2019 • 23 • 4

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 • 4

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

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Уважаемые коллеги, дорогие читатели! Предлагаем Вашему вниманию летний выпуск «Вавиловского журнала генетики и селекции», посвященный ряду актуальных направлений в области генетики растений, животных и человека, молекулярной и популяционной генетики и клеточной биологии.

Раздел «Молекулярная и клеточная биология» состоит из двух обзорных статей и экспериментального исследования. Открывает рубрику обзор, в котором изложены современные представления о роли мобильных элементов в процессах морфогенеза у растений и животных. Роль цистатина С и его терапевтическая функция в лечении нейродегенеративных заболеваний (болезней Альцгеймера и Паркинсона) рассмотрены в следующем обзоре. В экспериментальном исследовании раздела представлены результаты использования рекомбинантных белков для профилактики и лечения инфекций, вызванных ортопоксвирусами.

Разделы «Генетика растений» и «Генофонд и селекция растений» включают пять оригинальных исследований. Рассматриваемый в них спектр проблем достаточно широк: от влияния «вегетативных» генов *YABBY*, *MhyFIL1* и *MhyFIL3* и их гомологов на асимметрию листа у высших растений до биоинформатических подходов для оценки признаков продуктивности у сельскохозяйственных культур. В одной из экспериментальных статей предложены методические рекомендации по криоконсервации апексов растений культурных видов картофеля с пошаговым протоколом. Привлекает также внимание работа, в которой обсуждаются проблемы цитоплазматической мужской стерильности у сорго и механизмы восстановления фертильности.

Рубрика «Физиологическая генетика» представлена обзорной статьей и оригинальным исследованием. В обзоре литературных данных освещены вопросы патогенеза психических и нейродегенеративных заболеваний. Рассмотрены модели патологических состояний с участием серотонинергической системы, описаны генетические подходы по разработке фармакологических средств для коррекции болезней. В задачу экспериментальной работы раздела входило изучение влияния неонатального введения синтетического глюкокортикоидного гормона дексаметазона на когнитивные способности животных.

Два литературных обзора вошли в рубрику «Генетика человека». В одном из них обсуждаются результаты исследования депрессивных расстройств, полученные с помощью современных полногеномных и полноэкзомных подходов. Описаны ключевые процессы, принимающие участие в формировании депрессии, в том числе эпигенетические факторы. Второй обзор знакомит с молекулярными механизмами наследственных спиноцеребральных атаксий, входящих в число нейрогенеративных заболеваний, которые приводят к нарушению координации движений.

Проведение генетического мониторинга очень важно для выявления моногенных заболеваний у крупного рогатого скота. Предлагаем вниманию читателя оригинальное исследование по применению полногеномного поиска ассоциаций для выявления летальных гаплотипов у эмбрионов коров голштинской или черно-пестрой голштинизированной породной группы. Еще одна статья посвящена генетическому мониторингу жизнеспособных эмбрионов животных с целью создания системы ускоренного воспроизводства высокоценного племенного скота. Результаты представлены в разделе «Генетика животных».

Завершает журнал рубрика «Экологическая и популяционная генетика», в которой приведены экспериментальные данные по изучению фенотипической изменчивости листовой пластины липы мелколистной. Получены корреляции между вариациями признаков листа и условиями произрастания в городской среде и сельской местности.

Мы рады сообщить нашим читателям, что журнал вошел в третий (категория Agricultural and Biological Sciences) и четвертый (категория Biochemistry, Genetics and Molecular Biology) квартили базы данных Scopus (см. https://www.scopus.com/sourceid/21100822819?origin=resultslist#tabs=1).

Академик В.К. Шумный

Вавиловский журнал генетики и селекции. 2019;23(4):380-389
DOI 10.18699/VJ19.506

The role of transposable elements in the ecological morphogenesis under the influence of stress

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In natural selection, insertional mutagenesis is an important source of genome variability. Transposons are sensors of environmental stress effects, which contribute to adaptation and speciation. These effects are due to changes in the mechanisms of morphogenesis, since transposons contain regulatory sequences that have *cis* and *trans* effects on specific protein-coding genes. In variability of genomes, the horizontal transfer of transposons plays an important role, because it contributes to changing the composition of transposons and the acquisition of new properties. Transposons are capable of site-specific transpositions, which lead to the activation of stress response genes. Transposons are sources of non-coding RNA, transcription factors binding sites and protein-coding genes due to domestication, exonization, and duplication. These genes contain nucleotide sequences that interact with non-coding RNAs processed from transposons transcripts, and therefore they are under the control of epigenetic regulatory networks involving transposons. Therefore, inherited features of the location and composition of transposons, along with a change in the phenotype, play an important role in the characteristics of responding to a variety of environmental stressors. This is the basis for the selection and survival of organisms with a specific composition and arrangement of transposons that contribute to adaptation under certain environmental conditions. In evolution, the capability to transpose into specific genome sites, regulate gene expression, and interact with transcription factors, along with the ability to respond to stressors, is the basis for rapid variability and speciation by altering the regulation of ontogenesis. The review presents evidence of tissue-specific and stage-specific features of transposon activation and their role in the regulation of cell differentiation to confirm their role in ecological morphogenesis.
Key words: variability; morphogenesis; stress; transposable elements; evolution.

For citation: Mustafin R.N., Khusnutdinova E.K. The role of transposable elements in the ecological morphogenesis under the influence of stress. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):380-389. DOI 10.18699/VJ19.506

Стресс-индуцированная активация транспозонов в экологическом морфогенезе

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Инсерционный мутагенез, обусловленный транспозициями мобильных элементов, лежит в основе изменений геномов в естественном отборе. Транспозоны являются сенсором экологических стрессовых воздействий, благодаря чему воздействия стрессоров на организмы потенцируют изменения расположения транспозонов, что способствует адаптации и видообразованию. Это обусловлено изменением механизмов морфогенеза, так как транспозоны содержат в своем составе регуляторные последовательности, оказывающие *цис*- и *транс*-воздействие на экспрессию специфических белок-кодирующих генов. Мобильные генетические элементы способны также к сайт-специфическим перемещениям, которые приводят к активации генов стрессового ответа. Кроме того, транспозоны служат источниками микроРНК, siРНК, длинных некодирующих РНК и сайтов связывания с транскрипционными факторами. В эволюции благодаря мобильным генетическим элементам возникают новые белок-кодирующие гены путем одомашнивания, экзонизации и дупликации. Данные гены содержат нуклеотидные последовательности, которые взаимодействуют с процессированными из транспозонных транскриптов некодирующими РНК, в связи с чем они находятся под управлением эпигенетических регуляторных сетей с участием мобильных генетических элементов. Поэтому наследуемые особенности расположения и состава транспозонов могут иметь значение в характере реагирования на определенные экологиче-

ские стрессорные воздействия. Это служит основой для отбора и выживания особей со специфическим составом и характером расположения транспозонов, способствующих адаптации при определенных средовых условиях. В эволюции свойство транспозонов перемещаться в специфические сайты генома, регулировать экспрессию генов и взаимодействовать с транскрипционными факторами, наряду со способностью реагировать на экологические стрессоры, является основой для быстрой изменчивости и видообразования за счет модулирования управления онтогенезом. Роль транспозонов в экологическом морфогенезе подтверждена данными об их ткане- и стадиеспецифических особенностях активации и участии в управлении дифференцировкой клеток в эмбриогенезе и постнатальном развитии. Дополнительным источником изменчивости служит горизонтальный перенос транспозонов, способствующий изменению их состава в геномах.
Ключевые слова: изменчивость; морфогенез; стресс; транспозоны; эволюция.

Introduction

The concept of "ecological morphogenesis" includes the features of growth and development of organisms under the influence of key adaptation mechanisms that are formed under the influence of environmental factors (Curran, 2015). The basic principles of ecological morphogenesis are due to the differentiation and development of tissues under the control of genetic programs that have evolved under the influence of certain environmental factors with the selection of the optimal adaptive morphofunctional capabilities of the organism (Deng et al., 2014). In 1942, Conrad Waddington discovered that some phenotypic traits induced by heat shock in *Drosophila* pupae and then selected over several generations become inheritable in the presence of heat shock (Waddington, 1942). This means that changes in phenotypic traits that were acquired under the influence of environmental stressors (ES) can be inherited through the germ line. Waddington developed the concept of "canalization and assimilation" to explain his concept in the framework of Darwin's evolutionary theory. He called the "canalization" of stress resistance of organisms and their ability to maintain a constant phenotype. With these processes, genetic variations of genes of organisms of the same species that can change the phenotype remain hidden. If stress is strong enough to overcome this resistance, the path of development may change due to the expression of a particular gene from the existing hidden variations. This variant of the gene can be preserved during the selection and become inherited through the process of "assimilation" (Waddington, 1959).

The literature provides enough data to suggest that transposons (TE, transposable elements) can serve as hidden genetic structures capable of changing the expression of specific genes in response to certain ES (which overcome resistance to the constancy of the phenotype). This is due to the fact that TEs play an important role in the regulation of gene expression in ontogenesis (de Souza et al., 2013; Pavlicev et al., 2015; Wang et al., 2016; Ito et al., 2017; Ramsay et al., 2017; Saze, 2018) and serve as sources of changes in epigenetic (EG) factors (Li et al., 2011; Ito, 2012; Kapusta et al., 2013; Gim et al., 2014; Cho, 2018).

TEs are genetic elements that transpose from one locus in the genome to another. They can be simple (for example, insertion elements, IS) or complex (for example, TE, which contain genes involved in conjugation and drug resistance in prokaryotes). TE sizes range from 0.7 to 500 thousand base pairs (Zhang, Saier, 2012). The nomenclature system of IS-elements of prokaryotes is presented in the database (http://www-IS.biotoul.fr). The system proposed in 2008 is still used to classify transposons of bacteria and archaea (Roberts et al., 2008). TE classified into two classes. Class I includes retroelements (RE), which are moved by the "copy-and-paste" mechanism. They are divided into LTR-RE, which contain long terminal repeats (LTR, long terminal repeat) and non-LTR-RE (not containing LTR). Different members of RE contain transcriptase (RT, reverse transcriptase) for replication, but they differ in the composition of the catalytic components responsible for integration into the host genome. Class II includes DNA-TE, which are moved by "cut-and-paste" or "rolling circle". Their transposition is possible due to 3 different mechanisms: DDD/E transposase, tyrosine recombinase and endonuclease HUH in combination with helicase (Kojima, 2018). The most common TE classification is presented in Repbase (http://www.girinst.org/repbase/). TE occupy a significant part of the genomes of plants (more than 80 %), fungi (3–20 %) and animals (3–45 %) (Wicker et al., 2007).

A significant contribution to the study of TE, their systematization and the definition of a regulatory role was made by Russian researchers. R.B. Khesin in 1984 in his monograph "Genome inconsistency" described and systematized prokaryotic and eukaryotic TE in detail. The author noted the role of TE in genetic recombination, leading to an increase in the number of genes. Parallel variability Khesin explained the influence of TE with similar site-specificity of integration (Khesin, 1984). In further experimental works on *Drosophila*, the role of site-specific integration of TE as the adapting ability of genomes was confirmed (Kaidanov et al., 1996). If TEs are located near specific genes, which is associated with their peculiarities of germinative insertions, a specific reaction to ES is possible, which explains the concepts of "canalization and assimilation" at the genetic level. This is due to the activation of the TE in response to stress (Todeschini et al., 2005; Markel, 2008; Ito et al., 2016) and the effect of the TE on stress-sensitive genes due to the content of regulatory nucleotide sequences in transposons (de Souza et al., 2013; Feng et al., 2013). For example, the human genome contains 794 972 binding sites with transcription factors (TF) derived from LTR-RE, which are characterized by specific activation at different stages of development and depending on the tissue (Ito et al., 2017). Moreover, during evolution, the ability of TE to site-specific transposition was formed (Markel, 2008; Feng et al., 2013), thanks to which TE can dynamically regulate the expression of protein-coding genes (BCG) *in cis* and *in trans*, as well as stably alter gene regulation due to structural rearrangements (de Souza et al., 2013; Sahebi et al., 2018). The basis of the evolutionary processes is the variability, which is

induced by the ES (Markel, 2008), which caused the speciation of eukaryotes due to "outbreaks of transpositions" during interspecies hybridization, acting as a "genomic shock" (Cheresiz et al., 2008).

The activation of TE by stress is first described by Barbara McClintock. In her early observations, McClintock showed that TE transpositions are associated with heterochromatinization phenomena, and mobilization of heterochromatic domains can alter gene expression. McClintock suggested that the TE, which she called controlling elements, allows the genome to react more flexibly to stress (McClintock, 1984). Indeed, TEs play an important role in adaptation. TE insertions can affect host gene expression patterns and provide organisms with mechanisms for rapid genetic variation through the TE response to stress. For example, in *Arabidopsis thaliana*, the transposition of the heat-activated TE ONSEN causes a mutation of a gene that is sensitive to abscisic acid (ABA). As a result, plant insensitivity to ABA and tolerance to the stressor arise. That is, activation of TE under the action of ES can cause modulation of the host's reaction to environmental influences. Epigenetic mechanisms usually cause a silencing effect of a new insertion (for example, for ABA using IBM2), but they can selectively activate TE under stress (Ito et al., 2016). Induction of integration events under the influence of ES was detected in fungi. For example, in *Saccharomyces cerevisiae*, Re *Ty1* is activated under the influence of various ES, including the lack of adenine (Todeschini et al., 2005).

The role of TE in evolutionary variability is also associated with their ability to induce chromosomal rearrangements, including deletions, inversions, and replicon fusion – the central events of many evolutionary processes. Moreover, TEs contribute to a significant reorganization of genes from the initial random order under the pressure of selection. TE contributed to the emergence of adaptive mutations that occur randomly, but with increased speed under the influence of stress in eukaryotes in the course of evolution (Zhang, Saier, 2012). Large-scale changes in DNA methylation in response to stress (Dowen et al., 2012) may reflect the dynamics of expression of TE, sensitive to ES and to the stress response of the organism (Todeschini et al., 2005; Markel, 2008; Ito et al., 2016). This is due to the important role of TE as sources of non-coding RNA (ncRNA) (Li et al., 2011; Gim et al., 2014; Cho, 2018), which have a regulatory effect on chromatin structure and genome methylation (Ito, 2012; Zhang et al., 2015).

In addition, the ability of the animated TE to enhance the transcription of the stress response genes, as well as exert a regulatory effect *in cis* and *in trans* on various BCGs, makes the TE universal mediators of the interaction of the genome with ES (de Souza et al., 2013; Feng et al., 2013; Ito et al., 2016). For example, in the study of DNA demethylases in plants, it was revealed that their primary function is to regulate the expression of stress response genes due to the effect of TE on their promoters (Le et al., 2014). In addition, the key influence of the TE on the regulation of the genome is due to the origin of the transcription factors from the TE (Lin et al., 2007; Feschotte, 2008) and TF binding sites (TFBS) from the TE in the evolution. Specific activation of these TFBSs was detected by switching gene expression during cell differentiation during ontogenesis (de Souza et al., 2013; Ito et al., 2017).

