying 177 amplified fragments of 270–1800 bp length. Figure 1 shows the ISSR profile of samples obtained by amplification with primer 17899B.

According to the ISSR analysis results, 16 *Miscanthus* samples were divided into two clades: Sinensis and Sacchariflorus (Fig. 2), that is consistent with the species of each sample (the bootstrap support value is 55). At the same time, *M. purpurascens_I* and *II* were distributed inside the clade Sacchariflorus (see Fig. 2). Taking into account the admittedly hybrid origin of *M. purpurascens* species, the authors tend to evaluate the result as a confirmation of this hypothesis (Jiang et al., 2013).

The extremely close genetic relationship found for samples of *M. sacchariflorus_I* and *II* is remarkable (see Fig. 1, 2). These samples, collected in one population, have almost identical ISSR patterns, that may be evidence of these two individuals origin as a result of vegetative reproduction of the original plant.

Based on the principal component analysis (PCA) of ISSR-marking data, the distance between two groups of samples inside Sacchariflorus clade has been determined (Fig. 3). The first group includes *M. sacchariflorus_I* and *II*, the second – *M. sacchariflorus_I, II* and *M. purpurascens_I, II*.

Table 2. Characteristics of ISSR primers tested and selected (in bold) to study the genetic polymorphism of *Miscanthus* species

Primer	Nucleotide sequence, 5′-3′	Temperature of annealing, °C
814	$(CT)_8TG$	51
17898A	$(CA)_6AC$	45
17898B	$(CA)_6GT$	48
17899A	$(CA)_6AG$	48
17899B	$(CA)_6GG$	42
844A	$(CT)_8AC$	44
844B	$(CT)_8GC$	42
M1	$(AC)_8CG$	56
M2	$(AC)_8YG$	58
M7	$(GAC)_5$	46
M11	$(CA)_6AR$	39
M14	$(GACA)_4$	47
HB10	$(GA)_6CC$	48
HB12	$(CAC)_3GC$	41
HB14	$(CTC)_3GC$	42
UBS826	$(AC)_8C$	53

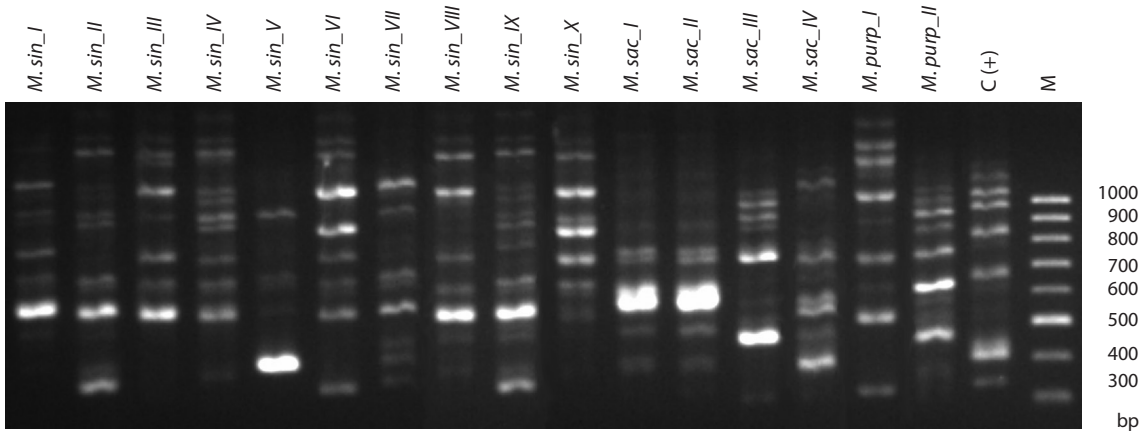

Fig. 1. ISSR-PCR profile of three *Miscanthus* species: ISSR primer 17899B was used for amplification.
C(+), positive control; M, weight marker. Designation of the samples of *Miscanthus* see in Table 1.

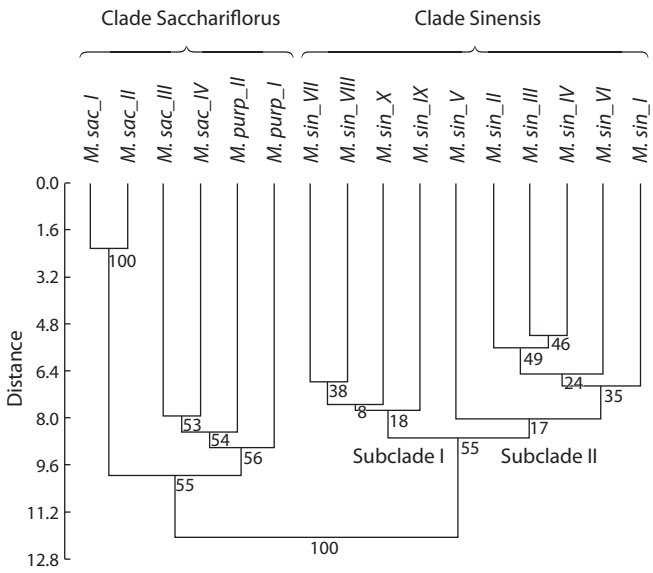

Fig. 2. Cluster dendrogram reflecting the value of genetic distances between 16 samples of three *Miscanthus* species.

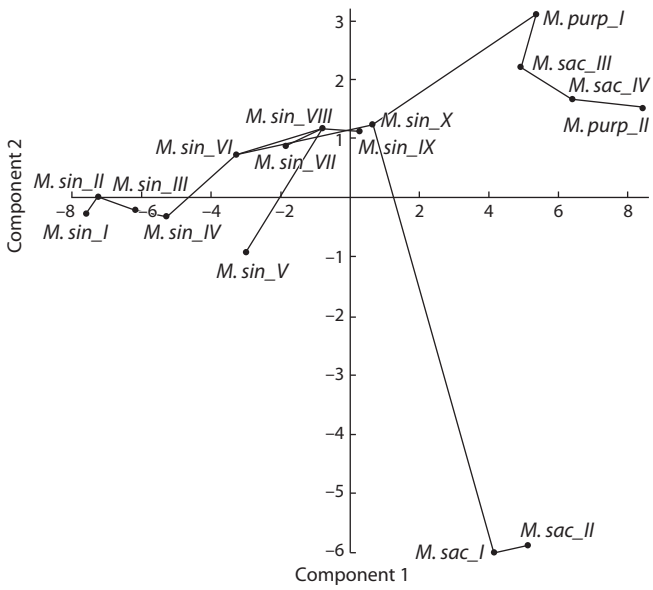

Fig. 3. The scheme of genetic relationships between 16 specimens of three *Miscanthus* species resulted by the Principal Component Analysis (PCA) of ISSR data.

Histochemical analysis

Histochemical studies aimed to investigate the seasonal dynamics and peculiarities of straw lignification were undertaken related to chemical analysis results carried out at the Institute of Problems of Chemical and Energetic Technologies SB RAS (IPChET SB RAS, Biysk city) showed some samples with elevated lignin content reaching 28.1 ± 0.5 % in terms of absolutely dry weight (Dorogina et al., 2018). It has been found that high lignin content decreases the technological value of the raw material (Dorogina et al., 2018).

Based on histochemical analysis, it is revealed that the shoot structure of *M. sinensis* representatives is similar to that of the family Poaceae. The straw outside is covered with thin single-layer epidermis; this species layers are radially spread mechanical tissue with thick lignified cell walls (Fig. 4). The thickened cell walls of the sclerenchyma are richest in lignin arranged in two layers around the conducting beams (see Fig. 4). The mechanical lignin-bearing tissue thickness differs in various plants: the less pronounced lignified sclerenchyma is developed in plant of population No. 3 compared with the sample of population No. 24 (see Fig. 4, *a*, *b*).

Discussion

Based on the clade Sinensis analysis, it was found that the clade was divided into two subclades (see Fig. 2). Subclade I included samples growing under the most unfavorable micro-

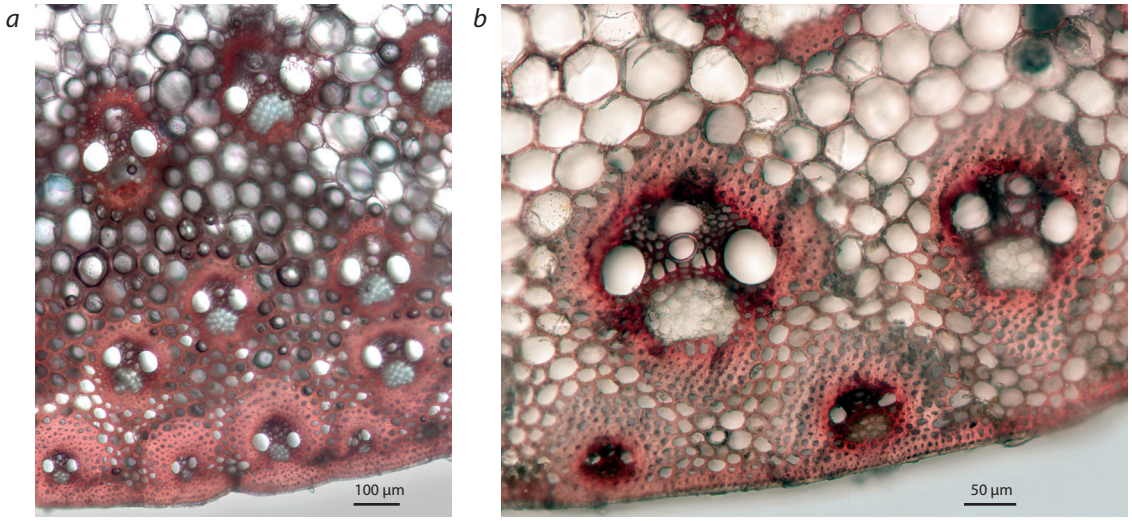

Fig. 4. The cut stem of the samples *M. sinensis_VI* (*a*) and *M. sinensis_IV* (*b*).
Staining with phloroglucinol – altianalis blue die.

ecological conditions. So, the sample of *M. sinensis_VII* is collected in the Telyakovsky Bay splash or supralittoral zones located on the sea-land border, above the maximum tide level, which implies more saline microecological conditions (Fig. 5).