TEs possess the sensitivity of their expression both to ES and to the stress reactions of the organism itself. At the same time, TEs can cause local regulation of certain genes, acting as enhancers (Makarevitch et al., 2015). In addition, TEs can alter the expression of specific genes using ncRNA (Cho, 2018). TEs can act as stress-sensitive regulators, affecting *in cis* and *in trans* the functioning of BCG (de Souza et al., 2013; Sahebi et al., 2018). In the course of evolution, host genomes have developed the ability to regulate the activity and specificity of integration for TE. This mitigates the instability of the genome caused by uncontrolled transpositions. Recent studies have shown the key role of small ncRNAs in the regulation of transpositions in fungi, plants and animals through a conservative mechanism. Signals about the presence of transposon RNA or about the integration of TE into specific loci of the genome are transmitted to small ncRNAs that send epigenetic modifications and gene silencing back to TE (Wheeler, 2013). The regulation of the host genome by means of TE is global with the involvement of all ncRNA species (Mustafin, Khusnutdinova, 2017). The origin of ncRNA from TE suggests that during the course of evolution, TE developed the ability to self-regulate with the help of processed products of their own transcription, which ensure their optimal interaction with the host genome. TEs can induce mutations under the control of host factors in a process that phenotypically and mechanistically corresponds to the definition of "directional mutations" that occur with high frequency when exposed to stress if they are beneficial to the host. Prototypes of "directed mutations" are found in bacteria, for example, *E. coli* strains with a deletion of the *crp* gene cannot grow on glycerol (Glp(–)). However, insertions of the IS5 into a specific region immediately prior to the glpFK promoter lead to the efficient use of glycerol (Glp(+)) at rates that are significantly increased by the presence of glycerin or loss of the glycerin repressor (GlpR) (Zhang, Saier, 2012). The non-random TE integration was found in the *Schizosaccharomyces pombe* genome, in which the *Tf1* LTR-PE is inserted into promoters with a preference for stress response genes (Feng et al., 2013). In addition, sites of preferred stress-induced TE integration in *D. melanogaster* were identified (Vasileva et al., 1997). In *D. melanogaster*, 94 % of all insertions of the *P*-element and all insertions of other elements (*jockey*, *gipsy*) in the field of gene promoters were in the heat shock gene *hsp70* (Walser et al., 2006). That is, transposon-mediated changes are "directed mutations" (Cheresiz et al., 2008).

The effect of stress on the activation of transposable elements in plants

Plants are characterized by the peculiarities of the composition of TE in their genomes, the distribution of TE, as well as the characteristics of microRNA, which is reflected in their morphogenesis under the influence of stress (Mustafin, 2018). The effect of TE on the dynamics of gene expression during tissue formation is mediated by interaction with epigenetic factors (ncRNA, DNA methylation and histone modification). In plants, siRNAs are responsible for RNA-dependent DNA methylation (RdDM), which suppresses TE when they are activated by stress and hybridization events (Ito, 2012). The ability to regulate gene activity by exposure to target methylation and histone modifications was also detected in

miRNAs and long ncRNAs (Zhang et al., 2015). Important sources of these non-coding RNAs are TE (Li et al., 2011; Kapusta et al., 2013; Lorenzetti et al., 2016; Cho, 2018). That is, in the course of evolution, TE acquired the ability of self-regulation by the products of processing their own transcripts, which is important in controlling the functioning of genes *in cis* and *in trans* (de Souza et al., 2013) and through site-specific transpositions (Markel, 2008; Feng et al., 2013; Sahebi et al., 2018).

NcRNAs, which are derived from TE, are able to exert a regulatory effect directly on the expression of BCG, which contain sequences identical to TE (Fig. 1). This is due to the important role of the TE in the emergence of BCG in the course of evolution due to their domestication (Zdobnov et al., 2005; Lin et al., 2007; Feschotte, 2008; Dupressoir et al., 2012), retrogenes generation (Kubiak, Makalowska, 2017) and exonization (Lin et al., 2008; Sela et al., 2010; Schmitz, Brosius, 2011; Tajnik et al., 2015). The correlation between stress induction of TE and activation of cell defense mechanisms against stress is associated with the presence of TF binding sites in TE, which induce the genes of cell defense systems and are often co-opted by host genomes for their own needs due to their adaptive role (Cheresiz et al., 2008). The conservation of TEs in the host genomes is due to their participation in the activation of responses to the stress response for survival (Feschotte, 2008; de Souza et al., 2013; Ito et al., 2017).

In plants, the major part of the genomes is occupied by LTR-RE, which are regulated by various ESs, displaying finely tuned responses to these stimuli, mainly in the form of activation. LTR regions contain regulatory motifs similar to cellular genes and are involved in the functional plasticity of host genomes, acting as dispersed regulatory modules that can reorient stress stimuli for neighboring genes (Grandbastien, 2015). In response to stress, both TE and genes located near insertions can be transcriptionally activated. TEs are important sources of epigenetic factors (Mustafin, Khusnutdinova, 2017), and most plant miRNAs are identical or homologous to TE (Lorenzetti et al., 2016; Cho, 2018). Therefore, to determine the response of plants to stress, miRNAs are investigated, which play an important role in controlling the activity of BCG and TE. For example, in rice microRNAs were identified that are subject to regulatory effects by stressors, such as drought, cold and insolation. Of these newly identified 227 miRNAs, 80 were derived from TE (Barrera-Figueroa et al., 2012). The activation of LTR-RE in rice was revealed under the influence of an excess of iron in the soil. It was shown that LTR-RE can enhance the expression of genes involved in iron homeostasis (Finatto et al., 2015).

The ideal system for studying the effect of TE on gene regulation is the maize genome, since it contains many different TEs alternating with BCG. An analysis of the location of genes near TE and stress-induced transcripts has shown that these TEs can perform enhancer activity that stimulates the expression of stress-sensitive genes. It was reported that TE are important sources of regulation of alleles of genes sensitive to abiotic ES (Makarevitch et al., 2015). Therefore, TFBS, which are derived from the TE, can rewrite existing transcriptional networks. This leads to the emergence of new adaptive traits, which is important for the formation of new species. For example, during speciation of cabbage, TE amplified binding sites with the transcription factor E2F, therefore

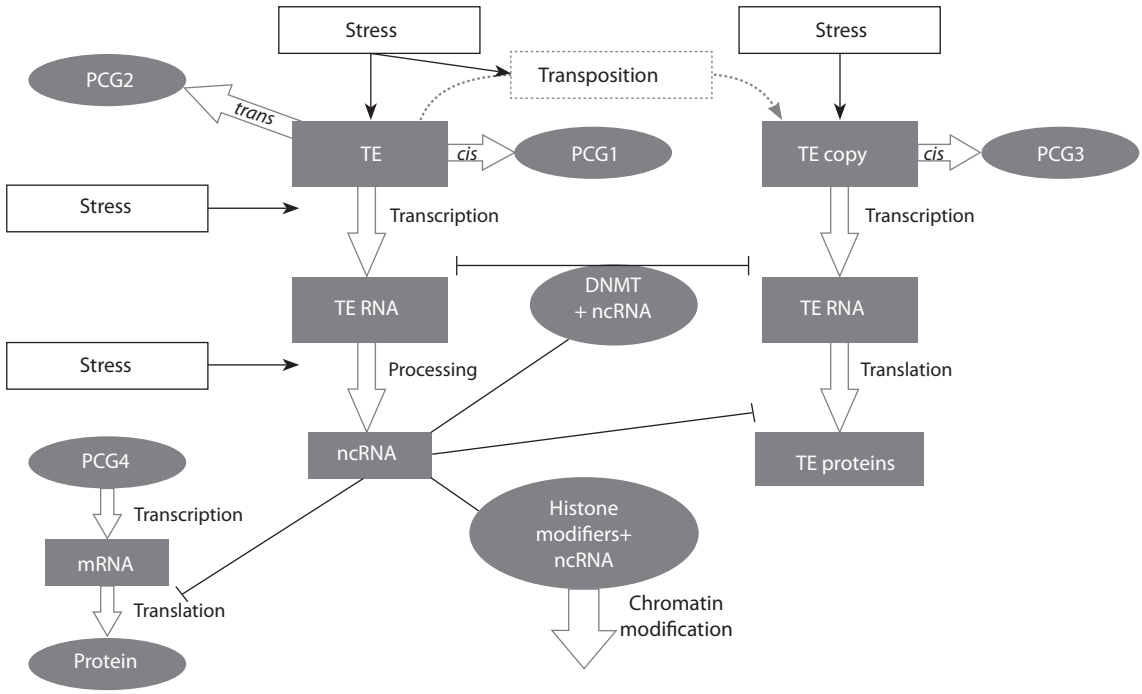

Fig. 1. Role of transposable elements in the regulation of genomic functioning under the action of stress.
TE, transposable elements; PCG, protein-coding gene; ncRNA, non-coding RNA; mRNA , messenger RNA; DNMT, DNA methyltransferase.

in some species of cabbage more than 85 % of all E2F are inside the TE (Henaff et al., 2014).

The effect of stress on the activation of transposable elements in animals

In 1985, it was shown that heat shock in *D. melanogaster* enhances the expression of RE *copia* and the *hsp-10* gene (Strand, McDonald, 1985). Further studies revealed that the features of *copia* activation are specific for different lines of *D. melanogaster* (Cheresiz et al., 2008). These results indicate the variation in TE sensitivity to stress, even in individuals within the same species. The probable causes of such variability may be a violation of the relationship of TE with various TFs. It can be suggested that the sensitivity of TE to environmental stressors and stressful changes in the organism may vary in different cell lines of one organism under the influence of varying gene expression during differentiation. In this regard, cells of different organs and tissues may exhibit the specific susceptibility of TE in the composition of their genomes to stress. The most highly stress sensitive cells are brain neurons (Hunter, McEwen, 2013). One of the ways that the ES affects the brain is the change in epigenetic marks at different stages of development in ontogenesis, thereby regulating the expression of specific TE depending on their location (Hunter, McEwen, 2013). The possible reason for the inheritance of stress resistance can be the peculiarities of the composition, distribution, and peculiarities of TE activation in individual development, which are important factors of EG regulation of morphogenesis (Mustafin, Khusnutdinova, 2018b). That is, individuals survive in natural selection, whose location and composition of TEs in the genome of which contribute to the effect of ES contributing to the adaptation and survival.

Epigenetic factors may explain the mechanism by which ES causes long-term changes in physiology and behavior. Stress, as well as steroid hormones, due to changes in epigenetic markers (DNA methylation and histone modifications) affect plasticity in a number of brain regions (Hunter et al., 2015). These areas include the hippocampus, which contains neuronal stem cells and is characterized by TE activity necessary for their differentiation into specific neurons for different areas of the brain (Mustafin, Khusnutdinova, 2018a). It has been shown, for example, that acute stress in rats causes significant and hippocampal-enhanced enhancement of H3K9me3 levels that target LTR-RE in order to contain potential genomic instability (Hunter et al., 2012). In addition, some TEs are activated under the influence of stress caused by viral infections of host cells. For example, infection of cell cultures with the herpes virus, human immunodeficiency and adenovirus induces the transcription of SINE elements (Cheresiz et al., 2008).

The relationship of hormones with stress-induced transposon activity

The expression pattern of TE is influenced by the type of cells and tissues (with particular importance in the placenta and germ cells), aging and differentiation factors, cytokines, agents and steroids disrupting the function of cells (Taruscio, Mantovani, 2004). TEs are highly sensitive to hormones, the level of which, in turn, reflects the body's response to stress. Based on this, it can be suggested that the management of ontogenesis with the participation of hormones was formed under the influence of environmental changes during the adaptation of organisms using TE, participating in the regulation of gene expression in various cells depending on the tissue and developmental stage. This assumption is based on data on the key role of epigenetic factors in the regulation of TE activity. At the same time, TE themselves are sources of ncRNAs (Lorenzetti et al., 2016; Cho, 2018), which are capable of being translated into functional peptides (miPEP) (Couzigou et al., 2016), that regulate gene expression. It is assumed that peptide hormones can affect the tissue- and stage-specific TE activation, which is used to control gene expression during the growth and development of the organism (Mustafin, Khusnutdinova, 2018a). This is due to the ability of miPEP to enhance the expression of its own miRNAs (Couzigou et al., 2016), which are involved in the epigenetic regulation of TE (Mustafin, Khusnutdinova, 2017) (Fig. 2).

Stress and steroid hormones affect brain structures, where a high TE activity has been identified (Hunter et al., 2015), presumably related to the regulatory effect of transposons on the differentiation of neuronal stem cells (Mustafin, Khusnutdinova, 2018a). The relationship of TE with hormones is determined both in their sensitivity to steroids and peptides, and in the regulatory effect of TE insertions on genes that affect hormone production. For example, the insertion of TE *Taguchi* occurs to the *cis*-regulatory region of the ecdysonoxidase gene, which encodes a key enzyme to reduce the level of the molt hormone 20-hydroxyecdysone. Phylogenetic analysis showed that the TE insertion occurred during the domestication of the silkworm and was adaptive for *Bombyx mori*, as it is used as an enhancer that induces the expression of 20-hydroxyecdysone (Sun et al., 2014). For the final stages of cortisol and aldosterone synthesis in humans, the *CYP11B1* and *CYP11B2* genes, respectively, are responsible. These two genes are homologous in the proximal regions, however, the insertions of *Alu* and L1 caused the divergence of promoters (Cheng et al., 2012). In the human genome, a micro-transcriptional mechanism regulating the expression of the *Tspo* gene was detected. In the intron of the *Tspo* gene, SINE B2 is located, which regulates steroidogenesis specific for the *Tspo* gene. High levels of TSPO are expressed in Leydig cells in the testes, and their expression levels dictate the ability of cells to form androgens (Fan, Papadopulos, 2012).

An *in vitro* transfection study with human *DMBT1* (deleted in malignant brain tumors 1) promoter constructs showed that an *Alu* site approximately 3000 nucleotides upstream of the gene mediates estrogenic regulation. Estrogen antagonists tamoxifen, raloxifene and ICI 182,780 also induce the expression of *DMBT1* gene through this *Alu* site (Tynan et al., 2005). It was found that the ORF-1p L1 element enhances the transcriptional activity of androgen receptors (AR) and the expression of the prostate-specific antigen (PSA) (Lu et al., 2013). Plants produce a large amount of hormonal substances – phytosterols, which have a diverse effect on their development. Currently, mutants of *Arabidopsis* for transposons are widely available for studying phytosterols (Suzuki, Muranaka, 2007). Thus, various independent studies have shown that hormones can affect TE expression, while transposons affect hormone production by insertions into specific loci and effects on the epigenetic regulation of morphogenesis.

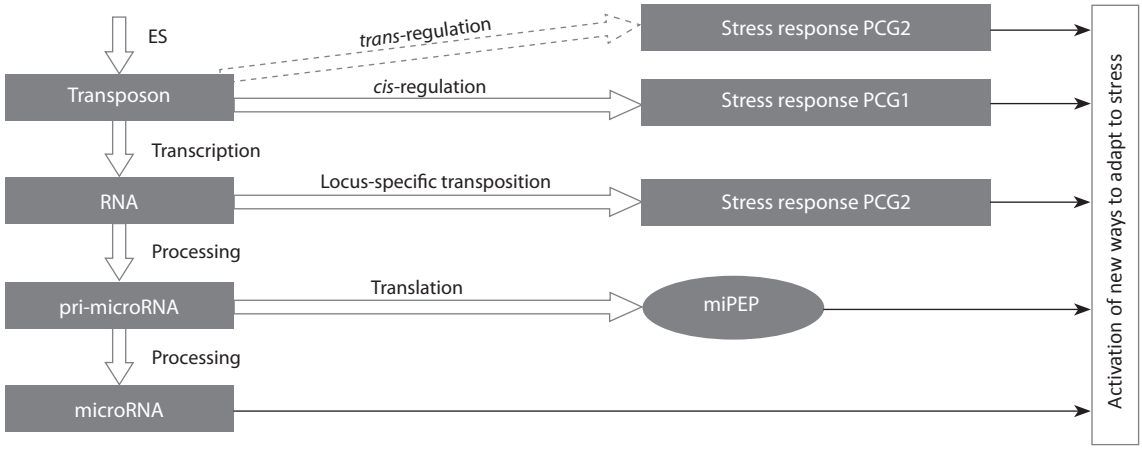

Fig. 2. Scheme of the role of transposon self-regulation in the regulation of stress-induced adaptive variability of genomes.
ES, environmental stressors; miPEP, product of microRNA translation; PCG, protein-coding gene.

Tissue-specific and stage-specific activation of transposons

TEs are successfully preserved in evolution, they respond to certain ES and stress changes in the organism, and also have a global regulatory effect on the genome. This indicates the universality of stress-induced TE activity in evolution and ontogenesis. As stress signals affecting the activity of certain TE, there may be changes in the intracellular environment during successive cell divisions, starting with the first division of the zygote. The specific composition and distribution of TE for each species suggests that activation of certain TE may cause activation of some BCG by *cis* and *trans* regulation (de Souza et al., 2013; Sahebi et al., 2018) and silencing of other BCG via ncRNA formed by processing TE transcripts (Gim et al., 2014; Cho, 2018). As a result, a cascade of sequential changes in BCG expression necessary for cell differentiation may form. The existence of such evolutionary-established mechanisms of regulation of ontogenesis with the help of TE can be confirmed if the suppression of the activity of specific TE required for further regulation of cell differentiation will stop the further development of the organism. Indeed, in experiments on mouse embryos, it was shown that depletion of the long *LincGET* ncRNA associated with LTR-RE leads to a complete arrest of further development at the two-cell stage (Wang et al., 2016). In this stage, many transcripts are initiated from the LTR-RE (Macfarlan et al., 2012) and are associated with enhancers that support the polypotency (Fort et al., 2014).

In embryogenesis, TEs can redistribute the regulation of BCG expression depending on the developmental stage and the type of tissue (Pavlicev et al., 2015). In mouse and human genomes, TE are sources of at least 30 % of transcription start sites with tissue-specific activation features (Gerdes et al., 2016). In mice, in early embryogenesis, up to 20 % of the transcriptome is initiated from RE. In evolution, specific transpositions of retroelements became the basis for the distribution of tissue-specific binding sites for TF (Mak et al., 2014). On the basis of experimental data on the accumulation of LINE-1 insertions in human embryonic stem cells, accompanied by the suppression of the activity of certain genes, the role of the TE in regulating the work of the genome in cell differentiation was confirmed (Garcia-Perez et al., 2007). Profiles of specific TEs may indicate cellular identity. This suggests the importance of TE in controlling the work of the genome in cell differentiation. The active transcription of TE, which contributed to the maintenance of pluripotency, as well as the reactivation of certain REs in the early embryonic development of animals along stem cell lines was proven (Gerdes et al., 2016).

TEs are important sources of TFBS, which are characterized by their ability to be activated depending on the tissue and developmental stage (Lowe, Haussler, 2012; de Souza et al., 2013). TFBS, which originated from LTR-RE, differ in tissue- and stage-specific activation features (Ito et al., 2017). In addition, TEs can control tissue-specific features of gene expression by providing alternative splicing sites and UTR regions, since the alternative variants of the translated proteins have different properties (Lin et al., 2008; Tajnik et al., 2015). It was shown that epigenetic regulation of the function of transposons located in the introns of genes contributes to a change in their regulation in ontogenesis and in the peculiarities of expression (Saze, 2018). In human tissue cultures, a difference in tissue-specific gene expression was detected for 62 different LTR classes in 18 types of tissue. These results allowed to propose the role of TE as a tissue-specific regulator of gene expression in ontogenesis (Pavlicev et al., 2015). Non-LTR retroelements have functions similar to long ncRNAs, regulating BCG expression in stem cell differentiation during embryogenesis (Honson, Macfarlan, 2018). In addition, in humans, various long ncRNAs derived from the retrovirus HERVH are expressed and necessary for stem cell pluripotency (Ramsay et al., 2017).

Horizontal transfer of transposable elements in evolution

In addition to the distribution of transposons in the genomes of eukaryotes, TE composition plays an important role in speciation, which is modified by horizontal transfer. Horizontal transfer plays a major role in the evolution of prokaryotes, in which more than 81 % of their genes are involved in this process (Palazzo et al., 2017). In the evolution of animals, the

character of TE spreading by means of horizontal transfer is noted, like interspecies viral pandemics. For example, about 46 million years ago there was a major surge in the activity of DNA-TE of the *SPIN* family, making the sequences of these TE identical by 96 % in a wide variety of animals (african clawed frog, lizard anole, senegal galago, rat, mouse, brown bat) (Pace et al., 2008). In animals, for the first time, horizontal transposon transfer was detected in 1990 in *D. melanogaster* (Daniels et al., 1990). Further studies have revealed the ability of horizontal transfer of most TEs in insects, reptiles, jaws, lamprey and mammals (Zhang et al., 2014).

It is assumed that the genes of RAG, forming the complex necessary for recombination of the V(D)J, in the jawed vertebrates have arisen due to the horizontal transfer of TE (Sniezewski et al., 2018). In the insect *Helicoverpa zea*, Transib transposase was found, which has properties similar to RAG. This suggests the possibilities of horizontal transfer in the evolution and phylogenetic relationship of the TE genes domesticated by the genomes of various types of eukaryotes (Hencken et al., 2012). Phylogenetic studies have revealed a horizontal transfer of retroelements of the *An-RTE* family in angiosperms from ancestors of arthropods. In 42 animal species, retroelements were significantly identical with *An-RTE* flowering plants (Gao et al., 2018). In the Horizontal Transposon Transfer DataBase (http://lpa.saogabriel.unipampa.edu.br:8080/httdatabase/), great attention is paid to horizontal transfer in eukaryotes (Dotto et al., 2018).

Recently, mechanisms of horizontal transfer between different taxa are being actively studied. One of the ways of spreading TE by horizontal transfer was demonstrated by the example of TE *Bari* belonging to the *Tc1-mariner* superfamily, which in evolution acquired the property of "diffuse promoters" and can move between different genomes (Palazzo et al., 2017). Viruses can act as vectors for horizontal transposon transfer. For example, for the *Chapaev* transposon, the *Bracovirus* virus can act as a vector. Due to the horizontal transfer, *Chapaev* elements are widely distributed in the genomes of many animal species (Zhang et al., 2014).

TEs are found in the genomes of giant viruses, where they participate in the functioning and evolutionary transformations, and their transfer to the host genomes is not excluded (Filee, 2018). For example, the transposon *Submariner* was found in the genome of *Pandoravirus salinus*. For this pandoravirus, the host is the ameba *Acanthamoeba castellanii*, in the genome of which *Submariner* and its associated DNA-TE are found, which indicates the presence of a horizontal transfer between the virus and the host (Sun et al., 2015). In addition, viruses can activate TE, for example, a cytomegalovirus infection can cause the expression of HERV (Assinger et al., 2013). Moreover, TEs can participate in the integration of viral genomes into host DNA, as well as in protection against viral infections (Speiseder et al., 2014; Tarocchi et al., 2014). For example, in animals, the ability of ERV to cause restriction of related exogenous retroviruses was identified (Malfavon-Borja, Feschotte, 2015). At the same time, the ability to transform ERVs into exogenous retroviruses, and exogenous viruses to ERV in the genomes of various hosts has been proven (Zhuo, Feschotte, 2015). *Caulimoviridae* viruses in plants have evolved from LTR retroelements (Llorens et al., 2009). Analysis of the results of experimental work by various authors has shown that the relationship between TE and viruses in the evolution of eukaryotes is global and plays a crucial role in the variability of the host genomes. A number of studies have shown the interconversions of viruses and TE (Zhuo, Feschotte, 2015), as well as the existence of elements that combine the properties of viruses and transposons. Intermediate evolutionary link between DNA-TE and viruses are virophages (Fisher, Suttle, 2011). *RVP* virophages are known, which are a hybrid of polytone virophages that can cause viral infections (Yutin et al., 2015). Polintons are also known which possess the properties of TE. Polintons exist as both autonomous and non-autonomous elements. They are common among various species of animals, mushrooms and protists. It is assumed that many eukaryotic viruses, including megaviruses and adenoviruses, originated from polintons (Krupovic, Koonin, 2016).

Conclusion

In the scientific literature accumulated enough experimental data confirming the sensitivity of TE to stress in all living organisms. The activation of TE under the action of stress leads to a change and the emergence of new regulatory gene networks that contribute to variability. This is an important factor in the natural selection of individuals with a specific composition and location in their genomes TE, whose activation under the influence of stress during ontogenesis facilitates adaptation.

The study of TE activation in different tissues and at different stages of development under the influence of stress may be the key to clarifying the mechanisms of ecological morphogenesis. These studies are promising for diagnostic and therapeutic models in biology, genetics and biochemistry. Determining the role of miRNAs and hormones in changing cell differentiation under the influence of TE is promising for planning targeted therapy of pathologies associated with dysfunction of epigenetic factors, as well as a possible impact on the aging process. The key to modulating the growth and development of organisms can be the study of various TEs capable of site-specific transposition. These studies are promising for the controlled use of stem cells in organ-substituting technologies. Moreover, to study changes in regulatory gene networks in evolution, it is promising to compare the sensitivity to stress of certain TE different taxa of eukaryotes.

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Conflict of interest. The authors declare no conflict of interest.
Received November 21, 2018. Revised December 17, 2018. Accepted January 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):390-397
DOI 10.18699/VJ19.507

The regulatory role of cystatin C in autophagy and neurodegeneration

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Autophagy is a dynamic cellular process involved in the turnover of proteins, protein complexes, and organelles through lysosomal degradation. It is particularly important in neurons, which do not have a proliferative option for cellular repair. Autophagy has been shown to be suppressed in the striatum of a transgenic mouse model of Parkinson's disease. Cystatin C is one of the potent regulators of autophagy. Changes in the expression and secretion of cystatin C in the brain have been shown in amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases, and in some animal models of neurodegeneration, thus proving a protective function of cystatin C. It has been suggested that cystatin C plays the primary role in amyloidogenesis and shows promise as a therapeutic agent for neurodegenerative diseases (Alzheimer's and Parkinson's diseases). Cystatin C colocalizes with the amyloid β-protein in the brain during Alzheimer's disease. Controlled expression of a cystatin C peptide has been proposed as a new approach to therapy for Alzheimer's disease. In Parkinson's disease, serum cystatin C levels can predict disease severity and cognitive dysfunction, although the exact involvement of cystatin C remains unclear. The aim: to study the role of cystatin C in neurodegeneration and evaluate the results in relation to the mechanism of autophagy. In our study on humans, a higher concentration of cystatin C was noted in cerebrospinal fluid than in serum; much lower concentrations were observed in other biological fluids (intraocular fluid, bile, and sweat). In elderly persons (61–80 years old compared to practically healthy people at 40–60 years of age), we revealed increased cystatin C levels both in serum and intraocular fluid. In an experiment on C57Bl/6J mice, cystatin C concentration was significantly higher in brain tissue than in the liver and spleen: an indication of an important function of this cysteine protease inhibitor in the brain. Using a transgenic mouse model of Parkinson's disease (5 months old), we demonstrated a significant increase in osmotic susceptibility of brain lysosomes, depending on autophagy, while in a murine model of Alzheimer's disease, this parameter did not differ from that in the appropriate control.
Key words: cystatin C; autophagy; neurodegeneration.

For citation: Korolenko T.A., Shintyapina A.B., Pupyshev A.B., Akopyan A.A., Russkikh G.S., Dikovskaya M.A., Vavilin V.A., Zavjalov E.L., Tikhonova M.A., Amstislavskaya T.G. The regulatory role of cystatin C in autophagy and neurodegeneration. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4): 390-397. DOI 10.18699/VJ19.507

Регуляторная роль цистатина С в аутофагии и нейродегенерации

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Аутофагия – динамичный клеточный процесс, связанный с оборотом белков, белковых комплексов и органелл посредством лизосомной деградации. Аутофагия особенно важна в нейронах, которые не имеют пролиферативного ресурса для клеточного восстановления. Одним из мощных регуляторов аутофагии является цистатин С. Изменения экспрессии и секреции цистатина С в головном мозге показаны при боковом амиотрофическом склерозе, болезни Альцгеймера и Паркинсона, а также на некоторых моделях нейроде-

генерации у животных, что подтверждает защитную функцию цистатина С. Высказано предположение, что цистатин С играет важную роль в амилоидогенезе и может рассматриваться как возможное терапевтическое средство для предупреждения и лечения ряда нейродегенеративных заболеваний (болезни Альцгеймера и Паркинсона). Цистатин С колокализуется с амилоидом β в головном мозге при болезни Альцгеймера. Контролируемая экспрессия пептида цистатина С предложена в качестве нового подхода к терапии болезни Альцгеймера. При болезни Паркинсона уровни цистатина С в сыворотке крови могут прогнозировать тяжесть заболевания и когнитивную дисфункцию, хотя конкретное участие цистатина С остается неясным. Рассмотрена роль цистатина С в нейродегенерации и проведена оценка результатов в связи с активностью аутофагии. У здоровых людей обнаружена высокая концентрация цистатина С в спинномозговой жидкости по сравнению с сывороткой крови; значительно более низкие концентрации наблюдали в других биологических жидкостях (внутриглазная жидкость, желчь, пот). При оценке влияния возраста обнаружено повышение концентрации цистатина С как в сыворотке, так и во внутриглазной жидкости у пожилых людей (61–80 лет) по сравнению с практически здоровыми людьми в возрасте 40–60 лет. В эксперименте на мышах C57Bl/6J концентрация цистатина С была значительно выше в мозговой ткани, чем в печени и селезенке, что указывает на важную функцию этого ингибитора цистеиновых протеаз в головном мозге. На трансгенной мышиной модели болезни Паркинсона (5 месяцев) найдено значительное увеличение осмотической чувствительности лизосом мозга, соответствующее усилению аутофагии, тогда как на мышиной модели болезни Альцгеймера этот показатель не обнаружил изменения аутофагии.
Ключевые слова: цистатин С; аутофагия; нейродегенерация.