*M. sinensis*_X sample grew in a technogenically disturbed habitat, in the gas station environs at the crossway to Andreevka village not excluding high exhaust gases effect (see Table 1).

It is noteworthy that the samples entering subclade II are plants with the most powerful habitus. *M. sinensis_V* and *M. sinensis_VI* growing in the shrub-grass grouping on a plateau above Vityaz Bay stood out by their morphological parameters (Fig. 6).

Conclusion

The foresaid suggests that there are plants characterizing with high resistant in subclades I samples, and that's why they might be donors of resistance, while subclades II samples – donors of productivity. The revealed molecular signs of *Miscanthus* various species should be used to identify and certify *Miscanthus* forms and lines perspective to get economically available plant materials suitable for applying as environmentally safe promising alternative biofuels.

Based on the histological analysis of shoots of *M. sinensis* representatives, it should be assumed that some *M. sinensis* specimens store a large amount of lignin in dry straws after vegetation, which could impede industrial processing, therefore additional studies of young shoots are needed to investigate the lignin dynamics accumulation. In this regard, feasibly earlier harvesting of the vegetative mass should be more productive, and selection of individuals accumulating less lignin (with the least developed sclerenchyma) is the most appropriate.

Fig. 5. General view of the natural habitat of *M. sinensis_VII* in the Telyakovsky Bay splash zone.

Fig. 6. General view of the plants *M. sinensis_V* and *M. sinensis_VI* growing in the shrub-grass group on the plateau above the Bay Vityaz (Primorsky Region).

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Acknowledgments. The studies were carried out according to the Complex Program of Fundamental Research of SB RAS II.1. Project block "Studying the morphogenetic potential of the genus *Miscanthus* Anderss., and possibilities of its use in the introduction and selection under West Siberian conditions", state registration No. AAAA-A17-117120640059-4, and applying materials bioresource scientific "Collection of living plants in open and closed ground" USU 440534.The authors thank PhD V.A. Bakalin, the head of the Laboratory of Cryptogamous Biota of the Botanical Garden-Institute of the Far Eastern Branch RAS, for his help in carrying out expeditionary work and advice in determining the elephant grass specificity.

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

Received May 27, 2019. Revised June 14, 2019. Accepted June 23, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):933-940
DOI 10.18699/VJ19.569

The Fifth International Scientific Conference PlantGen2019

Features of the resource species *Miscanthus sacchariflorus* (Maxim.) Hack. when introduced in West Siberia

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Here we provide a scientific justification and experimental support for the choice of easily renewable cellulosic feedstock *Miscanthus sacchariflorus* (Maxim.) Hack. in order to obtain high-quality nutrient broths therefrom for bacterial cellulose biosynthesis. The plant life-forms promising for breeding were screened under introduction conditions at the Central Siberian Botanical Garden, SB RAS, and this study was thus aimed at investigating the full and reduced ontogenetic patterns; cellulose and noncellulosic contents, including lignin; and duraminization of vegetative (feedstock source) organs throughout the seasonal development. The full ontogenetic patterns of the plants grown from seeds that had been collected in native habitats were compared to show that *M. sacchariflorus* and *M. sinensis* Anderss. accessions are distinguished by longer being at the most vulnerable developmental stages: seedlings and plantlets. Hence, it is preferable to cultivate seedlings on protected ground, and plantations are advisable to establish with more stable cloned vegetative material. The chemical compositions of the whole plant, leaf and stem separately, from seven *M. sacchariflorus* harvests were examined to reveal a rise in cellulose content and a drop in noncellulosic content with plantation age. The *Miscanthus* stem was found to contain more cellulose than the leaf, regardless of the plant age. The overall cellulose content was 48–53 %, providing a rationale for studies of bacterial cellulose biosynthesis in a *M. sacchariflorus*-derived nutrient medium. Since high lignin content is undesirable for technological processes concerned with biosynthesis of bacterial cellulose, we performed histochemical assays of transverse sections of the culms to monitor the seasonal course of lignification. Our results suggest that the specific time limits for harvesting the aboveground biomass as a feedstock be validated by histochemical data on the seasonal course of lignification of *M. sacchariflorus* sprouts. To sum up, the examined chemical composition of *M. sacchariflorus* grown in the Siberian climate conditions demonstrated its prospects as a source of glucose substrate, the basic component of good-quality nutrient media for biosynthesis of bacterial cellulose.
Key words: Miscanthus; *Miscanthus sacchariflorus*; ontogeny; chemical composition; cellulose; bacterial cellulose; histochemical analysis.

For citation: Gismatulina Yu.A., Budaeva V.V., Sakovich G.V., Vasilyeva O.Yu., Zueva G.A., Gusar A.S., Dorogina O.V. Features of the resource species *Miscanthus sacchariflorus* (Maxim.) Hack. when introduced in West Siberia. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):933-940. DOI 10.18699/VJ19.569

Особенности ресурсного вида *Miscanthus sacchariflorus* (Maxim.) Hack. при интродукции в Западной Сибири

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В настоящей работе представлено научно-теоретическое обоснование и экспериментальное подтверждение выбора легковозобновляемого целлюлозосодержащего сырья – *Miscanthus sacchariflorus* (Maxim.) Hack. – с целью получения из него доброкачественных питательных сред для биосинтеза бактериальной целлюлозы. Отбор форм, перспективных для селекции, проводили в условиях интродукции Центрального сибирского ботанического сада Сибирского отделения Российской академии наук. Цель наших исследований – изучение онтогенеза, массовой доли целлюлозы и нецеллюлозных компонентов, включая лигнин, а также процессов одревеснения вегетативных (сырьевых) органов в течение сезонного развития. Сравнительный анализ особенностей онтогенеза полного типа у растений, выращенных из семян,

собранных в естественных местообитаниях, показал, что образцы *M. sacchariflorus* и *M. sinensis* Anderss. отличаются продолжительным пребыванием в наиболее уязвимых онтогенетических состояниях – проростках и ювенильных растениях. Поэтому более успешное выращивание сеянцев лучше проводить в условиях защищенного грунта, а производственные площади целесообразно закладывать за счет более устойчивого вегетативного клонированного материала. При анализе химического состава растения в целом, а также листа и стебля отдельно семи урожаев *M. sacchariflorus* обнаружено, что по мере взросления плантации увеличивается массовая доля целлюлозы и уменьшается содержание нецеллюлозных компонентов. Выявлено, что вне зависимости от возраста растения в стебле мискантуса присутствует более высокая массовая доля целлюлозы, чем в листе. Массовая доля целлюлозы в целом составляет 48–53 %, что свидетельствует об актуальности изучения биосинтеза бактериальной целлюлозы на питательной среде из растений *M. sacchariflorus*. Поскольку для технологических процессов, касающихся биосинтеза бактериальной целлюлозы, высокое содержание лигнина нежелательно, нами были проведены гистохимические исследования поперечных срезов соломин для определения сезонной динамики лигнификации. На основании полученных результатов предложено при установлении конкретных сроков заготовки надземной массы в качестве технологического сырья подтверждать их данными гистохимического анализа сезонной динамики лигнификации побегов *M. sacchariflorus*. Таким образом, изучение химического состава *M. sacchariflorus*, выращенного в климатических условиях Сибири, представило перспективность его использования с целью получения глюкозного субстрата – основного компонента доброкачественных питательных сред для биосинтеза бактериальной целлюлозы.
Ключевые слова: мискантус; *Miscanthus sacchariflorus*; онтогенез; химический состав; целлюлоза; бактериальная целлюлоза; гистохимический анализ.

Introduction

Nowadays, search for economically accessible and environmentally safe energy sources for multipurpose use is topical (Jones, 2001; Shumny et al., 2010; Dorogina et al., 2018; Schroder et al., 2018). Of special interest are fast-growing herbaceous perennials that exhibit high gain of aboveground vegetative biomass, as they possess a number of ecological advantages over annual plants (Zhang et al., 2011; Iqbal et al., 2015). Alongside with common species, new plants, including *Miscanthus*, are being extensively put into practice.

Miscanthus, *Miscanthus sacchariflorus* (Maxim.) Hack., is a perennial, ecologically efficient crop with a high annual biomass gain of 10–15 t/ha over the span of 15–25 years (Slyn'ko et al., 2013; Bulatkin et al., 2015, 2017; Kapustyanchik et al., 2016; Gismatulina, Budaeva, 2017). *Miscanthus* features a special C4 photosynthetic pathway, which allows it to remain one of the most effective accumulators of solar energy, and ensures a high annual productivity even on badlands (Slyn'ko et al., 2013; Anisimov et al., 2016; Morandi et al., 2016; Xue et al., 2017).