Introduction

The search for new markers of aging, neurodegenerative diseases, and atherosclerosis is important for modern medicine (Ciechanover, Kwon, 2015). Cystatins are known to be potent endogenous inhibitors of cysteine proteases of the papain superfamily and form equimolar, tight, and reversible complexes with human cysteine proteases (cathepsins B, H, K, L, and S) (Stoka et al., 2005; Korolenko et al., 2011, 2015).

Cystatin C is a known extracellular endogenous cysteine proteinase inhibitor, which has been studied more thoroughly than other cystatins (cystatin SN et al.) and has been evaluated as a possible marker of several pathological processes (tumor growth and metastasis) (Poteryaeva et al., 2000; Maxfield, 2014). Increased serum cystatin C concentration is well known as an alternative measure of renal function (Keppler, 2006). According to the recent findings cystatin C is regulated at both transcriptional and post-translational levels (Xu et al., 2015), moreover, cystatin C production by haematopoietic cells is significant in the systematic pools of this inhibitor.

Neurodegenerative diseases combine disturbances in certain structures of brain cells (especially in endosomes and lysosomes), which, in the presence of the signs of such a disease, include some common molecular and cellular mechanisms (Harris, Rubinsztein, 2011). Protective mechanisms of action of cystatin C in several neurodegenerative diseases (Alzheimer's disease, Parkinson's disease) were suggested (Gauthier et al., 2011). Cystatin C was shown previously implicated in the process of neurodegeneration (Kaur, Levy, 2012). An *in vivo* study on dopaminergic-neuron survival revealed that administration of cystatin C to rats with a neurotoxin 6-hydroxydopamine-induced lesion partially rescues substantia nigra dopaminergic neurons. 6-Hydroxydopamine-mediated lesioning induces relatively slow but sustained upregulation of cystatin C and suggests that this inhibitor may exert a neuroprotective action on dopaminergic neurons (Xu et al., 2005). Those authors show that cysteine proteinase inhibitors may be new candidates for neuroprotective treatment of Parkinson's disease (Cuervo, Wong, 2014).

We tried to evaluate the biological role of cystatin C in neurodegeneration, especially as autophagy inducer, which remains unclear and controversial until now. Destruction of the nigrostriatal dopaminergic pathway can trigger neuroinflammation and increase the synthesis of neural growth factors, both in the striatum and in the substantia nigra. In general, the pathological processes involved in such responses are poorly characterized and may contribute to secondary damage and/or regeneration in the central nervous system (Ling, Salvaterra, 2009; Harris, Rubinsztein, 2011). Increased levels of p62 (also called as sequestosome 1 (SQSTM1) and higher sensitivity to 7-oxysterol-mediated lysosomal membrane damage was shown in macrophages isolated from cystatin C knockout ($CysC^{-/-}$) mice (Li et al., 2016).

Cystatin C as a possible diagnostic marker of neurodegenerative diseases

Cystatin C has a broad spectrum of biological roles, including modulation of inflammatory response (Li et al., 2016). Using immunohistochemical methods in Alzheimer's disease Zhong et al. (2013) have revealed that cystatin C co-localizes with amyloid-β in amyloid-laden vascular walls and in the senile plaque cores. These authors suggested that cystatin C revealed protection against neurodegeneration by inhibition of cysteine proteases (cathepsin B), suppression of amyloid-β aggregation and induction of autophagy (Zhong et al., 2013). Low cystatin C level in cerebrospinal fluid of patients with Alzheimer disease and dementia confirmed this hypothesis. In elderly men (free of dementia at the baseline) low levels of serum cystatin C can precede clinical manifestation of Alzheimer's disease and may be an early marker of future risk of Alzheimer's disease development (Sundelöf et al., 2008). Plasma Cys C levels were significantly correlated with dementia development in Alzheimer's disease and combination (ratio) of elevated cystatin C and decreased HDL concentrations were suggested recently as potential diagnostic value test in differential diagnosis of Alzheimer's disease and vascular dementia (Wang et al., 2017).

In the brain, Mathews and Levy (2016) have demonstrated that cystatin C is playing protective roles via several different pathways that depend upon the inhibition of endosomal-lysosomal proteolysis (cysteine proteases inhibition), the induction of cellular autophagy, via the induction of cell proliferation and the inhibition of amyloid-β (Aβ) aggregation. However,

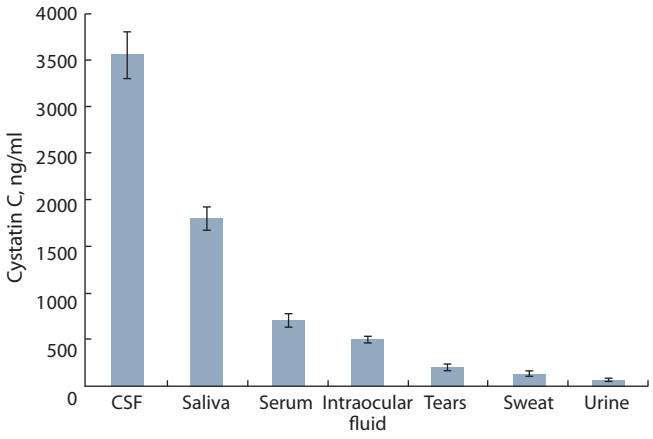

Fig. 1. Distribution of cystatin C in biological fluids of healthy persons, aged 30–50 years old ($M \pm m$), n = 7–10 per group.

Samples of CSF (cerebrospinal fluid) were obtained from the Federal Center for Neurosurgery, Novosibirsk, when puncturing patients for a diagnostic purpose that were considered as practically healthy individuals according to the cellular composition of CSF revealed. Cystatin C concentration was measured by Cystatin C Human ELISA kits, BioVendor (Czechia).

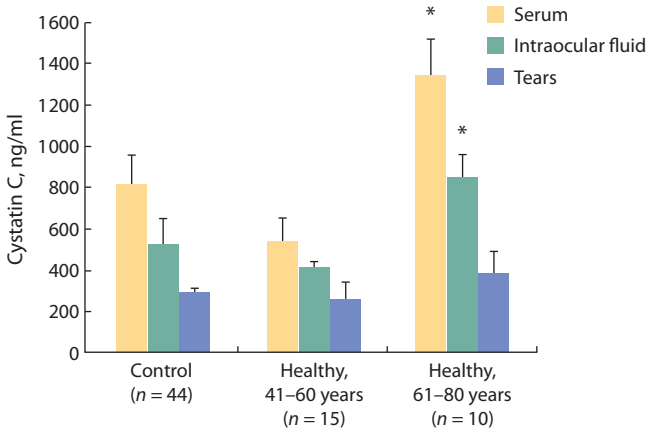

Fig. 2. Concentration of cystatin C (ng/ml) in biological fluids of healthy persons depending from age, n = 7–10 per group.

Data are presented as $M \pm m$. $^*p < 0.05$ vs. control (group of healthy 20–29 years old individuals) or practically healthy persons of 41–60 years old. Cystatin C concentration was measured by Cystatin C Human ELISA kits, BioVendor (Czechia).

there are opposite points of view of researchers suggesting that brain amyloid-β level depends mainly on a balance between its formation from the amyloid precursor protein (APP) and its removal by proteolysis, with a significant role of several zinc-proteases including neprilysin, the endothelin converting enzymes (ECE-1 and -2), and the insulin-degrading enzyme (Nalivaeva, Turner, 2017). Moreover, cathepsin B, as the most important cysteine protease, might be also anti-amyloidogenic, helping in amyloid-β clearance or, instead, might be involved in amyloid-β production (Zerovnik, 2009).

Parkinson's disease is characterized by a decreased motor activity resulting from the death of dopaminergic neurons in the substantia nigra. α-synuclein, located in presynaptic terminals of neurons, is considered as the main pathogenic protein in Parkinson's disease; another strong pathogenic factor is oxidative stress (Hara et al., 2006; Huang et al., 2015). Dopamine can induce autophagic cell death with upregulation of α-synuclein in human neuroblastoma cells (Gomez-Santos et al., 2003).

An increased serum level of secreted cystatin C was demonstrated during the process of aging (Cuervo, Wong, 2014; Mathews, Levy, 2016) and in various diseases like tumors (Gashenko et al., 2013) or atherosclerosis (Korolenko et al., 2011, 2012, 2015). In biological fluids of healthy persons, the highest concentration of cystatin C in our study was noted in cerebrospinal fluid (CSF), with the following ranking of concentrations: CSF > saliva > serum > intraocular fluid > tear fluid > sweat > urine (Fig. 1).

Results similar to our data concerning cystatin C levels in CSF, saliva, and serum were obtained by other authors (Bjornstad et al., 2015). According to the data that we obtained in practically healthy persons, the concentration of cystatin C is increased in the serum and intraocular fluid of people aged 61–80 years (Fig. 2).

At early stage of Parkinson's disease, increased serum cystatin C was associated with sleep-disordered breathing problems (Xiong et al., 2018). In CSF of patients with amyotrophic lateral sclerosis cystatin C level was significantly decreased, there was a correlation between cystatin C levels and G73A polymorphism in *CST3* gene encoding cystatin C (Yamamoto-Watanabe et al., 2010). In the brain of patients with Alzheimer's disease cystatin C revealed protective role as a strong endogenous inhibitor of cysteine proteases (cathepsin B), inducer of cellular autophagy related to amyloid-beta aggregation (one of the several pathways of protection) (Mathews, Levy, 2016).

Autophagy is a dynamic cellular process involved in the turnover of proteins, protein complexes, and organelles through lysosomal degradation (Chen, Klionsky, 2011). Autophagy is particularly important in neurons, which do not have a proliferative option for cellular repair (Lee, 2009; Son et al., 2012). Positive and some negative effects (like the "Janus-faced" role) of increased autophagy have been uncovered during development of neurodegeneration (Viscomi, D'Amelio, 2012; Wang, Hiesinger, 2012).

The experimental model of neurodegenerative diseases, autophagy, and cystatin C

A common feature of neurodegenerative diseases is the accumulation of proteins prone to aggregation and protein inclusions, which are, on the one hand, the markers of these diseases and, on the other hand, the cellular tools for combating these diseases; one of the important therapeutic targets in this process is autophagy (Ciechanover, Kwon, 2015; Torra et al., 2018). Autophagy is weakened in various ways in these diseases and decreases with ageing. The removal of the accumulating toxic proteins and structures is carried out by the mechanisms of chaperone-mediated autophagy, macroautophagy, and mitophagy during interactions with the ubiquitin–proteasome system.

Changes in autophagy are involved in the development of various age-dependent degenerative disorders such as neurodegeneration (Tizon et al., 2010b), cancer (Gammoh et al., 2016), tissue atrophy, alcohol neurointoxication (Luo,

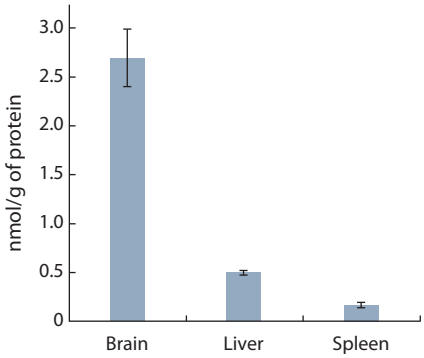

Fig. 3. Cystatin C concentration in different organs in intact C57Bl/6J mice.

Data are presented as $M \pm m$, $n = 7$ per group. Cystatin C assay was performed with ELISA kit for mice (BioVendor, Chechia).

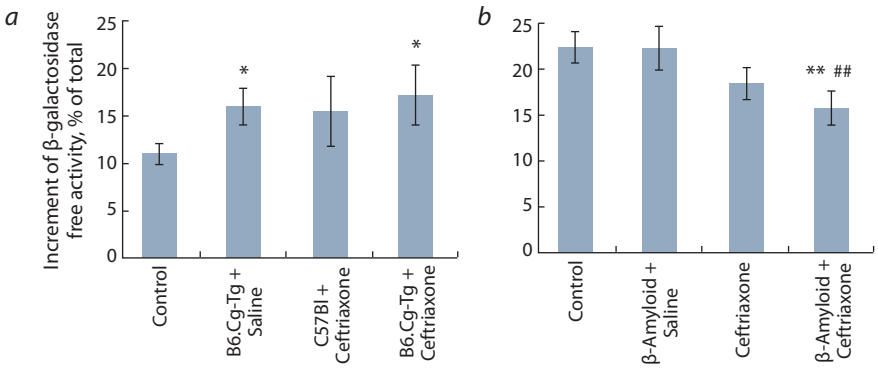

Fig. 4. Autophagy activation in the brain of murine models of Parkinson's (*a*) and Alzheimer's (*b*) diseases.

Data are presented as $M \pm m$, $n = 5–6$ per group. Autophagy activation was measured by permeabilization of lysosomal membranes as an increment of free activity of β-galactosidase (in percentage of the total activity of the enzyme) after treatment of brain homogenate in hypotonic media as described in (Pupyshev et al., 2005). Ceftriaxone (100 mg/kg/day for 5 weeks, intraperitoneally) was applied as a neuroprotective treatment since it demonstrated a marked neuroprotective potential at experimental neurodegeneration in the models of Parkinson's and Alzheimer's disease (Weng et al., 2016; Tikhonova et al., 2017).
a, Transgenic 5-month-old mice with overexpression of α-synuclein of B6.Cg-Tg(Prnp-SNCA*A53T) 23Mkle/J strain (B6.Cg-Tg) were used as a model of Parkinson's disease (Pupyshev et al., 2018). $^{*}p < 0.05$ vs. Control (mice of wild-type genotype); *b*, To induce a pharmacological model of Alzheimer's disease, mice of C57Bl/6J strain were administered with an amyloid beta fragment (Aβ 25–35) (Sigma) bilaterally i.c.v. as described earlier (Park et al., 2011; Choi et al., 2013). $^{**}p < 0.01$ vs. Control (mice with vehicle (sterile water) i.c.v. injections); $^{\#\#}p < 0.01$ vs. β-amyloid + saline group.

2014) and accelerated aging (Coria et al., 1987; Kovács et al., 2017). Decreased concentration of cystatin C in CSF was reported to have diagnostic significance in neurodegenerative diseases, such as amyotrophic lateral sclerosis, which is characterized by progressive motor neuron degeneration (Mathews, Levy, 2016). Low concentration of cystatin C was detected in the serum of patients with Alzheimer's disease, correlating with conversion from mild cognitive disturbances to dementia; in CSF of patients with Alzheimer's disease, the cystatin C level was found to be lower as compared to patients with dementia; therefore, it is possible that changes of cystatin C in CSF may serve as a biomarker of disease (Mathews, Levy, 2016). However, according to some data (Přikrylová Vranová et al., 2010) cystatin C level in CSF of patients with Parkinson's disease was not changed significantly.

Many studies indicate that the weakening of autophagy and/or the incompleteness of protein degradation are important initiators of Parkinson's disease (Wang, Hiesinger, 2012; Maxfield, 2014). *In vitro* studies have revealed that pharmacological induction of autophagy, for instance, by trehalose (Dehay et al., 2010) or by resveratrol (Wu et al., 2013), leads to an improvement in the molecular patterns of Parkinson's disease. In general, it is believed that a decrease in autophagy and in recognition of the segregated material is the key factor of Parkinson's disease, and possibly, activation of autophagy is a good therapeutic strategy against this disease.

Recently new pharmacological modulators of autophagy with a therapeutic potential were introduced (Galluzzi et al., 2017a, b). Among modulators of autophagy in brain cells for prevention of neurodegeneration (Cheung, Ip, 2011), various authors suggested activators of autophagy, like rapamycin (Malagelada et al., 2010), trehalose, valproate (Harris, Rubinsztein, 2011), and cystatin C (Watanabe et al., 2018).

According to our data obtained in an experimental study, the cystatin C level was significantly higher in the brain than in the liver or spleen of C57Bl/6J untreated mice (Fig. 3), indicating the significant role of this inhibitor in brain tissue.

As demonstrated in our previous study on an experimental murine genetic model of Parkinson's disease, autophagy (according to LC3-II expression) was decreased in the striatum and substantia nigra as compared to other regions of the brain, thus reflecting injury of motor neurons (Pupyshev et al., 2018). This model of Parkinson's disease manifested increased osmotic susceptibility of lysosomes in the brain (according to a release of lysosomal enzyme β-galactosidase) (Fig. 4, *a*) in conjunction with secondary changes of their membranes as a result of dysfunction of lysosomes and intralysosomal accumulation of toxic material. In a model of Alzheimer's disease, we did not notice the changes in the permeabilization of lysosomal membranes in brain tissue (Fig. 4, *b*).

Stabilization of lysosomal membrane by a neuroprotector ceftriaxone (see Fig. 4, *b*) can be related to anti-inflammatory effect of this drug. In the pharmacological model of Alzheimer's disease caused by the central administration of amyloid β in mice, amyloid β toxicity produced an almost 3-fold increase in LC3-II expression (as result of activation of autophagy related to neuroinflammatory process in the frontal cortex) (Fig. 5), and ceftriaxone significantly reduced the rate of autophagy, which apparently reflects the anti-inflammatory effect of ceftriaxone on the cerebral cortex and thereby weakening the autophagic response.

Regulation of autophagy is a comlex process, depending on the cell types and diseases, including neurodegeneration (Kiriyama, Nochi, 2015; Zou et al., 2017). Current state of knowledge concerning transcriptional, post-transcriptional, and post-translational regulation of autophagy in yeast and mammals were discussed (Feng et al., 2015; Hwang et al., 2017). Novel pharmacological modulators of autophagy were revealed under recent investigations (Ha, Kim, 2016; Gao et al., 2017; Johnston et al., 2017). Several other approaches have been suggested for restoration of autophagy (Martini-Stoica et al., 2016). One of them is introduction of

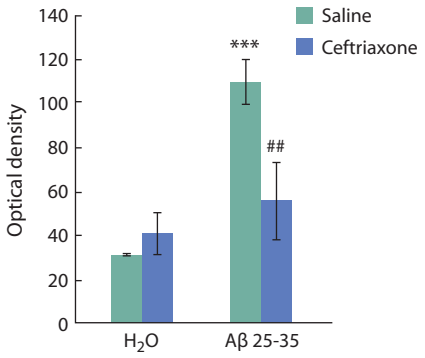

Fig. 5. Effect of ceftriaxone on LC3-II expression in the frontal cortex of C57Bl/6J mice with pharmacological model of Alzheimer's disease.

Data are presented as $M \pm m$, n = 3–5 per group. Autophagy activation was measured using immunohistochemical analysis of LC3-II expression as described in (Pupyshev et al., 2018). $^{***}p < 0.001$ vs. control group (H_2O + saline) of mice treated with vehicle (sterile water, i.c.v.) and intraperitoneal saline injections; $^{\#\#}p < 0.01$ vs. β-amyloid + saline group.

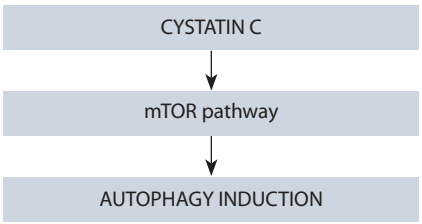

Fig. 6. Possible mechanism of regulation of autophagy in murine primary cortical neurons. The mammalian target of rapamycin (mTOR) regulates cell proliferation, cell motility, cell survival, autophagy (Tizon et al., 2010b; Zou et al., 2017).

AUTEN-99 (autophagy enhancer 99), which activates autophagy in cultured cells and animal models (Kovács et al., 2017). AUTEN-99 possibly can effectively penetrate the blood–brain barrier and somehow protect the progression of neurodegenerative changes in the experiment. Nonetheless, further research is necessary in this direction, especially a study on *in vivo* models of neurodegeneration.

Cystatin C produced by all nucleated cells and has a stable production rate; it is freely filtered by the glomerulus and metabolized after tubular reabsorption (Svechnikova et al., 1998; Bjornstad et al., 2015). Earlier cystatin C knockout mice were found to be fertile and have no gross pathological abnormality or signs of neurodegeneration up to 6 months of age (Huh et al., 1999). The absence of neurodegeneration in that study might be attributed to young age of mice (less than 6 m.o.) and strong compensatory mechanisms. Later cystatin C was shown to inhibit amyloid-beta deposition in Alzheimer's disease mouse models (Mi et al., 2007). Moreover, induction of autophagy by cystatin C may be a mechanism that protects murine primary cortical neurons and neuronal cell lines (Lee et al., 2007; Maetzler et al., 2010; Tizon et al., 2010a). In the brain, multiple *in vitro* and *in vivo* findings have demonstrated cystatin C protective roles via pathways that depend upon the inhibition of endosomal-lysosomal pathway cysteine proteases (cathepsin B), via the induction of cellular autophagy, via the induction of cell proliferation, or via the inhibition of amyloid-β (Aβ) aggregation (Gauthier, Liu, 2016; Mathews, Levy, 2016).

There are some controversial results on the effect of certain drugs on serum cystatin C concentrations in humans for estimation of glomerular filtration rate (GFR) in patients with renal disease. Corticosteroids (prednisolone, repeated administration) can significantly increase serum cystatin C concentration from 1.24 ± 0.40 mg/L at baseline to 1.61 ± 0.80 mg/L at the end of a study period ($p < 0.05$). This finding needs to be considered when interpreting cystatin C levels in patients with heart failure receiving corticosteroid therapy (Zhai et al., 2016).

An additional property of cystatin C (except as a protease inhibitor) that makes this protein clinically relevant is that it can form aggregates by the mechanism known as domain swapping, similar to that involved in the formation of amyloid β plaques in Alzheimer's disease (Xu et al., 2011; Kaminskyy, Zhivotovsky, 2012). Secretion of IL-10 in response to inflammatory stimuli downregulates IRF-8 and consequently cystatin C synthesis *in vivo*. The serum concentration of cystatin C decreases in an IL-10-dependent manner in mice treated with the TLR9 agonist CpG (synthetic analog of bacterial DNA, activator of dendrite cells). Cystatin C synthesis is therefore more tightly regulated than hitherto recognized. The mechanisms underlying this regulation may be targeted to alter cystatin C production, with potential therapeutic benefits (Xu et al., 2005, 2011). Transcription factor – IFN regulatory factor 8 (IRF-8) is critical for cystatin C expression in primary dendritic cells. Only the cells with IRF-8 bound to the *CST3* gene promoter express high levels of this inhibitor abundantly. Cystatin C can prevent formation of amyloid β plaques associated with Alzheimer's disease and can itself form toxic aggregates. Cystatin C regulates NO secretion by macrophages and is a TGF-β antagonist.

New approaches to Parkinson's disease treatment

According to data obtained by Chen et al. (2015), Hu et al. (2016), changes in the expression of cystatin C in Parkinson's disease are related to mild cognitive dysfunction. Recently, it was shown that intracerebroventricular administration of cystatin C ameliorates amyotrophic lateral sclerosis-like disturbances in mice (Watanabe et al., 2018). Induction of autophagy by cystatin C was suggested as a potential mechanism of prevention of cerebral vasospasm during several neurological disturbances in an animal experiment (after subarachnoid hemorrhage in mice) (Liu et al., 2013). Possible mechanism of stimulation of autophagy by cystatin C is suggested via the mammalian target of rapamycin (mTOR) signaling pathway (Fig. 6).

It has been found that the injections of cystatin C into the s. nigra of A53T mutant alpha-synuclein transgenic mice revealed neuroprotective effect *in vivo* in Parkinson's disease model with increased autophagy markers LC3B in different brain regions (Zou et al., 2017). Authors concluded, that neuroprotective effect of cystatin C in A53T transgenic mice was related to upregulating the autophagy and VEGF pathways. It presents a new approach to the treatment of Parkinson's disease through neuronal-vascular protection mediated by cystatin C.

Conclusion

The roles of autophagy in the maintenance of cellular survival and in suppression of neurodegeneration have been evaluated in Alzheimer's, Parkinson's, and Huntington's diseases, which are accompanied by the accumulation of amyloid β, α-synuclein, and huntingtin, respectively. Autophagy is down regulated in various ways in these diseases and decreases with ageing. Cystatin C is one of the potent regulators of autophagy. Changes in the expression and secretion of cystatin C in the brain have been shown in amyotrophic lateral sclerosis, Alzheimer's and

Parkinson's diseases, and in some animal models of neurodegeneration, thus proving a protective function of cystatin C. It has been suggested that cystatin C plays the primary role in amyloidogenesis. Controlled expression of a cystatin C peptide has been proposed as a new approach to therapy for Alzheimer's disease. Neuroprotective cystatin C effect *in vivo* (in A53T transgenic mice) was connected with upregulation in autophagy and VEGF pathways, opening a new approach to the treatment of Parkinson's disease through neuronal-vascular protection mediated by cystatin C. Administration of cystatin C as a regulator of autophagy holds promise as one possible approach to the treatment of neurodegenerative diseases.

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Acknowledgements. This work was supported partially by grant No. 16-04-01423-a from the Russian Foundation for Basic Research (Russia), to T.A.K., budget from the project No. 0538-2014-0009 of the Scientific Research Institute of Physiology and Basic Medicine (SRIPhBM) and a unique scientific resource "Biological collection – Genetic biomodels of neuro-psychiatric disorders" (No. 493387) at the SRIPhBM. The studies were implemented using the equipment of the Center for Genetic Resources of Laboratory Animals at ICG SB RAS, supported by the Ministry of Education and Science of Russia (unique identifier of the project: RFMEFI62117X0015). The English language was corrected and certified bu shevchuk-editing.com.

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

Received September 24, 2018. Revised February 11, 2019. Accepted February 11, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):398-404
DOI 10.18699/VJ19.508

Изучение иммуногенности рекомбинантного фрагмента ортопоксвирусного белка p35

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Несмотря на ликвидацию натуральной оспы, ортопоксвирусы продолжают оставаться источником биологической опасности для людей, так как в природе циркулируют вирусы оспы коров и оспы обезьян, причем последний способен вызывать не только спорадические случаи заболеваний человека, но и вспышки оспоподобной инфекции. Кроме того, периодическая вакцинация необходима для представителей определенных профессий (ученые, изучающие патогенные ортопоксвирусы, медицинские работники и др.). Оспопрививание – вакцинация живым вирусом осповакцины, которое широко использовалось при ликвидации натуральной оспы, обеспечивает формирование у вакцинированных людей длительного иммунитета. Однако, давая достаточно надежную защиту, оспопрививание нередко сопровождается серьезными поствакцинальными осложнениями, вероятность возникновения которых особенно велика для лиц со сниженным иммунным статусом. В связи с этим разработка препаратов для профилактики и лечения инфекций, вызванных ортопоксвирусами, актуальна и в настоящее время. Цель данного исследования – оценка иммуногенности в мышиной модели рекомбинантного белка p35Δ12, сконструированного на основе белка p35 вируса оспы коров. Ранее было показано, что белок p35Δ12 связывается с высоким сродством с полноразмерным вируснейтрализующим анти-ортопоксвирусным антителом человека. В настоящей работе рекомбинантный белок p35Δ12, наработанный в клетках *E. coli* XL1-blue и очищенный хроматографически, использовали для двукратной иммунизации мышей. Через две недели после второй иммунизации у мышей брали образцы крови и анализировали находящиеся в сыворотке антитела. Методами иммуноферментного и вестерн-блот анализа было показано, что сыворотки иммунизированных животных содержали антитела класса IgG, направленные к рекомбинантному белку p35Δ12. Методом конфокальной микроскопии показано, что антитела, индуцированные белком p35Δ12, способны узнавать клетки Vero E6, зараженные вирусом осповакцины ЛИВП-GFP. Кроме того, находящиеся в сыворотках иммунизированных мышей антитела могут нейтрализовать инфекционность вируса осповакцины ЛИВП-GFP в реакции ингибирования бляшкообразования *in vitro*.
Ключевые слова: белок p35 ортопоксвирусов; вирус оспы коров; рекомбинантный белок; иммунизация; конфокальная микроскопия.

Для цитирования: Хлусевич Я.А., Матвеев А.Л., Гончарова Е.П., Байков И.К., Тикунова Н.В. Изучение иммуногенности рекомбинантного фрагмента ортопоксвирусного белка p35. Вавиловский журнал генетики и селекции. 2019;23(4):398-404. DOI 10.18699/VJ19.508

Immunogenicity of recombinant fragment of orthopoxvirus p35 protein in mice

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Despite the elimination of smallpox, orthopoxviruses continue to be a source of biological danger for humans, as cowpox and monkey pox viruses circulate in nature and the last virus can cause both sporadic cases of human diseases and outbreaks of smallpox-like infection. In addition, periodic vaccination is necessary for representatives of some professions (scientists studying pathogenic orthopoxviruses, medical personnel, etc.). Vaccination against smallpox virus with live vaccinia virus, which was widely used during the elimination of smallpox, induces the formation of long-term immunity in vaccinated people. However, providing a high level of protection, the vaccination is often accompanied by serious post-vaccination complications, the probability of which is particularly great for individuals with compromised immunity. In this regard, the development of preparations for the prevention and treatment of infections caused by orthopoxviruses remains important today. The aim of this study was to assess the immunogenicity in the mouse model of recombinant protein p35Δ12, designed previously on the base of the cowpox virus protein p35. It was previously shown that the

protein p35Δ12 was recognized by fully human neutralizing anti-orthopoxviral antibody with high affinity. In this work, recombinant protein p35Δ12 produced in *E. coli* cells XL1-blue and purified by chromatography was used for two-time immunization of mice. Two weeks after the second immunization, blood samples were taken from mice and serum antibodies were analyzed. It was shown by ELISA and Western-blot analysis that immunized mice sera contained IgG antibodies specific to recombinant protein p35Δ12. Confocal microscopy showed that antibodies induced by the p35Δ12 protein were able to recognize Vero E6 cells infected with the LIVP-GFP vaccinia virus. In addition, the antibodies in the serum of immunized mice were able to neutralize the infectivity of the vaccinia virus LIVP-GFP in the plaque reduction neutralization test *in vitro*. These experiments have demonstrated promising properties of the p35Δ12 protein if it were used as a component of vaccine for prophylaxis of orthopoxvirus infections.
Key words: p35 orthopoxvirus protein; cowpox virus; recombinant protein; immunization; confocal microscopy.