In the context of the search for carbon sources to derive unique microbial synthesis products, *Miscanthus sacchariflorus* (Maxim.) Hack. can be a promising feedstock for good-quality nutrient media and specifically for biosynthesis of bacterial cellulose (BC). The features of this species grown in West Siberia include its ability to build up biomass within a short-term vegetative phase in the continental climate with short arid summer; frost-resistance; pest and disease resistance; and no need for fertilizers during the vegetative phase.

West Siberian forest steppe is a region of risky agriculture for many farm crops; thus, in choosing study methods, emphasis should be placed on overall stability, yielding capacity (fruit yield, seed yield, vegetative biomass yield), and feedstock quality. Besides, given the short vegetative phase, the plants of seed and vegetative origins demand that the durations of different ontogenetic states be assessed to reveal the most vulnerable ones.

Within the generic family of *Miscanthus*, which is studied at the Central Siberian Botanical Garden (CSBG) SB RAS and Institute for Problems of Chemical and Energetic Technologies (IPCET) SB RAS, the bioenergy resource species *M. sacchariflorus* is of special interest. As its domestication and screening of life-forms promising for breeding had been performed outside its native habitat and climatic zone, our efforts were focused on examining the full and reduced ontogenetic patterns, cellulose and noncellulosic contents, and on histochemical assays of duraminization in vegetative (feedstock source) organs during seasonal development.

Thus, the present study aimed to investigate the ontogeny, chemical composition, and duraminization in vegetative organs of *M. sacchariflorus* in the context of producing good-quality broths for subsequent microbiological synthesis of bacterial cellulose.

Materials and methods

The study was conducted with accessions of *M. sacchariflorus* (Maxim.) Hack. and, as developmental reference, *M. sinensis* Anderss. Plants of both species were sampled in the Russian Far East and introduced at the CSBG SB RAS in 2012. The developmental stages were identified using the periodization of ontogeny (Uranov, 1967, 1975; Plants Cenopopulations…, 1976, 1988) and studies on crop ontogeny (Ontogenetic Atlas…, 1997, 2013).

Chemical compositions were measured for the whole plant and for leaf and stem separately in seven *Miscanthus* harvests. For this purpose, *Miscanthus* was harvested annually within October 3 to 8 over the span of seven years, from 2011 to 2017. The aboveground part of the plant was cut

off at 10–15 cm above the ground. After the plant was harvested, the rhizomes were left in soil to winter. Thus, the plantation was established once in 2011 and harvested many times (one year after the preceding harvest had been cut). Prior to the *Miscanthus* chemical composition analysis, the feedstock was dried to a humidity no more than 8 % and chopped with scissors into 5–10-mm cuts.

The chemical composition was quantified as follows: Kürschner cellulose (a weighed sample treated with ethanolic HNO_3 solution); pentosans were quantified by standard analytical procedures for raw materials (Obolenskaya et al., 1991) (a weighed sample was heated in HCl solution, and distilled furfural was assayed spectrometrically); ash content was quantified by incineration of the weighed sample in a porcelain crucible, followed by calcination of the residue in a muffle furnace; acid-insoluble lignin was assayed by TAPPI T222 om-83; and the wax-fat fraction (a weighed sample was extracted with methylene chloride, the extract was evaporated, and the nonvolatile residue was dried) was measured by TAPPI 204 cm-97. The moisture content was determined on an OHAUS MB-25 moisture analyzer (USA). The chemical composition measurements were performed with equipment of the Biysk Regional Shared Access Center of Scientific Equipment of the SB RAS (IPCET SB RAS, Biysk).

For histochemical assays, we collected vegetative shoots in late August and dry shoots in late September past final vegetation of the plants that had earlier been brought in as live rhizomes from three natural populations in the Chuguyev and Khasan raions of Primorsky Krai. They were then grown under monotypic conditions as part of the Bioresource Scientific Collection of the CSBG SB RAS, USU No. 440534. For analysis, 10-cm long stem samples were taken at the minimum industrial height 10 cm above the ground level.

Further studies were conducted at the Shared Access Center of Scientific Equipment, CSBG SB RAS. The shoots were cut lengthwise with a scalpel into bars of about 3 mm and placed into a freezing microtome to make longitudinal sections of 60–90 μm. The samples were stained with phloroglucinol by the standard procedure (Barykina et al., 2004). Microscopic photos were taken with a Carl Zeiss Axio Scope A1 optical microscope.

a

b

Fig. 1. Rhizome fragments of immature plants: *a – Miscanthus sacchariflorus* (Maxim.) Hack.; *b – Miscanthus sinensis* Anderss.

Results

The ontogenetic features of *M. sacchariflorus* were examined against the other species, *M. sinensis*, as these species have different biomorphs in their native habitats in Primorsky Krai. Investigation of the genesis of a plant life-form during development under new growing conditions allows a set of growing methods to be devised and optimum time limits for feedstock preparation to be identified.

Since the literature lacks data on the ontogenetic features of the genus *Miscanthus*, we were first to investigate comprehensively the ontogeny of *M. sacchariflorus* specimens versus *M. sinensis*. The enforced nondeep dormancy of *M. sacchariflorus* seeds was found to constitute the latent stage. The caryopses were very small, 2.3 to 2.6 mm long, narrow ellipsoidal and loose, and the germ was one-half as long as the seed.

Pregenerative stage. When sown under lab-scale conditions, the seeds produced shoots on day 3 or 4. The vigor and germinating capacity of freshly collected (in native habitats) seeds were very high, 80.0–85.5 %. The seedlings had a primary shoot and an embryo root. Second leaves appeared after seven days. Further formation of next leaves suspended, whereas the root system composed of secondary roots was intensely developing. The plants remained in the seedling state for a long time, up to 2.5 months.

The juvenile plants lost the contact with the caryopsis. The primary sprout and the roots remained and developed further. The plants had 9 or 10 green assimilating leaves, and they were smaller and narrower than those of adult plants. The *M. sinensis* scale-like leaves were tightly adjacent to the base of the shoot. This state lasted more than two months. It should be noted that the seedlings and plantlets were in a greenhouse prior to the vegetative phase.

The shoot and root systems of immature plants (Fig. 1, *a*, *b*) acquired signs of transition from juvenile to adult. The type of leaves was also transitional: they rapidly grew and increased in length and width. The basic diagnostic marker sign of this state in cereals was that plants entered the tillering stage. The bottom leaves obliqued from the stem, assumed the horizontal position, and died off, exposing intravaginal lateral shoots. The shoot formation in *M. sacchariflorus* was more intense (see Fig. 1, *a*) (which, later on, would allow it to build up vegetative biomass), and lateral shoots totaling 2–3 developed, whereas *M. sinensis* had only one

lateral shoot (Fig. 1, *b*). This ontogenetic state lasted 4–4.5 months.

Virginile plants. The sod diameter of virginile *M. sinensis* plants was considerably smaller than in *M. sacchariflorus* (15–20 vs. 25–30 cm, respectively). The plants had adult-type stems, leaves and roots. The lateral shoots were fast to develop, keeping up with the primary shoot in height. The bottom leaves of the primary shoot died off, and by that time the green assimilating leaves numbered 7–9. The life forms typical of adult plants were noted to establish (*M. sinensis*: a perennial, summer-green, grassy, short-rhizome, loose-sod, sympodially accrescent polycarpic plant with a semi-rosulate upright shoot and *M. sacchariflorus*: a perennial, summer-green, grassy, thin-long-rhizome, sympodially accrescent, polycarpic plant with an elongated upright shoot). Most of the plants had no generative shoots. In September, by the end of the vegetative stage of the first life year, the plants produced 8 to 20 shoots. The average number of shoots per plant exceeded 10, and the average plant height was 57.5 cm. The root system was well developed, with the length exceeding 15 cm.

Occasional samples passed to the generative stage, the G1 phase. Yet the young generative plants formed only one generative shoot each, on which seeds did not ripen. However, this fact had earlier been ascertained for *Miscanthus* in the continental climate of West Siberia forest steppe and attributed to temperatures too low for the formation of seeds in October, as opposed to the monsoon climate of southern Primorsky Krai.

Thus, this study into the features of the full ontogeny in plants grown from seeds collected in native habitats demonstrated that the specimens of the two *Miscanthus* species are distinguished by longer being in the most vulnerable ontogenetic states, seedlings and plantlets.

By expert evidence, a feedstock that is abundant, available, annually renewable, low-cost, highly ecologically effective, and storable will become a major raw material for many biotechnological industries in the nearest future (Mussatto et al., 2010). *Miscanthus* meets these criteria completely.

The study of the chemical composition of *M. sacchariflorus* grown in Siberia would allow one to evaluate the expediency of its processing for BC biosynthesis. Bacterial cellulose is an organic nanomaterial produced extracellularly by microorganisms under static (on the culture medium surface) or dynamic (agitated) conditions. Diverse nutrient media for BC culturing have been reported (Goelzer et al., 2009; Hong et al., 2012; Chen et al., 2013; Sakovich et al., 2017; Velásquez-Riaño, Bojacá, 2017; Revin et al., 2018; Hussain et al., 2019), and food-industry waste and agricultural residues are reckoned among preferential ones. The global trend is to substantiate the use of low-cost nutrient media for BC synthesis. However, it is seldom that *Miscanthus* is enlisted among potential nutrient media. Table summarizes the chemical compositions of seven *M. sacchariflorus* harvests: the whole plant, leaf and stem separately.