For citation: Khlusevich Ya.A., Matveev A.L., Goncharova E.P., Baykov I.K., Tikunova N.V. Immunogenicity of recombinant fragment of orthopoxvirus p35 protein in mice. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):398-404. DOI 10.18699/VJ19.508 (in Russian)

Введение

Представители рода *Orthopoxvirus* (семейство Poxviridae) – это сложные ДНК-содержащие вирусы, реплицирующиеся в цитоплазме зараженных клеток и имеющие свои собственные механизмы регуляции репликации и транскрипции (Shchelkunov et al., 2005; Moss, 2013). Ортопоксвирусы различаются по спектру хозяйской специфичности: существуют вирусы с широкой хозяйской специфичностью – вирус оспы коров (ВОК), вирус оспы обезьян (ВОО); есть вирусы, способные заражать только одного хозяина – вирус эктромелии (ВЭ), вирус натуральной оспы (ВНО) (Buller et al., 1986; Shchelkunov et al., 2005). Несколько представителей этого рода являются патогенными для человека; ВНО и ВОО могут вызвать генерализованное заболевание (Jezek et al., 1986; MacNeil et al., 2009; Smith, 2013; McCollum, Damon, 2014; Nakazawa et al., 2015), тогда как инфекция вирусом осповакцины (ВОВ) или ВОК обычно приводит лишь к локальному повреждению кожи (Moss, 2013).

Несмотря на то что естественная трансмиссия ВНО была прекращена, ортопоксвирусы продолжают оставаться источником биологической опасности для людей, так как в природе циркулируют ортопоксвирусы, способные инфицировать человека. Так, ВОО может вызывать как спорадические случаи оспоподобного заболевания человека, так и вспышки этой инфекции (Di Giulio, Eckburg, 2004; Rimoin et al., 2010; Reynolds et al., 2013). Кроме того, постоянно регистрируются случаи заболеваний людей оспой коров и вакциноподобным заболеванием (Zafar et al., 2007; Campe et al., 2009; Carletti et al., 2009; Ninove et al., 2009; Silva-Fernandes et al., 2009; Trindade et al., 2009; Bhanuprakash et al., 2010; Ducournau et al., 2013; Riyesh et al., 2014; Hobi et al., 2015; Kinnunen et al., 2015). Однако в связи с ликвидацией натуральной оспы массовое оспопрививание (вакцинация живым ВОВ) было прекращено во второй половине 1970-х гг., и в настоящее время большинство населения не имеет иммунитета к ортопоксвирусным инфекциям. Прививка живым ВОВ приводит к формированию у вакцинированных людей длительного иммунитета (Crotty et al., 2003). Вместе с тем, давая достаточно надежную защиту, оспопрививание нередко сопровождается серьезными поствакцинальными осложнениями, вероятность возникновения которых особенно велика для лиц со сниженным иммунным статусом (Fulginiti et al., 2003; Kawakami et al., 2009). В связи с этим не прекращаются разработки препаратов для профилактики и лечения инфекций, вызванных ортопоксвирусами.

С появлением рекомбинантных технологий возникло новое направление в вакцинологии – применение отдельных антигенов вместо целых патогенов. Такой подход позволяет сфокусировать иммунный ответ на более значимых мишенях. Предполагается, что иммунизация определенным рекомбинантным белком, несущим наиболее иммуногенные эпитопы, могла бы индуцировать наработку эффективных антител, как при классической вакцинации. Действительно, субъединичные вакцины в настоящее время активно разрабатываются и уже используются в практике (Bazzill et al., 2018; Medina et al., 2018; Shi et al., 2018; Wang et al., 2018).

Ранее для исследования эпитопной специфичности анти-ортопоксвирусного полноразмерного антитела человека fh1A, способного нейтрализовать инфекционность вируса осповакцины, был сконструирован рекомбинантный белок p35Δ12 (Khlusevich et al., 2018), представляющий фрагмент белка p35 вируса оспы коров. Характер взаимодействия вируснейтрализующего антитела с белком p35Δ12 позволил предположить наличие вируснейтрализующего эпитопа в последовательности этого рекомбинантного белка. Цель данного исследования – оценка способности белка p35Δ12 индуцировать образование вируснейтрализующих антител у мышей.

Материалы и методы

Рекомбинантный белок p35Δ12, сконструированный ранее (Khlusevich et al., 2018), нарабатывали в клетках *Escherichia coli* XL1-blue, трансформированных плазмидой pQE30-Δ12 и индуцированных 0.2 мМ изопропил тиогалактозидом (ИПТГ). Белок p35Δ12 очищали из цитоплазмы трансформированных клеток с помощью аффинной хроматографии с использованием носителя Ni-NTA Sepharose. Мономерную фракцию белка отделяли хроматографически на колонке с носителем Superdex 75 10/300 GL (GE Healthcare). Очищенный белок p35Δ12 концентрировали в фосфатно-солевом буферном растворе (ФСБР, 100 мМ NaCl, 50 мМ Na_2HPO_4, pH 7.4) с использованием фильтров Amicon Ultra-4 10K (Millipore).

Эксперименты с животными проводили в соответствии с рекомендациями по защите животных, используемых в научных целях (Директива EU2010/63/EU). Все экспе-

рименты с животными были одобрены комитетом по биоэтике Института химической биологии и фундаментальной медицины Сибирского отделения Российской академии наук, Новосибирск, Россия. Мышей размещали в пластиковых клетках при нормальном цикле день/ночь. Вода и еда были предоставлены *ad libitum*.

Для иммунизации использовали самок мышей BALB/c возраста 5–6 недель, приобретенных в питомнике Государственного научного центра вирусологии и биотехнологии «Вектор» (р.п. Кольцово, Россия). Очищенный белок p35Δ12 разводили в ФСБР и вводили интраперитонеально в дозировке 10 мкг на мышь одновременно с полным адъювантом Фрейнда (Sigma, США), общий объем инъекции – 500 мкл. Через 2 недели иммунизацию повторяли, заменив полный адъювант Фрейнда неполным адъювантом (Sigma). Через 14 дней после второй иммунизации собирали кровь из лицевой вены.

Сыворотку выделяли из свернувшихся образцов крови центрифугированием, после чего образцы сыворотки инкубировали 30 мин при 56 °C для инактивации белков комплемента. Эффективность иммунизации оценивали иммуноферментным анализом (ИФА) и вестерн-блот анализом.

Для выявления анти-p35Δ12 антител в сыворотках иммунизированных мышей в лунки 96-луночного полистиролового планшета («Медполимер», Россия) сорбировали белок p35Δ12 в ФСБР, 200 нг/лунку. После удаления несвязавшегося антигена лунки промывали ФСБР, участки неспецифического связывания блокировали 5 % раствором обезжиренного молока в ФСБР в течение 1 ч, после чего лунки снова промывали ФСБР с 0.1 % Tween 20. Затем в лунки вносили последовательные разведения сывороток мышей в ФСБР, начиная с разведения 1:200, с шагом разведения 1:2. Планшеты инкубировали 1 ч при 37 °C. Образовавшиеся иммунные комплексы выявляли конъюгатом поликлональных антител козы против IgG (H+L) мыши со щелочной фосфатазой (Sigma) в разведении 1:8000. Затем лунки последовательно промывали ФСБР с 0.2 % Tween 20 и АР-буфером (100 мМ NaCl, 5 мМ $MgCl_2$, 100 мМ Трис-HCl, pH 9.5); иммунные комплексы окрашивали пара-нитрофенилфосфатом в АР-буфере.

Для проведения вестерн-блот анализа лизат клеток *E. coli*, продуцирующих белок p35Δ12, фракционировали электрофоретически в 12.5 % полиакриламидном геле с 0.1 % додецилсульфатом натрия и переносили на нитроцеллюлозную мембрану (Sigma), которую после блокирования сайтов неспецифического связывания 5 % раствором обезжиренного молока в ФСБР инкубировали с мышиными сыворотками, разведенными 1:200 в ФСБР с 0.1 % Tween 20. Связавшиеся антитела выявляли конъюгатом поликлональных антител козы против IgG (H+L) мыши со щелочной фосфатазой (Sigma) в разведении 1:8000. Визуализацию иммунного комплекса проводили, добавляя 5-бромо-3-индолил фосфат и нитро-тетразолиевый голубой. В качестве положительного контроля использовали полноразмерное антитело человека fh1A, специфичное к белку p35 ортопоксвирусов (Khlusevich et al., 2018), которое инкубировали аналогично мышиным сывороткам, но выявляли с помощью поликлональных антител козы против IgG (H+L) человека (Sigma).

В экспериментах по оценке взаимодействия сывороток мышей с жизнеспособными вирионами ВОВ использовали штамм ЛИВП-GFP, в котором ген, кодирующий зеленый флуоресцентный белок (GFP), встроен в состав гена тимидинкиназы (Петров и др., 2013).

Для проведения лазерно-сканирующей микроскопии суспензию ВОВ (280 БОЕ), штамм ЛИВП-GFP (Петров и др., 2013), разведенную в 100 мкл питательной среды DMEM с 2 % эмбриональной сывороткой, добавляли к монослою клеток Vero E6, выращенных в 35 мм чашках Петри для микроскопии (Ibidi, Германия), и инкубировали в течение 1 ч при 37 °C. Затем клетки три раза отмывали питательной средой. Через 2 дня зараженные клетки фиксировали 10 % раствором формалина, промывали ФСБР и блокировали 3 % бычьим сывороточным альбумином 1 ч при 37 °C. Далее к фиксированным клеткам добавляли сыворотки иммунизированных мышей в ФСБР в разведении 1:200 и инкубировали 1 ч при 37 °C. После промывания клеток стерильным ФСБР связавшиеся антитела выявляли поликлональными антителами козы против IgG (H+L) мыши, конъюгированными с флуоресцентной меткой Alexa Fluor 633. Через час инкубации клетки промывали стерильным ФСБР и добавляли 300 нМ раствор DAPI (Life Technologies, США) в ФСБР для окрашивания клеточных ядер. В качестве отрицательного контроля использовали нормальную мышиную сыворотку.

В качестве положительного контроля использовали полноразмерное анти-p35 антитело человека fh1A (Khlusevich et al., 2018), которое инкубировали аналогично мышиным сывороткам, но выявляли с помощью поликлональных антител козы против IgG (H+L) человека, конъюгированных с флуоресцентной меткой Alexa Fluor 633.

Изображения специфического взаимодействия антител с клетками, зараженными ВОВ ЛИВП-GFP, получали с помощью конфокального микроскопа LSM 710 (Carl Zeiss, Германия) с 20-кратным объективом. Флуоресцентные метки DAPI, GFP и Alexa Fluor 633 возбуждали на длинах волн 405, 488 и 633 нм соответственно; эмиссию детектировали на длинах волн 440–480, 490–530 и 630–700 нм соответственно. Получение и обработку изображений выполняли в пакете программ ZEN black edition (Carl Zeiss).

Для оценки вируснейтрализующей активности сывороток мышей, иммунизированных очищенным белком p35Δ12, суспензию ВОВ, штамм ЛИВП-GFP (Петров и др., 2013), разведенную в питательной среде DMEM с 2 % сывороткой телят, смешивали в равном объеме с различными разведениями мышиных сывороток и инкубировали 1 ч при 37 °C. После этого смесь наслаивали на монослой клеток Vero E6 в 24-луночных культуральных планшетах (TPP, Швейцария). Через 2 ч смесь удаляли, клетки промывали средой DMEM и культивировали в питательной среде DMEM с 2 % сывороткой телят при 37 °C. В контрольные лунки вносили суспензию вируса без добавления мышиной сыворотки. Через несколько дней клетки окрашивали 0.1 % кристаллическим фиолетовым в 10 % растворе формальдегида и подсчитывали бляшки. Уровень нейтрализации рассчитывали по формуле $N = (V_o - V_n)/V_o \times 100\ \%$, где V_o – среднее количество бляшек в контрольных лунках, а V_n – количество бляшек в экспериментальных лунках.

Результаты

Рекомбинантный белок p35Δ12, наработанный в клетках *E. coli* XL1-blue, очищали из фракции растворимых цитоплазматических белков (рис. 1, *а*). При очистке целевого белка с помощью аффинной хроматографии с использованием Ni-NTA сефарозы вместе с белком p35Δ12 с молекулярной массой около 30 кДа соочищался клеточный белок с молекулярной массой около 25 кДа (см. рис. 1, *а*). Вестерн-блот анализ очищенного белка p35Δ12 подтвердил его способность взаимодействовать с полноразмерным антителом человека fh1A, направленным к ортопоксвирусному белку p35; дополнительный белок с молекулярной массой около 25 кДа антителом fh1A не выявлялся (см. рис. 1, *б*).

Очищенный белок p35Δ12 использовали для иммунизации мышей BALB/c. Иммунизацию проводили по так называемой короткой схеме: белок вводили дважды в дозировке 10 мкг на мышь с интервалом в 2 недели. Из образцов крови, собранных через 7 дней после второй иммунизации, отделили сыворотку и оценили наличие в ней антител против белка p35Δ12. Данные ИФА подтвердили наличие специфических антител в сыворотках иммунизированных мышей (рис. 2). Кроме того, эти сыворотки выявляли белок p35Δ12, перенесенный на нитроцеллюлозную мембрану, в вестерн-блот анализе (см. рис. 1, *б*).

Для визуализации взаимодействия находящихся в сыворотках иммунизированных мышей анти-p35Δ12 антител с клетками, зараженными ортопоксвирусом, использовали лазерно-сканирующую микроскопию. В экспериментах применяли сконструированный ранее ВОВ, штамм ЛИВП-GFP (Петров и др., 2013), при размножении которого в цитоплазме зараженных клеток накапливается белок GFP и клетки окрашиваются в зеленый цвет при возбуждении светом с длиной волны 488 нм и детекции в диапазоне длин волн 490–530 нм. Клетки Vero E6 заражали ВОВ ЛИВП-GFP и обрабатывали сыворотками иммунизированных мышей и нормальной мышиной сывороткой (отрицательный контроль). Мышиные антитела выявляли конъюгатом антивидовых антител с флуоресцентной меткой Alexa Fluor 633. Этот флуорофор возбуждается светом с длиной волны 633 нм, и объекты, меченные Alexa Fluor 633, окрашиваются красным цветом при детекции в световой области 630–700 нм.