The contents of chemical elements in different plant organs are known to be variable and dependent on the plant species and age, regional climatic features, seasonal development, and forest growth conditions in the habitats (Torlopova, Robakidze, 2012). The *Miscanthus* biomass yield also depends on many factors: genotype, soil type, nutrients applied, plantation age, bioclimatic location, and weather during the agricultural season (Brosse et al., 2012).

The studies on the relationship between the chemical composition and plant age by the example of three harvests (2-, 3- and 4-year old plantations) revealed no considerable changes in any constituent except ash (Allison et al., 2011; Arnoult, Brancourt-Hulmel, 2015). In addition, the chemical compositions of different morphological parts of the plant were reported in (Krotkevich et al., 1983; Bergs et al., 2019). However, those studies were conducted in the temperate climate rather than in extremely continental Siberia. Therefore, investigation of the effects of plantation age and morphological part on the chemical composition of *Miscanthus* bred in Siberia remains highly relevant.

By the example of seven *M. sacchariflorus* harvests (see Table), chemical composition was found to depend not only on the plant age but also on plant morphology. The whole *Miscanthus* plant contained 41.7–53.6 % Kürschner cellulose, 3.2–6.3 % ash content, 20.1–23.8 % acid-insoluble lignin, 18.6–25.3 % pentosans, and 2.8–5.7 % fat-wax. The findings are consistent with the data reported for different *Miscanthus* genotypes in terms of the major constituents, cellulose and lignin (Jones, Walsh, 2001; Somerville et al., 2010; Brosse et al., 2012). The fat-wax fraction exceeds the values reported overseas, 0.5−0.6 % (Villaverde et al., 2009).

According to these literature reports, the major constituents of *Miscanthus* are cellulose (40–60 %), which forms the plant framework; hemicellulose (20–40 %), which is a matrix substance consisting of different polysaccharides; and lignin, which provides the structural rigidity and integrity (10–30 %). We found that the cellulose content increased and the noncellulosic content decreased with plantation age. A substantial growth in cellulose content was observed from the first (41.7 %) to the fifth (53.6 %) life year of the plantation, a nearly 12 % increase. Past the five-year age of the plantation, no significant increment in cellulose content was noticed; the cellulose content of 50 % remained stable over two years (*Miscanthus* harvests from plantations aged 6 and 7 years). Thus, 5-year-old *Miscanthus* was found to have the highest cellulose content, 53.6 %.

Among noncellulosic ingredients, high lignin content has an adverse effect during pretreatment of the feedstock for subsequent biosynthesis of BC. Therefore, lignin, which provides structural rigidity and integrity, should be controlled. In this regard, histochemical assays of transverse sections of the culms were performed to determine the seasonal course of lignification.

Representatives of the genus *Miscanthus* pertain to monocotyledon plants of the Poaceae family, most of

Chemical compositions of seven *M. sacchariflorus* harvests: whole plant, leaf and stem separately

Harvest year, plantation age	Morphological part of *Miscanthus*	Content*, %				
		Kürschner cellulose	Ash	Lignin	Pentosans	Wax-fat fraction
2011, 1 year	Whole plant	41.7	6.3	22.2	25.3	5.7
	Leaf	38.7	11.5	23.9	20.7	7.7
	Stem	48.1	3.0	20.5	27.9	4.3
2012, 2 years	Whole plant	44.5	6.2	23.8	23.6	4.8
	Leaf	40.5	8.7	25.3	20.7	6.1
	Stem	50.2	2.1	18.4	26.6	4.0
2013, 3 years	Whole plant	47.8	4.6	21.1	25.1	2.8
	Leaf	43.7	7.5	23.9	20.8	4.6
	Stem	50.7	2.0	17.2	27.4	1.8
2014, 4 years	Whole plant	53.1	5.9	22.0	21.0	5.0
	Leaf	43.3	9.2	23.6	20.3	6.3
	Stem	55.7	2.1	14.9	23.0	2.7
2015, 5 years	Whole plant	53.6	3.6	20.1	18.6	3.6
	Leaf	43.6	6.7	22.8	17.0	6.1
	Stem	56.6	2.2	16.0	20.9	2.1
2016, 6 years	Whole plant	50.1	3.2	22.3	20.4	4.5
	Leaf	43.8	9.9	26.6	19.5	6.7
	Stem	55.9	1.7	18.9	21.5	2.5
2017, 7 years	Whole plant	50.2	5.1	23.1	20.4	4.9
	Leaf	45.2	7.5	27.2	19.5	5.7
	Stem	53.6	1.5	18.7	22.5	3.1

* On an oven-dry basis.

which possess closed fiber vascular bundles scattered over the entire stem thickness. The constitution of the *Miscanthus* stem is close to that in rye and barley: parenchymal cells and vascular bundles gravitate to the periphery (Fig. 2, *a*).

The bundles are interspaced with the basic parenchyma tissue. Under epidermis, one can clearly see a rich pink ring of sclerenchyma, which imparts strength to the culm, partly owing to gradual duraminization. It is lignification that hinders feedstock treatment and pulping. In Fig. 2, *b*, the cells and tissues having a pink color of different intensity are stained with phloroglucinol, which exposes the extent of duraminization. Each fiber vascular bundle is made up of xylem vessels surrounded by woody parenchyma and of phloem and sclerenchymatous fibers. It is seen in Fig. 2, *b* that the bundle sheath exhibits a high level of duraminization as well.

It should be noted, though, that those sections were made upon completion of the vegetative stage, when the lignification process was the most pronounced in all tissues. The studies done at an earlier stage of feedstock preparation (see Fig. 2, *c*) showed that culm duraminization was at the initial stage.

Discussion

The study into the features of full ontogeny in plants grown from seeds that had been collected in native habitats demonstrated that the accessions of both *Miscanthus* species are distinguished by longer duration of the most vulnerable ontogenetic states: seedlings and plantlets. It can thus be inferred that the seed propagation method is of interest when working with a breeding material in good agricultural conditions, including cultivation of seedlings in protected ground. Industrial and pilot plantations should

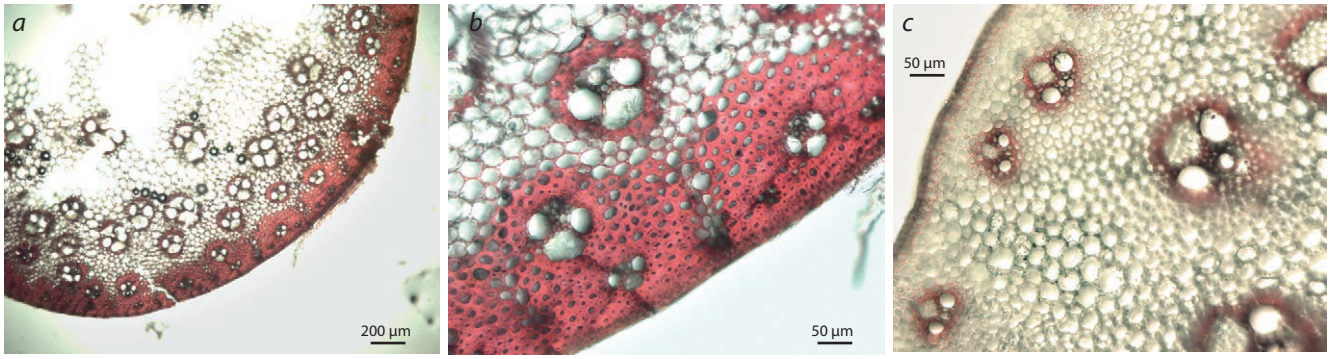

Fig. 2. Anatomical structure of the transverse section of *Miscanthus sacchariflorus* (Maxim.) Hack. stem: (*a*) typical (general view), (*b*) in late September, (*c*) in late August.

be established with cloned vegetative material, which is more stable.

The analyses of the chemical compositions of whole *M. sacchariflorus* plants and of leaves and stems separately form seven harvests showed that the cellulose content increased and the noncellulosic content decreased with plantation age. Beyond the 5-year age of the plantation, no growth in the cellulose mass fraction was observed. The cellulose content within 50.2–53.6 % indicates that this *Miscanthus* species is a promising cellulosic resource. We infer therefrom that it is more advisable to use a mature plant for the processing, as the cellulose yield is higher in this case. Generally, *Miscanthus* plantations aged 4–7 years exhibited high cellulose contents, and they are preferable for different chemical and biotechnological conversions.

The rise in cellulose percentage in the whole plant throughout five years is due to the fact that the *Miscanthus* seedling density becomes larger year after year, while the contribution of leaves, containing less cellulose, declines.

Irrespective of the plantation age, the highest cellulose content was found in the *Miscanthus* stem (48.1–56.6 %) and in the leaf it was 38.7–45.2 %. Despite the earlier recommendations given for different *Miscanthus* genotypes (Brosse et al., 2012), it was found herein that it is most advisable to use the *Miscanthus* stem in order to obtain a higher yield of cellulose and/or glucose. The prevailing content of cellulose in cereal crop stems is reported for wheat straw, oat, barley and rice (Lengyel, Morvay, 1973; Sun, 2010).

The histochemical assay revealed that since the transverse sections were made after the vegetative phase was completed, lignification was predominant in all tissues. The data on earlier feedstock preparation (see Fig. 2, *c*) demonstrated that culm duraminization was at the initial stage in August. As the hydrothermal conditions of the vegetative phases in terms of plant introduction vary considerably from year to year, we suggest here for the first time that the histochemical assay of the seasonal course of lignification of *Miscanthus* shoots be performed annually in determining specific time limits for the harvesting of aboveground biomass as the feedstock.