Результаты конфокальной микроскопии, представленные на рис. 3, однозначно свидетельствуют о способности сыворотки иммунизированных мышей выявлять зараженные клетки. Видно, что меченные Alexa Fluor 633 антитела, направленные к суммарному IgG мыши, локализуются на поверхности зараженных клеток (окрашены зеленым) и не выявляют незараженные клетки Vero E6. Полноразмерное антитело fh1A, выявленное меченными Alexa Fluor 633 антителами, направленными к суммарному IgG человека, также выявляет клетки, зараженные ВОВ ЛИВП-GFP.

Для проверки способности рекомбинантного белка p35Δ12 вызывать наработку антител, обладающих вируснейтрализующими свойствами, исследовали вируснейтрализующую активность сывороток иммунизированных мышей. В экспериментах для заражения клеток Vero E6 также использовали вирус ВОВ ЛИВП-GFP. При инкубации этого вируса с различными разведениями мышиных сывороток обнаружено снижение инфекционности вируса более чем на 50 % при разведении сывороток 1:20 и 1:100 (рис. 4). Нормальная мышиная сыворотка, полученная от неиммунизированного животного, такой способностью не обладала (см. рис. 4).

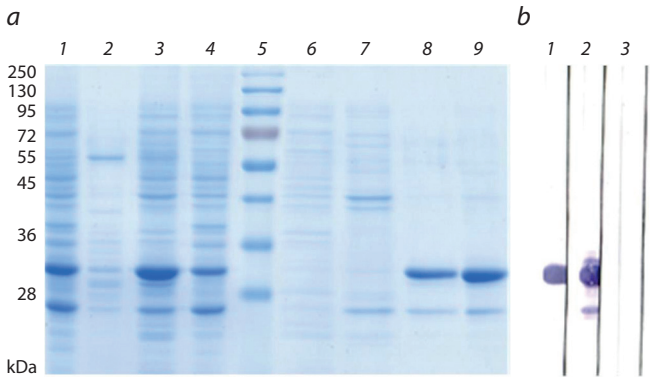

Fig. 1. (*a*) Electrophoretic resolution (12.5 % SDS-polyacrylamide gel) of cellular and protein fractions of *E. coli* XL1Blue/pQE30-Δ12. (*b*) Western blot analysis of purified p35Δ12 protein.

Lanes in the gel: (*1*) induced cell culture of *E. coli* XL1Blue/pQE30-Δ12, (*2*) periplasmic fraction, (*3*) fraction of soluble proteins of the cytoplasm, (*4*) inclusion body fraction, (*5*) molecular weight markers, (*6*) fraction of proteins not bound to Ni-NTA, (*7*) fraction eluted with 25 mM imidazole, (*8*) fraction eluted with 100 mM imidazole, (*9*) concentrated purified p35Δ12 protein. Strips of nitrocellulose membrane with transferred p35Δ12 protein probed with: (*1*) fh1A, (*2*) pooled serum of immunized mice, (*3*) normal mouse serum.

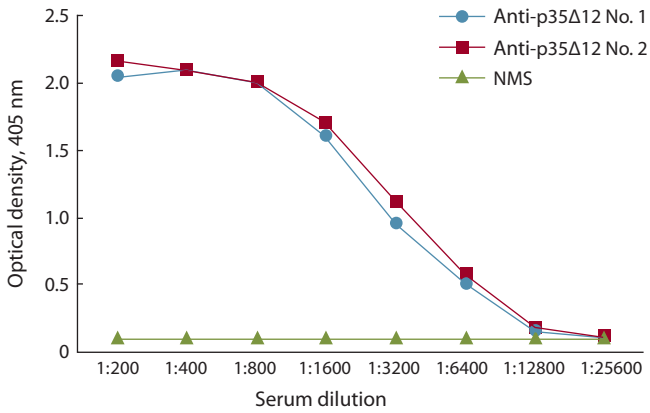

Fig. 2. ELISA evaluating the binding of sera from immunized mice and a normal mouse (NMS) to p35Δ12 protein.

Обсуждение

Широкое применение вакцин на основе живого ВОВ позволило победить натуральную оспу – одно из самых опасных инфекционных заболеваний в истории человечества. Однако после ликвидации натуральной оспы Всемирная организация здравоохранения в 1980 г. рекомендовала прекратить применение вакцин на основе живого вируса осповакцины из-за относительно большого числа серьезных осложнений и побочных эффектов. Использование классических вакцин для массовой вакцинации, вероятно,

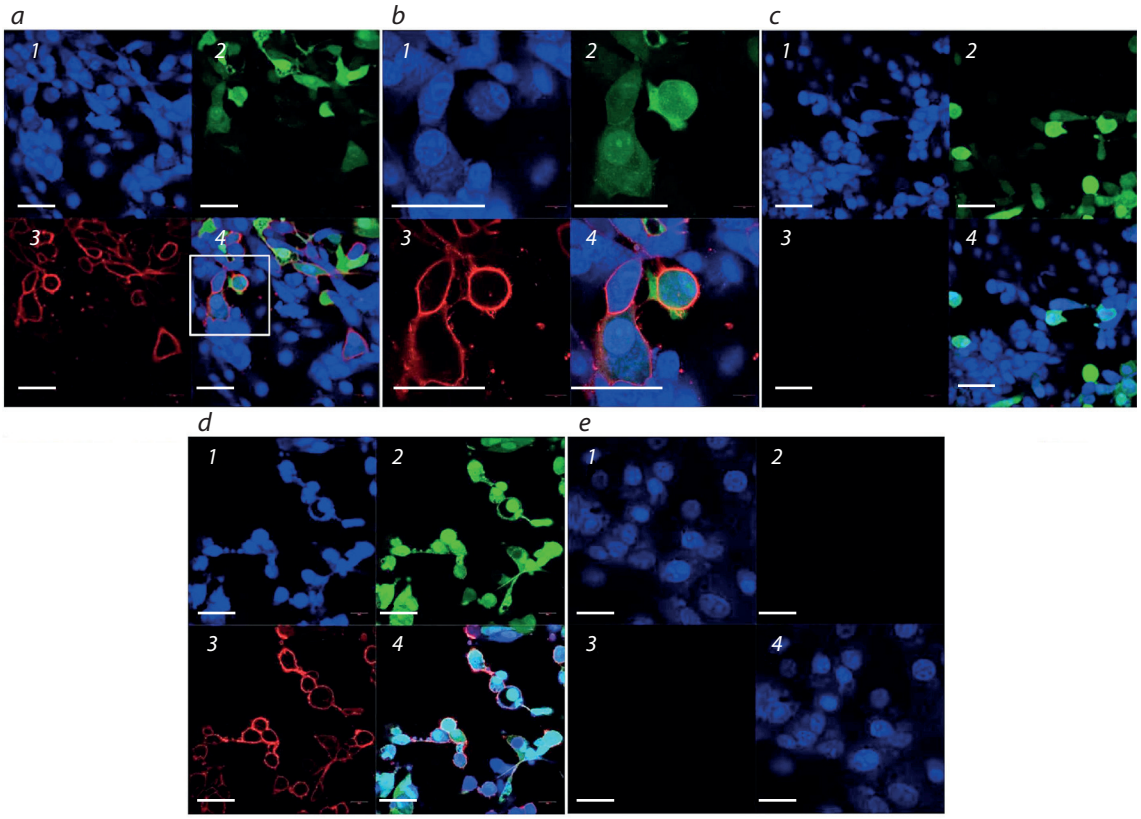

Fig. 3. Confocal microscopy of Vero E6 cells infected with vaccinia virus LIVP-GFP.

Infected monolayer cells were incubated with sera of mice immunized with p35Δ12 protein (*a*, *b*), normal mouse serum (*c*), and full-length human fh1A antibody (*d*). Monolayer non-infected cells incubated with sera of mice immunized with p35Δ12 protein were used as negative control (*e*). Bound antibodies were detected with anti-species goat anti-mouse IgG or human antibodies conjugated with Alexa Fluor 633. In addition, preparations were stained with DAPI for cell nuclei. Images of the antibody binding with cells were obtained using a LSM 710 confocal microscope (Carl Zeiss, Germany). Scale bar – 10 μm. *1* – DAPI; *2* – GFP; *3* – Alexa Fluor 633; *4* – merged.

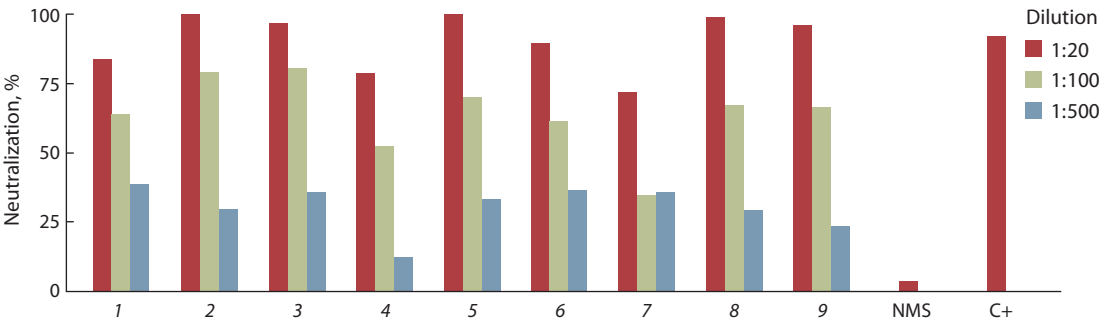

Fig. 4. Neutralization of plaque formation by smallpox vaccinia virus LIVP-GFP on Vero E6 cell culture by different dilutions of sera from p35Δ12-immunized mice.

The virus-neutralizing activity of twentyfold diluted sera of 9 immunized mice, normal mouse serum (NMS, negative control), and serum from a volunteer repeatedly vaccinated with vaccinia LIPV virus (positive control) are presented.

привело бы к возрастанию числа осложнений, поскольку в последние годы у все большего числа людей регистрируются различного рода нарушения иммунитета. Вместе с тем необходимость в вакцинах, защищающих от ортопоксвирусов, не исчезла, поскольку в природе по-прежнему существуют патогенные для человека ортопоксвирусы, и вспышка оспы обезьян в США из-за вируса, чей природный резервуар находится на другом континенте, доказала это. Кроме того, периодическая вакцинация необходима для представителей определенных профессий (ученые, изучающие патогенные ортопоксвирусы, медицинские работники и др.) (Petersen et al., 2016).

Разработка вакцин на основе высокоаттенуированных вирусов осповакцины, не способных размножаться в клетках человека (Meyer et al., 1991), привела к получению вакцин MVA и LC16m8 (Meyer et al., 1991; Kidokoro et al.

2005), которые крайне редко вызывают побочные реакции и могут применяться даже для вакцинации индивидуумов с ослабленным иммунитетом (Olson, Shchelkunov, 2017). Эти вакцины не вызывают длительного напряженного иммунитета, и целесообразно их использовать для первой вакцинации (Olson, Shchelkunov, 2017; Shchelkunova, Shchelkunov, 2017). В связи с этим продолжаются попытки создания различных вариантов противооспенных вакцин, включая ДНК-вакцины и вакцины на основе генно-инженерных вариантов вируса осповакцины (детально рассмотрено в обзоре (Shchelkunova, Shchelkunov, 2017)). Одним из перспективных подходов является получение субъединичных вакцин для разработки стратегии безопасной вакцинации, включающей на первом этапе вакцинацию субъединичной вакциной, после чего можно было бы применять вакцины на основе аттенуированных ортопоксвирусов. Для создания субъединичных вакцин необходимо детальное исследование потенциальных иммуногенов.

При оспопрививании или инфицировании ортопоксвирусами в организме человека нарабатываются антитела против широкого спектра белков, экспрессируемых на разных стадиях инфекции, причем наряду с мембранными оболочечными белками, которые доступны благодаря их расположению на поверхности вирусной частицы, нарабатываемые антитела специфически взаимодействуют и с коровыми белками вириона (Jones-Trower et al., 2005). В случае ортопоксвирусов наиболее значимыми для формирования иммунитета у человека являются белки B5R, A27L и p35 (Pütz et al., 2006). Первый белок экспонирован на поверхности EEV (внеклеточный оболочечный вирус), а белки A27L и p35 – на поверхности IMV (внутриклеточный зрелый вирус). Антитела против этих белков обладают вируснейтрализующими и протективными свойствами (Pütz et al., 2006). Ранее нами был сконструирован химерный белок p35 вируса оспы коров, содержащий на N-конце β-галактозидазу (Дубровская и др., 2007). Позднее был получен рекомбинантный белок p35Δ12, содержащий с 1 по 239 аминокислотные остатки белка p35 вируса оспы коров, и показано, что вируснейтрализующие антитела человека распознают этот белок с высокой эффективностью (Khlusevich et al., 2018).

Заключение

В настоящем исследовании доказано, что белок p35Δ12, созданный на основе белка p35 вируса оспы коров, при введении в мышей индуцирует у них образование антител, которые способны не только узнавать клетки, зараженные вирусом осповакцины, но и нейтрализовать инфекционность вируса осповакцины в экспериментах *in vitro*. При изучении иммунологических свойств полноразмерной формы рекомбинатного белка p35 осповакцины (Lin et al., 2000; Davies et al., 2005) обнаружено, что сыворотки, полученные от иммунизированных животных, также обладали вируснейтрализующими свойствами. Однако в указанных работах исследователи использовали для иммунизации рекомбинантный полноразмерный белок p35 в дозировках, превышающих на порядок дозировку, использованную в нашей работе. Следует отметить, что даже так называемая короткая схема иммунизации – двукратное введение рекомбинантного белка p35 в низкой дозировке, обеспечила индукцию вируснейтрализующих антител.

Проведенные эксперименты продемонстрировали высокую иммуногенность белка p35Δ12 и целесообразность его возможного использования в качестве компонента субъединичной вакцины или вовлечения гена, кодирующего p35, в ДНК-вакцину. Вместе с тем требуются дальнейшие детальные исследования *in vivo* для оценки протективных свойств белка p35Δ12 и предпочтительных схем иммунизации, обеспечивающих защиту мышей от ортопоксвирусных инфекций.

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Acknowledgements. This work was supported by the Russian Science Foundation, project 16-14-00083.