Conclusion

Thus, the analysis of the chemical composition of *M. sacchariflorus* grown in the Siberian climate demonstrated its high potential in the production of glucose substrate as the basic component of nutrient broths for biosynthesis of bacterial cellulose. The suggestion is innovative. It reduces the need for food-grade carbon sources for microbiological transformation.

Our findings prove the high relevance of the study into bacterial cellulose biosynthesis in a nutrient medium derived from *M. sacchariflorus*, the science-based engineering solution in the bacterial cellulose technology. *Miscanthus*, producing much cellulose, 48–53 % of the biomass, has a great resource potential for the manufacture of bacterial cellulose. Further studies are required for the most efficient solution of key technological issues. These issues include the development of cost-effective modes of chemical pretreatment and enzymatic hydrolysis of *Miscanthus* in a high glucose yield, enabling the use of such a glucose nutrient broth for bacterial cellulose synthesis.

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Acknowledgements. The study was supported by the Russian Science Foundation, project No.17-19-01054. Use was made of the materials of the bioresource scientific "Collection of Live Plants in Open and Protected Grounds", USU 440534.

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

Received June 18, 2019. Revised July 25, 2019. Accepted August 9, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):941-948
DOI 10.18699/VJ19.570

The Fifth International Scientific Conference PlantGen2019

Impact of growing conditions on the gum properties of different genotypes of guar (*Cyamopsis tetragonoloba* (L.) Taub.)

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Galactomannan (gum), a water-soluble polysaccharide, is widely used as a gelling agent in liquids, including in the oil and gas industry for hydraulic fracturing. The most effective source of this valuable plant material is seeds of guar (*Cyamopsis tetragonoloba* (L.) Taub.), a legume crop new for Russia. Although in recent years progress has been made in the selection of guar varieties adapted to the conditions of the Russian Federation, the question of the most appropriate region for the cultivation of this crop remains open. The purpose of the study was to investigate how a region and technology of guar cultivation can affect the main indicators of the final target product: the content and viscosity of guar gum extracted from the seeds of various guar genotypes. To understand this, ecogeographical tests of 13 guar accessions from the VIR collection were conducted at the experimental stations of the Vavilov Institute (VIR), where climatic conditions correspond to the temperature requirements of the crop. To compare the properties of gum extracted from the seeds of various genotypes, a fast-tracked laboratory method was suggested allowing gum extracts to be obtained for assessing their viscosity. The method allows fast screening of the breeding material and selecting guar genotypes with beneficial properties of guar gum which are in demand by the oil industry. Applying the fast laboratory method for assessing the properties of gum in seeds of 13 guar varieties showed that the content and viscosity of gum of the same variety vary greatly depending on growing conditions. The same set of 13 guar accessions was grown in 2018 at the Volgograd, Astrakhan, Dagestan and Kuban VIR experimental stations. As a result, the maximum viscosity values were obtained for the seeds reproduced at the Astrakhan region, where the guar was grown on irrigated lands. On the other hand, the maximum gum content in the seeds of all accessions was recorded when they were grown in the Volgograd region. The results showed that the guar gum extracted from seeds of guar plants grown in the Russian Federation can be used as a gelling agent in the processes of intensification of oil production by the method of hydraulic fracturing. This experience is new to the Russian Federation.
Key words: *Cyamopsis tetragonoloba* (L.) Taub.; guar; method for assessing guar gum content and viscosity; guar varieties; ecological testing; regions of propagation.

For citation: Kruchina-Bogdanov I.V., Miroshnichenko E.V., Shaukharov R.A., Kantemirova E.N., Golovina M.A., Abdullaev K.M., Balashov A.V., Rusinova E.V., Rusinov P.G., Potokina E.K. Impact of growing conditions on the gum properties of different genotypes of guar (*Cyamopsis tetragonoloba* (L.) Taub.). Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(7):941-948. DOI 10.18699/VJ19.570

Влияние условий выращивания различных генотипов гуара (*Cyamopsis tetragonoloba* (L.) Taub.) на свойства камеди семян

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Галактоманнан (камедь) – растворимый в воде полисахарид, используется в качестве гелеобразующего агента в жидкостях, в том числе в нефте- и газодобывающей промышленности для гидравлического разрыва пласта. Самым эффективным источником этого ценного растительного сырья (гуаровой камеди) являются семена гуара (*Cyamopsis tetragonoloba* (L.) Taub.), новой для Российской Федерации сельскохозяйственной культуры. Хотя за последние годы были достигнуты определенные успехи в селекции сортов гуара, адаптированных к условиям РФ, вопрос о наиболее подходящем регионе возделывания этой культуры остается открытым. Цель проведенных исследований заключалась в том, чтобы установить, в какой степени регион и технология выращивания гуара могут влиять на основные показатели конечного целевого продукта – содержание и вязкость камеди в семенах различных генотипов гуара. Для решения этого вопроса в 2017–2018 гг. были проведены эколого-географические испытания образцов гуара коллекции ВИР на опытных станциях института, климатические условия которых отвечают критериям теплообеспеченности этой культуры. Для сравнительного изучения свойств камеди в семенах различных генотипов гуара

впервые предложен ускоренный лабораторный метод получения вытяжек камеди для вискозиметрической оценки. Разработанный метод позволяет проводить ускоренный скрининг исходного материала для селекции сортов гуара, перспективных с точки зрения использования камеди из их семян в нефтедобывающей промышленности. В результате лабораторной оценки содержания и вязкости камеди, содержащейся в семенах 13 образцов гуара, обнаружено, что выход и свойства камеди одного и того же сорта сильно варьируют в зависимости от условий произрастания. Эксперимент 2018 г. по выращиванию одного и того же набора 13 образцов на Волгоградской, Астраханской, Дагестанской и Кубанской опытных станциях ВИР показал, что максимальные показатели удельной вязкости получены для семян репродукции Астраханской ОС ВИР, где гуар выращивался на поливе. С другой стороны, максимальное процентное содержание камеди в семенах у всех образцов гуара было зафиксировано при их выращивании в условиях Волгоградской ОС ВИР на капельном орошении. Результаты дополнительного тестирования образцов порошковой формы гуаровой камеди, полученной из семян сортов отечественной селекции, позволяют сделать вывод о пригодности продукта для использования в качестве гелеобразователя в процессах интенсификации добычи нефти методом гидравлического разрыва пласта. Данный опыт является уникальным для России.
Ключевые слова: гуар; камедь; содержание и вязкость; методика оценки; сорта; экологические испытания; регионы выращивания.

Introduction

Seeds of guar (*Cyamopsis tetragonoloba* (L.) Taub.), the new agriculture legume crop of the Russian Federation, serve as a source of a valuable vegetable product – guar gum. The latter mainly consists of the polysaccharides galactomannans which are able to increase the viscosity of the solution, even at low concentrations. Galactomannans, along with cellulose, starch, xyloglucan, xanthan gum and dextran, are the most economically demanded polysaccharides. Galactomannan is found in the seed endosperm of many plants. While several species are considered as a possible source of this substance, mainly three legume species – *Cyamopsis tetragonoloba* (L.) Taub., *Ceratonia siliqua* L. and *Caesalpinia spinosa* (Molina) Kuntze are used for the industrial production of plant gum (Thombare et al., 2016). With that, guar gum is extracted by the easiest and cheapest way, stimulating increased interest in guar, as an agricultural crop (McArdle et al., 2011).

Guar belongs to a rare group of legume species whose seeds contain a developed endosperm instead of large cotyledons, which are common for most legumes (Hanson, 2015). In the cell walls of the guar endosperm, as well as within the cells of this storage tissue, galactomannan inclusions are concentrated to provide energy to the developing plant during germination. All components of the guar seed are used in industry, and the raw materials obtained from them have a specific name: the hull (churi) covers two endosperm halves (splits) in the cells of which galactomannan is stored. The endosperm in turn surrounds two cotyledons including the embryo (korma).

The technology for producing powdered gum from guar seeds is the know-how of many commercial companies. The general scheme of the process usually includes several main stages: seeds are divided into two halves (split) which are dried at high temperature. As a result, the cotyledons with the embryo are easily separated from the endosperm. The separated endosperm halves are heated, the seed hull is softened, and then separated from the endosperm using various devices, such as mechanical grinders, attrition and roller mills. As soon as the endosperm is separated from other tissues of the seed, it is ground into a powder, from which galactomannan is extracted by the precipitation with ethanol, then dried. The final product is a homogeneous white powder, similar in texture to wheat flour. The most important criteria of the quality of the product are mesh size and viscosity of the aqueous solution, which is measured under standard conditions, often using a Brookfield viscometer (Abidi et al., 2015). The amount and molecular weight of the galactomannan found in the endosperm extract can vary significantly, depending on the source of the seed and the growing conditions (Ellis et al., 2001). Thus, for the successful introduction guar in the Russian Federation, the correct choice of regions and environmental conditions for the cultivation of this crop is just as important as breeding of new varieties.

In all areas of the Russian Federation, the main limiting factor for guar cultivation is the sum of effective temperatures during the growing season. From the analysis of temperature and humidity parameters of the agro-climatic zones of the Russian Federation it follows that a number of regions of the Crimea, Dagestan, Krasnodar, Stavropol and Rostov as well as the southern Volga, are suitable for guar cultivation since in those regions the sum of effective temperatures above 10 °C exceeds 3400–3500 °C (Lebed et al., 2017).

Although guar breeding in Russia started just recently, by 2018 the State Register of Breeding Achievements had registered five new guar varieties allowed for cultivation in the Russian Federation. Nevertheless, the question of the most suitable region for the crop cultivation still remains open, in particular, due to the lack of information on the quality of guar gum obtained from seeds that were produced in different ecogeographical regions. To solve this problem, in 2017–2018 the Vavilov All-Russian Institute of Plant Genetic resources (VIR) carried out ecological testing of its guar germplasm collection at the VIR experimental stations where climatic conditions corresponded to the temperature requirements of the crop. The purpose of the tests was primarily to establish to what extent the region and a growing technology can affect the main quality indicators of the final target product – the content and viscosity of the gum extracted from the seeds of different guar genotypes.

To date, there is no generally accepted standard or any arbitration method for assessing the capability of seeds of various guar genotypes to serve as a good raw material the production of gum of a certain viscosity. In the published reports two independent processes are usually described: 1) the isolation and purification of gum from seeds to estimate the gum yield,

and 2) the preparation of a solution from the obtained guar gum followed by a measurement of its viscosity (Cerqueira et al., 2009; Eldirany et al., 2015). These techniques are usually executed by different performers and are separated in time. Here, for the first time, we propose a method for assessing the quality of guar seeds for the both indicators – gum yield and viscosity – in one step, which opens up the possibility for extensive screening of various guar genotypes using compact and affordable means.

Material and methods

13 accessions promising in their agrobiological characteristics according to the results of their preliminary evaluation in 2017 at the Kuban branch of the VIR (Krasnodar region) were included in the ecological tests (Table 1). In 2018, the same set of seed accessions of a single reproduction of the Kuban branch was distributed across all geographical locations where ecological trials were planned. The amount of seeds in each location was enough to conduct field trials on an area of 10 m^2 for each accession.

Determination of the content and viscosity of gum extracted from guar seeds. Mass screening was carried out according to the newly developed method of laboratory assessment of the content and viscosity of gum in guar seeds. The average seed sample taken in an amount of 2.0 g (exact weight) was placed in a wide laboratory beaker with a capacity of 150 cm^3. Seeds in the beaker were poured into 15 cm^3 of distilled water and placed in an autoclave. Boiling of the soaked seeds was carried out by holding for 30 min at a temperature of 121 °C.

Then, after removing the water, the boiled seeds were placed in a beaker with 50 cm^3 of distilled water and stirred on a magnetic stirrer for 2 hours at room temperature. The obtained extract was separated by centrifugation at 7000 min^{-1} for 15 min. The separated precipitate was re-extracted into 50 cm^3 of distilled water on a magnetic stirrer for 1 hour at room temperature. The centrifugation and extraction procedure was repeated one more time, and clarified solutions after centrifugation were combined to obtain a final extract volume of 140–160 cm^3. Using the measuring cylinder, the resulting final volume of the gum extract was estimated with an accuracy of ±1 ml.

The dynamic viscosity of the resulting clarified solution was measured at room temperature on a Brookfield rotational viscometer (spindle L2, rotation speed 100 min^{-1}). The viscosity was recorded for 40 min, scoring the initial and final value. In the solution after measuring the viscosity, the concentration of "raw gum" was determined as the solids content according to standards (GOST 33977–2016, http://protect.gost.ru/).

The final viscosity estimation result was taken at the end of the measurement. The estimated relative viscosity values were calculated from the measured values of viscosity and gum concentration as follows:

$$\eta_{er} = \frac{\eta - \eta_0}{\eta_0 c},$$

where: η – the measured value of the gum solution viscosity, mPa·s; η_0 – viscosity of solvent (for water η_0 = 1 mPa·s); c – "raw gum" concentration in the extract, %.

Table 1. List of guar accessions from the VIR collection involved in ecological trials in 2018

No.	No. in VIR Catalogue	Where from the seed material was obtained	Variety
1	52568	Argentina	Unknown
2	52569	Pakistan	Unknown
3	52571	Crimea	Unknown
4	52572	Krasnodar region	Vavilovskii 130
5	52573		Kubanskii
6	52574		Vector
7	52575		Sinus
8	52580	Rostov region	Unknown
9	52581		Unknown
10	52584	USA	Santa Cruz
11	52585		Kinman
12	52586		Lewis
13	52742	Krasnodar region	Kubanskii Jubileinyi

The gum content (GC) in the seeds (in %) was calculated as the ratio of the amount of extracted gum in the solution to the initial seed weight:

$$GC = \frac{c \cdot \rho \cdot V}{a},$$

where: ρ – the density of the gum extract (at a concentration of gum less than 0.6 % should be taken ρ = 1 g/cm^3); V – measured final volume of gum extract, cm^3; a – seed sample taken for extraction, g.

The properties of dry powder guar gum samples, including stability and operating parameters as a gelling agent for hydraulic fracturing fluid, were analyzed in the laboratory of NIKA PetroTech company. A common oil industry method for measuring the viscosity of a 0.48 % guar solution was used. This solution would be commonly called a "40 lb gel" since contains 40 lb of guar mixed into 1000 gal of water and at 511 s^{-1} gives a viscosity of 30–45 centipoise (cP), depending on the quality of the guar gum (Abidi et al., 2015).

Statistical data analysis was carried out using the Statistica 12.

Results

Ecological trials of guar accessions at VIR experimental stations

Growing experiments with the set of 13 guar accessions (see Table 1) were carried out in 2018 at four branches of VIR: Kuban (Gulkevichsky District, Krasnodar region), Astrakhan (Astrakhan), Volgograd (Volgograd), Dagestan (Derbent, Daghestan). Peculiarities of agro-climatic conditions in four geographical points where the tests were carried out are given below.

Kuban branch. Seeds were sown in the last decade of May. Irrigation was not applied. During germination period

and further from May 28th to July 26th, abnormally high air temperatures ranging from 30 to 37.6 °C were recorded, with no any precipitation and strong dry winds, which led to the drying out of the soil horizon to a depth of 20–25 cm. The soil temperature at a depth of 20 cm in the first ten days of June was 21.8 °C, in the second – 23.8 °C, causing cracking of the soil arable horizon with wide crevices up to 2–5 cm to a depth of 50–60 cm. Such climatic indicators deviate from long-term averages, they occur with a frequency of once every 15–20 years and are stressful for the guar culture.

Daghestan branch. In the soil and climatic conditions of Dagestan (semi-dry subtropics) guar was grown for the first time. Irrigation (irrigation furrows) was used. Before the maturing phase, the plants developed quickly showing vigorous young growth. Then, however, heavy precipitation fell in mid-August, after which plants in the phase of the maturation of lower beans were affected by *Alternaria* pathogen. As the result, epiphytotic injury was recorded by September (4–5 points of defeat at 5- and point scale). This negatively affected all indicators of productivity.

Volgograd branch. The climate of the Lower Volga region, where the experimental station is located, is extremely continental. Spring is dry with a rapid increase in daytime temperatures and frequent strong winds. Summer is hot and dry with the scorching heat (air temperature is often above 40 °C, no precipitation). Throughout the growing season of guar, a drip irrigation system was used. Guar seeds were sown in mid-May in a well-heated soil at a depth of 2–3 cm, after the soil furrows were well moistened with water. Pronounced cooling in late May – early June negatively affected the development of guar, the plants turned yellow, some of them died. Nevertheless, after that, under fairly dry weather, high air temperature and drip irrigation, guar plants grew quite strong and branched (up to 123 cm in height). During the growing season of guar, *Aphid* and *Alternaria* lesions were monitored – only a small injury of individual plants was recorded.

Astrakhan branch. The branch is located in the southern part of the Astrakhan region in the delta of Volga river. The Astrakhan region is the most droughty area in Russia, generally characterized as extremely continental. Seeds were sown in the first decade of May. Sowing was carried out in irrigation furrows, the seeds were planted in moist soil manually. In order to preserve moisture and prevent the appearance of soil crust, manual row harrowing was carried out 5–6 days after sowing. Subsequently, the care of the crops consisted of timely manual weeding, cultivation and drip irrigation.

The rapid laboratory assessment of the content and viscosity of gum in guar seeds

For mass screening of breeding guar material for the content and quality of gum, we propose a rapid laboratory method for producing gum extracts from guar seeds with subsequent viscometric evaluation, without isolating the gum itself in its pure form. The developed method is based on the extraction of gum from boiled guar seeds. The boiling conditions (121 °C, 30 min) were chosen experimentally to ensure sufficient swelling of the endosperm polysaccharides to perforate the seed coat, but at the same time to prevent loss of gum when the seeds are soaked in water. Thus, instead of many hours of seed soaking process followed by isolation and drying of the endosperm halves (Eldirany et al., 2015), the whole procedure takes 6 hours, including measuring the viscosity and calculating the solids content.

The viscosity of the obtained extract with a solids concentration of 0.35–0.55 % is measured using a rotational viscometer. The measured value is adjusted to the solids concentration of the extract, obtaining the value of the specific viscosity. Other water-soluble components of guar seeds do not interfere with determination.

The generally accepted assessment of the viscosity of guar gum solutions involves the use of rotational viscosimetry at gum concentrations of about 0.5 % (see ECFA FAO Monographs 5, 2008). The amount of seeds (2.0 g) taken for analysis (considering the content of gum in the seeds from 30 to 50 % and the final extract volume of 150–200 cm^3) allows to ensure the concentration of gum in the aqueous extract at the level of the desired 0.5 %.

The availability of endosperm polysaccharides for extraction is determined by the completeness of their hydration. Previously, it was reported that hydration seed treatment requires a long-term soaking of about 16 hours (Eldirany et al., 2015). During the developing of our method, we experimentally established that the degree of hydration of the seeds (by their volume and residual hardness of the swollen phase), corresponding to 16 hours of soaking at room temperature, can be achieved in 2 hours of boiling at atmospheric pressure or in 30 min of boiling at 121 °C.

To prove this, we estimated the yield of gum polysaccharides in solution by changing the viscosity of the extract depending on the duration of extraction (Fig. 1).

The extraction conditions that were established experimentally (3 cycles of 4 hours) provide a gum output of at least 95 % of those determined by the conventional method after endosperm halves isolation, and allow the entire analysis procedure to be completed in 6 hours, including boiling and measuring the viscosity. Gum in a solution purified by centrifugation makes up at least 90–95 % by weight of the solids content. That allows, with sufficient accuracy for the express method, to equate content of gum in the extract to the total solids content.

The proposed method allows to estimate the content of gum in guar seeds and its specific viscosity based on only two measurements – the viscosity of the extract and the dry matter content in it – with knowing the weight of the seed sample and the volume of the obtained extract. The specific viscosity index is dimensionless. For its calculation, the concentration of solids in the extract is taken into account, the dynamic viscosity of which is measured by a rotational viscometer in cP (or mPa·s). This allows the use of a single scale for a comparative assessment of extracts from various unknown samples of guar seeds with different concentrations of gum.

To verify the effectiveness of the proposed method, we estimated the viscosity of the gum samples for two guar varieties – cv. Kubanskii and Kubanskii Jubileinyi using the traditional method, after isolation of the endosperm and preparation of dry guar gum powder. The results coincided with those obtained according to the method proposed by the authors within the metrological relative error of ±10 %, which can serve as a good reason for the introduction of the express method in everyday laboratory practice.

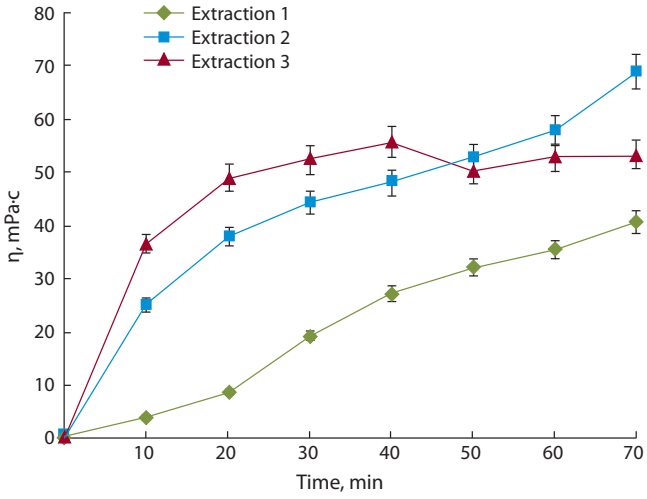

Fig. 1. Kinetics of viscosity changes in three successive extracts from boiled guar seeds.

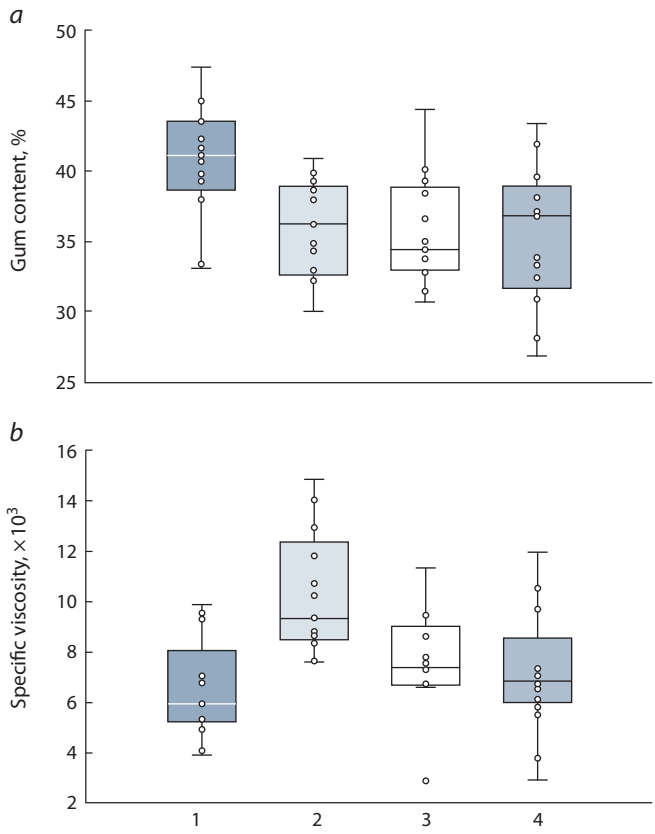

Fig. 2. Comparison of average percentages of gum (*a*) and specific viscosity of gum extracts (*b*) in seeds of 13 guar accessions grown at the Volgograd (1), Astrakhan (2), Dagestan (3) and Kuban (4) branches of VIR.

Assessment of gum content and viscosity in the seeds of guar accessions grown under different environmental conditions

The developed method for the rapid assessment of the content and viscosity of gum extracts allowed us to analyze the seeds of 13 guar accessions grown in 4 geographical points of ecological testing. The average percentage of gum in the seeds of 13 test samples varied from 35.4 % (Kuban) to 40.7 % (Volgograd). According to the results of the Student's *t*-test (*t*-test, dependent samples), guar seeds grown under the conditions of Volgograd region contained a significantly higher percentage of gum compared to seeds grown in Astrakhan ($p < 0.008$), Dagestan ($p < 0.011$) and Kuban ($p < 0.009$) (Fig. 2, *a*). The average specific viscosity of gum extracts was maximal for seeds propagated in the Astrakhan branch (10305), and significantly exceeded this indicator compared to seeds obtained in Volgograd ($p < 0.0005$), Dagestan ($p < 0.003$) and Kuban ($p < 0.001$) (see Fig. 2, *b*).

When comparing seeds of the same accession grown under different ecological conditions in terms of gum content and specific viscosity, the correlation turned out to be not significant (Fig. 3, *a*, *b*). Thus, environmental conditions during cultivation of guar significantly affect the level of accumulation and properties of gum as a reserve polysaccharide of guar seeds.

Preparation of dry powder gum samples from seeds of different guar varieties in laboratory conditions

In addition to the laboratory assessment of the content and specific viscosity of gum extracts by the express method developed, we also attempted to produce in laboratory conditions samples of powder gum from the seeds of 3 out of 13 tested guar accessions. The accessions selected were the first guar varieties bred in Russian Federation: Kubanskii, Kubanskii Jubileinyi and Sinus. The seeds propagated in the Astrakhan branch of VIR were used.

At the first stage of preparation of powdered gum, after soaking, the guar seeds were fractionated manually, isolating the endosperm (split) from the hull and germ (cotyledons with the embryo). The separated endosperms were then dried at 105 °C for 20 min as suggested by Sabahelkheir et al. (2012) (Fig. 4, *a*). Further isolation of the gum from the endosperm was carried out after 100 g of split were boiled (30 min, 121 °C), and three rounds of extraction at room temperature (1 hour each) were conducted. Insoluble residues were removed by centrifugation. After each extraction, the gum fraction was precipitated with acetone, dried at 105 °C in air and ground. The resulting dry powdered gum samples obtained after three successive extraction steps are shown in Fig. 4, *b*. The obtained dry powder guar gum samples (accessions No. 5, 7 and 13, see Table 1) were then transferred to the laboratory of NIKA PetroTech company for the further analysis.

Assessing of the powder guar gum samples as a gelling agent for hydraulic fracturing fluid

In the oil industry one of the most actual methods for increasing the efficiency of producing wells is hydraulic fracturing, which results in increased oil recovery from the oil reservoir (Silin et al., 2013). Hydraulic fracturing is a process in which fluid pressure acts directly on the oil-bearing rock until it collapses and a crack occurs.

To create hydraulic fractures in oil-bearing rock and to pump proppant there, an aqueous polysaccharide gel is commonly used. For more than 50 years, guar-based fluids and guar derivatives have been used as thickeners to create linear gels. Since the late 1960s and in present the so-called

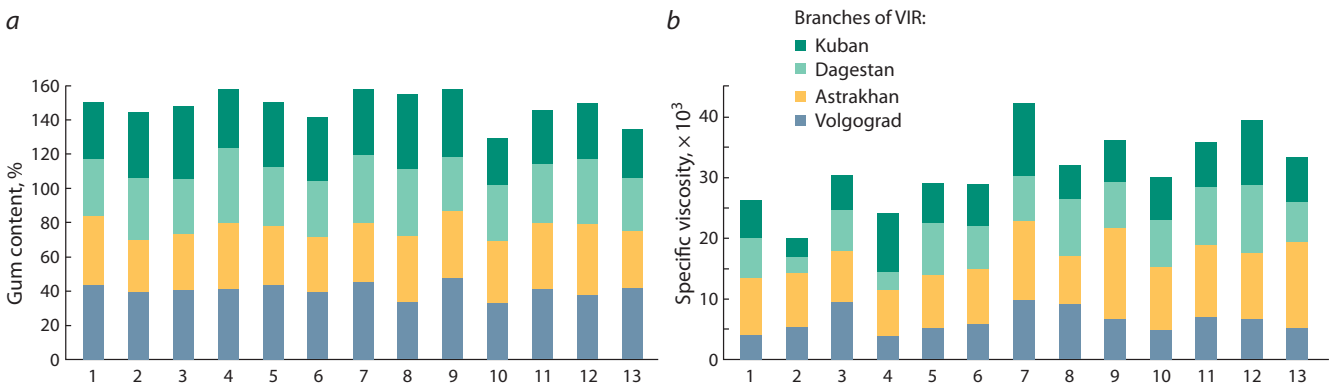

Fig. 3. Comparison of the quantity and quality of gum in the seeds of 13 guar accessions grown at different VIR experimental stations in 2018: *a*, gum content (%) in the seeds of the accessions; *b*, specific viscosity of gum extracts from seeds of the accessions.

The numbers on the X-axis correspond to the numbers of the accessions in Table 1.

Fig. 4. Preparation of dry powder gum samples from guar seeds in laboratory conditions. *a*, fractions of seeds: embryo and cotyledons (korma), seed coat (churi) and endosperm (split) containing gum; *b*, dry gum powder obtained from endosperm: from left to right – after three successive extraction (purification) steps.

"cross-linked" hydraulic fracturing fluids are used – solutions of polysaccharides that are complexed with multivalent ions of boron and other metals (cross-linkers). When a linear gel interacts with a cross-linker, complex bonds form between polymer chains. This interaction creates a cross-linked system, turning a low-viscosity fluid (linear gel) into a highly viscous structured fluid (cross-linked gel) that can hold and transport proppants into the oil-bearing formation to increase conductivity.

In NIKA PetroTech company three samples of powder gum obtained in the laboratory conditions were tested to their ability to provide a stable gel cross-linked with boron-containing agents with high sand-bearing characteristics. An estimated qualitative characteristic of a cross-linked hydraulic fracturing fluid is the ability to form a "tongue" (Fig. 5). The presence of the "tongue" indicates the sufficient viscosity of the obtained cross-linked gel. The viscosity of a linear gel is considered to be working at 40–45 mPa·s; while the viscosity of the cross-linked system should be in the range of more than 300–400 mPa·s, depending on the particular requirements.

As follows from the Table 2, the final viscosity of the gum powder solution (linear gel) obtained from the seeds of varieties Kubanskii and Kubanskii Jubileinyi reaches the required threshold level after 30 min (41.1 and 36.2 cP, respectively). This indicates the potential possibility of obtaining powder gum with desired properties from guar seeds produced in the Russian Federation. By the speed of viscosity increase, which is determined by the ratio of the viscosity index after 3 min of hydration to the maximum value of viscosity, the Sinus guar variety was distinguished.

Figure 6 shows that the obtained cross-linked gel meets the minimum quality requirements for fracturing fluid. A decrease in viscosity during the first 35 min is a typical thermal attenuation of the system and is acceptable; from 35 to 60 min, the average viscosity stabilizes and reach a value of 350 mPa·s. The standard concentration of dry gel in the hydraulic fluid is 3.6 kg/m^3. A higher concentration of the obtained product (4.8 kg/m^3) indicates that it is necessary to refine the cleaning process of the dry powder gum, as well as to conduct additional investigations of the molecular weight distribution of the resulting polymer.

Discussion

Galactomannan (guar gum), a water-soluble reserve polysaccharide of guar seeds, is used as a gelling agent in liquids, including in the oil and gas industry for hydraulic fracturing. In

Fig. 5. Appearance of a cross-linked gel obtained from a laboratory sample of powder gum (cv. Kubanskii).

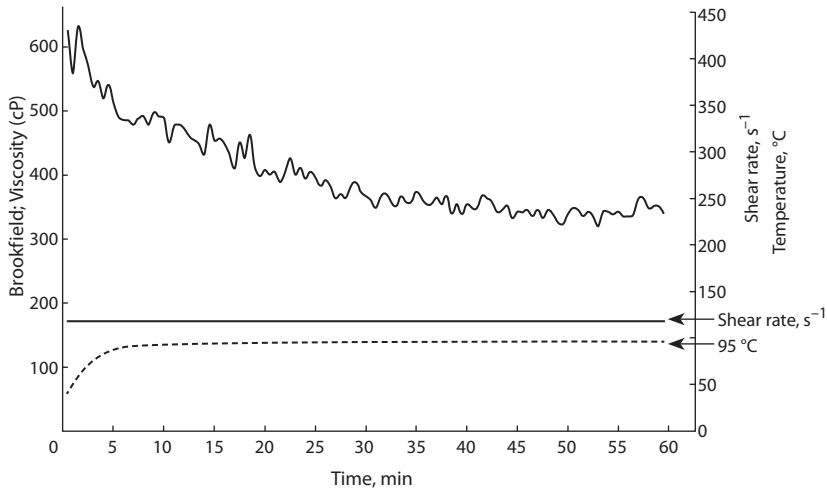

Fig. 6. Graph of the temperature stability of a cross-linked guar-borate gel obtained at a temperature of 95 °C from a powdered gum sample prepared from seeds of guar accession No. 5, propagated at the Astrakhan branch of VIR (upper broken line).

The left ordinate axis represents the viscosity index in cP (Brookfield; Viscosity). Two constant values are plotted on the right axis: a solid straight horizontal line reflects the constant speed of rotation of the beaker with the sample (Shear rate, 117.6 rotations per sec); the dashed line represents the temperature of the system (95 °C).

our study it was found that the different guar varieties possess distinct values of the total content and specific viscosity of gum extracts from seeds, and these indicators can vary significantly depending on the guar variety and the conditions of its cultivation.

For a comparative study of properties of gum contained in the seeds of various guar genotypes for the first time we proposed a rapid laboratory method for obtaining gum extracts for the gum quantity and viscosity evaluation. The developed method allows fast screening of the breeding material for the selection of guar varieties, promising from the point of view of using gum manufactured from their seeds in the oil and gas industry. The results of applying of the new method for the laboratory assessment of the content and viscosity of gum in the seeds of 13 guar samples showed that the yield and properties of the gum of the same variety vary greatly depending on the growing conditions.

Until now, attempts to grow guar as an industrial crop have been carried out in the Krasnodar region (Lebed et al., 2017), and in the Crimea, where guar is usually grown without irrigation (on a dry land). Although some success in obtaining yield in these regions was achieved, the risks of yield loss in the absence of rainfall during critical periods of plant development were also revealed. Thus, the idea of the possibility of guar production in the Lower Volga region using irrigation has now received support according to our results obtained from environmental tests of guar genotypes in different regions of the country.

So, the 2018 experiment on growing the same set of 13 guar accessions at four VIR experimental stations (Volgograd, Astrakhan, Dagestan and Kuban branches) showed that the maximum specific viscosity of gum was detected for seeds obtained in the Astrakhan branch, where irrigation is usually applied. Subsequent analysis of powder gum samples obtained in laboratory conditions from the seeds of three guar varieties grown at the Astrakhan branch also showed their potential suitability for industrial use. On the other hand, the maximal gum content among the all guar accessions tested was recorded for the seeds propagated under the conditions of the Volgograd branch of VIR with drip irrigation. The results are preliminary, as they are based on the results of environmental tests of just one year. However, they indicate that, given the appropriately developed technology for propagation of this crop under unusual growing conditions, the idea of cultivating guar with irrigation

Table 2. Physical and chemical parameters of the guar gum powder obtained from the seeds of three guar varieties reproduced in the Astrakhan branch of VIR

Parameter	Kubanskii	Temperature, °C	Kubanskii Jubileinyi	Temperature, °C	Sinus	Temperature, °C
Viscosity of gum powder solution, mPa·s						
initial (3 min)	4.7	20.7	2.3	21.9	10.2	21.2
final (30 min)	41.1	21.4	36.2	22.4	29.2	19.0
pH	7.16	20.7	6.59	22.1	6.65	19.0

in the Astrakhan and Volgograd regions can be promising and get support from the agribusiness.

Conclusion

Assessing of the powder guar gum samples prepared from seeds of guar varieties developed in Russian Federation allow us to conclude that the product is suitable for use as a gelling agent for hydraulic fracturing fluid used intensify oil and gas production. This experience is new to the Russian Federation.

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Acknowledgements. The study was financially supported by the Ministry of Equation and Science of the Russian Federation, project RFMEFI60417X0168, Agreement No. 14.604.21.0168.

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

Received June 4, 2019. Revised September 4, 2019. Accepted September 5, 2019.

Вавиловский журнал генетики и селекции. 2019;23(7):949
DOI 10.18699/VJ19.571

DOI 10.18699/VJ19.571

Исправление к статье «Применение полногеномной амплификации для генетической оценки эмбрионов коров»

К.С. Пантюх, И.В. Рукин, С.М. Портнов, А. Хатиб, С.Л. Пантелеев, А.М. Мазур

Вавиловский журнал генетики и селекции. 2019;23(4):489-495

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Оригинальная статья может быть найдена по DOI 10.18699/VJ19.518

DOI 10.18699/VJ19.571

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K.S. Pantiukh, I.V. Rukin, S.M. Portnov, A. Khatib, S.L. Panteleev, A.M. Mazur

Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):489-495 (in Russian)

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Издатель: Федеральное государственное бюджетное научное учреждение
«Федеральный исследовательский центр Институт цитологии и генетики
Сибирского отделения Российской академии наук»,
проспект Академика Лаврентьева, 10, Новосибирск, 630090.
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 25.10.2019. Выход в свет 29.11.2019. Формат 60 × 84 $^1/_8$. Усл. печ. л. 18.13.
Уч.-изд. л. 19.6. Тираж 150 экз. Заказ № 212. Цена свободная.

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