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

Received November 28, 2018. Revised March 04, 2019. Accepted March 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):405-411
DOI 10.18699/VJ19.509

Transcription factors MhyFIL1 and MhyFIL3 (*Monotropa hypopitys*) determine the asymmetric development of above-ground lateral organs in plants

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It is believed that the complete mycoheterotroph pinesap *Monotropa hypopitys* adaptively evolved from a photosynthetic mycorrhizal ancestor, which had lost its photosynthetic apparatus and vegetative organs (stem and leaves). The aerial part of the plant is a reproductive axis with sterile bracts and inflorescence with a flower type canonical for higher plants. The origin of leaves and leaf-like lateral organs is associated, among other factors, with the evolution of the *YABBY* genes, which are divided into "vegetative" and evolutionarily recent "reproductive" genes, with regard to their expression profiles. The study of the vegetative *YABBY* genes in pinesap will determine whether their functions (identification of cell identity on the abaxial surface of the lateral organs) are preserved in the leafless plant. In this study, the structural and phylogenetic analysis of the pinesap vegetative genes *MhyFIL1* and *MhyFIL3* is performed, the main conserved domains and motifs of the encoded proteins are characterized, and it is confirmed that the genes belong to the vegetative clade *YABBY3*/*FIL*. The effect of heterologous ectopic expression of the *MhyFIL1* and *MhyFIL3* genes on the phenotype of transgenic tobacco *Nicotiana tabacum* is evaluated. The leaves formed by both types of plants, 35S::*MhyFIL1* and 35S::*MhyFIL3*, were narrower than in control plants and were twisted due to the changed identity of adaxial surface cells. Also, changes in the architecture of the aerial part and the root system of transgenic plants, including aberrant phyllotaxis and arrest of the shoot and root apical meristem development, were noted. Some of the 35S::*MhyFIL1* and 35S::*MhyFIL3* plants died as early as the stage of the formation of the first leaves, others did not bloom, and still others had a greatly prolonged vegetation period and formed fewer flowers than normal ones. The flowers had no visible differences from the control except for fragile pedicles. Thus, the absence of structural changes from the *M. hypopitys* flower in comparison to autotrophic species and the effect of *MhyFIL1/3* heterologous expression on the development of tobacco plants indicate the preservation of the functions of the vegetative *YABBY* genes by the *MhyFIL1/3* genes in pinesap. Moreover, the activity of YABBY transcription factors of the FIL clade in *M. hypopitys* is not directly related to the loss of the ability of pinesap to form leaves during the evolutionary transition from autotrophic nutrition to heterotrophy.
Key words: *Monotropa hypopitys*; mycoheterotroph; heterologous gene expression; abaxial-adaxial asymmetry; transcription factors; YABBY; "vegetative" YABBYs; FILAMENTOUS FLOWER.

For citation: Shchennikova A.V., Kamionskaya A.M., Nezhdanova A.V., Gavrilova K.S., Filyushin M.A., Kochieva E.Z., Skryabin K.G. Transcription factors MhyFIL1 and MhyFIL3 (*Monotropa hypopitys*) determine the asymmetric development of above-ground lateral organs in plants. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):405-411. DOI 10.18699/VJ19.509

Факторы транскрипции MhyFIL1 и MhyFIL3 (*Monotropa hypopitys*) определяют асимметричное развитие боковых органов надземной части растения

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Считается, что полный микогетеротроф, подъельник *Monotropa hypopitys,* адаптивно эволюционировал из фотосинтезирующего микоризного предшественника, потеряв при этом аппарат фотосинтеза и вегетативные органы (стебель и листья). Надземная часть растения представляет собой цветонос со стерильными прицветниками и соцветием с каноническим для высших растений типом цветка. У растений происхождение плоского листа и других листоподобных латеральных органов связывают с эволюцией генов *YABBY*, которые, в зависимости от профиля экспрессии, разделяются на «вегетативные» и эволюционно более поздние «репродуктивные» гены. Изучение «вегетативных» генов *YABBY* подъельника позволит выяснить, сохранились ли их функции (определение идентичности клеток абаксиальной по-

верхности латеральных органов) у растения без листьев. В настоящем исследовании проведен структурно-филогенетический анализ генов подъельника *MhyFIL1* и *MhyFIL3*, охарактеризованы основные консервативные домены и мотивы кодируемых ими белков и подтверждена принадлежность генов к «вегетативной» кладе *YABBY3/FIL*. Проведена оценка влияния гетерологичной эктопической экспрессии генов *MhyFIL1* и *MhyFIL3* на фенотип трансгенных растений табака *Nicotiana tabacum*. Показано, что оба типа растений, 35S::*MhyFIL1* и 35S::*MhyFIL3*, формируют листья более узкие, чем в норме, и скрученные за счет измененной идентичности клеток адаксиальной поверхности. Выявлены также изменения архитектуры надземной части и корневой системы растений, включая аберрантный филлотаксис и подавление развития апикальных меристем побега и корня. Часть растений 35S::*MhyFIL1* и 35S::*MhyFIL3* погибала еще на стадии формирования первых листьев, часть не цвела, остальные имели сильно увеличенный период вегетации и при цветении формировали меньше цветков, чем в норме. Цветки не имели видимых отличий от контроля, за исключением ломких цветоножек. Таким образом, отсутствие изменений в строении цветка подъельника в сравнении с автотрофными видами, а также особенности влияния гетерологичной экспрессии генов *MhyFIL1/3* на развитие растений табака говорят о сохранении генами подъельника *MhyFIL1/3* функции «вегетативных» генов *YABBY*. При этом у *M. hypopitys* активность YABBY-факторов транскрипции группы FIL напрямую не связана с потерей способности формировать листья при эволюционном переходе подъельника от аутотрофного питания к гетеротрофии.
Ключевые слова: *Monotropa hypopitys*; микогетеротроф; гетерологичная экспрессия гена; абаксиально-адаксиальная асимметрия; транскрипционные факторы YABBY; «вегетативные» YABBY; FILAMENTOUS FLOWER.

Introduction

The most significant event in plant evolution is considered to be the emergence of photosynthesis, due to which most modern plants are autotrophs and only about 1 % of flowering plants are heterotrophic. Among the latter, a special place is occupied by complete mycoheterotrophs, which, in the course of adaptation to adverse environmental conditions, have acquired the ability to obtain nutrients through mycorrhiza (a symbiotic association of roots with fungi). The range of adaptation consequences due to photosynthesis incapability includes the degradation and rearrangement of the genome, large-scale loss of functional genes, etc. (Wicke et al., 2016; Graham et al., 2017).

The monotropoid type of mycorrhiza is characteristic only for members of the subfamily Monotropoideae of the Ericaceae family (Leake, 1994), including *Monotropa hypopitys* (syn. *Hypopitys monotropa*). Compared with the related photosynthetic species *Pyrola rotundifolia*, achlorophyllous *M. hypopitys* is characterized by considerable structural rearrangements in the genome, an increased rate of accumulation of nucleotide substitutions in the genes, a significant reduction in the plastome, and a loss of the photosynthesis apparatus from both the plastome and the nuclear genome (Ravin et al., 2016; Graham et al., 2017). Such changes often lead to degradation and/or modification of vegetative structures (Graham et al., 2017). Thus, pinesap is not only deprived of chlorophyll but it does not form aboveground vegetative organs. The reproductive axis bearing sterile bracts and inflorescence develops bypassing the vegetative stage, from adventitious buds in the pinesap mycorrhiza root system (Wallace, 1975; Merckx et al., 2013).

The development of photosynthesis is closely related to the evolution of the leaf, which changed from radially symmetric to asymmetric, thus increasing the insolation of its surface (Stewart, Rothwell, 1993; Cronk, 2001; Bowman et al., 2002; Beerling, Fleming, 2007). It is believed that the asymmetric leaf of seed plants originated in part due to the duplication and diversification of *YABBY* genes (Eckardt, 2010). The evolution of the ancestral *YABBY* gene produced a family of genes with different specializations, which could be associated with further transformations of the leaf and the emergence of other asymmetric organs that formed the flower (Mathews, Kramer, 2012).

The abaxial-adaxial asymmetry of all lateral organs is characteristic of most extant plants. One of the main factors determining the identity of the abaxial surface of organs is the family of YABBY transcription factors (Bowman et al., 2002; Bartholmes et al., 2012). In angiosperms, this family is divided into five subfamilies: three "vegetative" – YABBY1/YABBY3 (FILAMENTOUS FLOWER (FIL)), YABBY2/FASCIATED (FAS) and YABBY5, and two "reproductive" – CRABS CLAW (CRC) and INNER NO OUTER (INO) (Yamada et al., 2011; Bartholmes et al., 2012; Finet et al., 2016). "Reproductive" YABBYs have a narrow specialization, while "vegetative" YABBYs are involved in determining the polar development of vegetative and reproductive organs and are also important for proper organization and phyllotaxis of the shoot apical meristem (McConnell, Barton, 1998; Bartholmes et al., 2012). Thus, the "vegetative" *YABBY* genes preserve the expression profile of the ancestral gene, although they cannot completely replace the "reproductive" *YABBY*s (Yamada et al., 2011; Bartholmes et al., 2012).

The study of the *YABBY* genes of the complete mycoheterotroph *M. hypopitys* could clarify the possibility of preserving the ancestral functions by the "vegetative" *YABBY*s upon loss of the vegetative organs. The YABBY5 (*MhyYAB5*) and YABBY3/FIL (*MhyFIL1*, *MhyFIL2*, and *MhyFIL3*) genes with opposite expression patterns have been identified in pinesap (Shchennikova et al., 2018). In bracts, which are evolutionarily closer to leaves than to floral organs, only trace amounts of *MhyFIL2* transcripts are observed, and the expression levels of *MhyFIL1* and *MhyFIL3* are 5–10 times lower than that of *MhyYAB5* (Shchennikova et al., 2018). In the absence of leaves in pinesap, the reduced *MhyFIL1* and *MhyFIL3* expression in bracts suggests a loss of part of the "vegetative" *YABBY* function.

In this study, we perform a functional analysis of the vegetative *YABBY* genes, *MhyFIL1* and *MhyFIL3*, in leafless pinesap

M. hypopitys. The study of homologs of genes determining leaf asymmetry in higher plants in a complete mycoterotroph can expand the understanding of the evolution of the YABBY transcription factor family in the course of dramatic adaptive rearrangement of the plant.

Materials and methods

We invoked data from the transcriptome analysis of *M. hypopitys* roots, sterile bracts, and flowers (at the stage of anthesis) (Beletsky et al., 2016). To amplify and clone the coding sequence of the pinesap *YABBY* genes *MhyFIL1* and *MhyFIL3*, primers were designed on the basis of previously identified gene transcripts (Shchennikova et al., 2018): forward – 5'-catcatgtcctcctcaaattctt-3' (for both genes), and reverse – 5'-cttcttgattagtaggggacaca-3' (*MhyFIL1*) and 5'-cttcttgattagtaggggagacc-3' (*MhyFIL3*). Total RNA was isolated from pinesap flowers, where the expression of the *MhyFIL* genes was highest (the RNeasy Plant Mini Kit, QIAGEN, USA), and used for cDNA synthesis (Reverse Transcription System, Promega, USA). The complete coding sequences of the *MhyFIL1* and *MhyFIL3* genes were amplified at the following PCR conditions schedule: denaturation 95 °C 5 min; 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and elongation (72 °C, 1 min); extension (72 °C, 7 min). Amplificates of the expected size were purified (MinElute Gel Extraction Kit; QIAGEN, USA), cloned into the pGEM®-T Easy (Promega, Madison, WI, USA), and sequenced (Core Facility "Bioengineering", FRC "Fundamentals of Biotechnology" RAS). Sequence analysis of the fragments confirmed the cloning of the *MhyFIL1* and *MhyFIL3* cDNAs. The nucleotide and amino acid sequences were analyzed with the following software: Clone Manager 7.11 (http://clone-manager-professional.software.informer.com/), NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and MEME 5.0.1 (Bailey, Elkan, 1994). Alignment of sequences of genes and proteins encoded by them was performed using ClustalX (Larkin et al., 2007) and MEGA 6.0. (Tamura et al., 2013). For phylogenetic analysis, NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) and MEGA 6.0. (Tamura et al., 2013) were applied with tree generation by the maximum likelihood method based on the JTT model (Zuckerkandl, Pauling, 1965). To analyze the function of the MhyFIL1 and MhyFIL3 transcription factors, two types of transgenic *Nicotiana tabacum* plants with constitutive expression of the *MhyFIL1* and *MhyFIL3* genes were obtained. Two binary vectors were constructed based on the pBin19 plasmid, containing the cDNA (*MhyFIL1*/*MhyFIL3*) expression cassette in sense orientation under the control of the 35S CaMV promoter and NOS terminator. *Agrobacterium tumefaciens* AGL0 strains carrying the constructed plasmids were used for tobacco leaf disc transformation with further regeneration according to the previously described protocol (Goloveshkina et al., 2012). Regenerants were analyzed for the transgene presence in the genome by PCR with primers specific for the 3'-sequence of each gene (see above) and the 35S promoter (5'-caatcccactatccttcgcaagaccc-3'). The phenotypes of plants that gave a positive PCR signal were compared with the control (nontransgenic tobacco). The following parameters were assessed: the vegetation period (from planting in the greenhouse to budding), the structure of the aboveground vegetative part of the plant, and the phenotypes of the roots, leaves, and floral organs.

Results

Structural and phylogenetic analyses of MhyFIL proteins (Shchennikova et al., 2018) by NCBI-BLAST, NCBI-CDD and MEGA 6.0 confirm that MhyFILs belong to the YABBY3/FIL clade (Fig. 1). MhyFIL3 is closer to the ancestor than two other proteins, MhyFIL1 and MhyFIL2. As expected, the closest relatives of MhyFIL are representatives of the YABBY3/FIL clade in species of the Ericales order (basal Asterids), which include pinesap (see Fig. 1). Members of the YABBY3/FIL clade of other asterids form a sister subcluster (see Fig. 1). Inside the clade YABBY3/FIL, proteins of rosids (*Arabidopsis thaliana*) form a basal subcluster to analyzed asterid proteins (see Fig. 1). Analysis of putative conserved motifs (MEME 5.0.1) in the analyzed proteins reveals two sequences characteristic of all YABBY transcription factors and corresponding to the zinc finger and YABBY domains (Bartholmes et al., 2012). YABBY3/FIL proteins differ from members of other clades by the presence of six clade-specific (interdomain and C-terminal) motifs, and proteins of asterids, including Ericales, have an N-terminal motif, which is absent from *A. thaliana* YABBY3/FIL proteins (Rosids) (see Fig. 1). According to the conserved motif scheme obtained, all three MhyFILs are structurally closer to FIL than to YABBY3 (*A. thaliana*) (see Fig. 1).

For functional analysis of transcription factors MhyFIL1 and MhyFIL3, transgenic *N. tabacum* plants with individual constitutive expression of the cDNA of each of the *MhyFIL1* and *MhyFIL3* genes were obtained. Independent transgenic regenerants T_0 35S::*MhyFIL1* (3 plants) and 35S::*MhyFIL3* (12 plants), which rooted and formed true green leaves, were adapted to greenhouse conditions and then compared with the control (nontransgenic tobacco).

In contrast to the control, the obtained tobacco plants, 35S::*MhyFIL1* and 35S::*MhyFIL3*, developed the bushy structure (instead of a single stem), had a significantly longer vegetation (on average, 282 days vs. the control 48 days), and formed abaxially twisted leaves (with an altered identity of the adaxial surface), and a strongly thickened and shortened root with abnormal leaf-like outgrowths (instead of an extensive root system) (Fig. 2).

Reproductive axes that developed on one of the shoots of the bushy 35S::*MhyFIL1* and 35S::*MhyFIL3* plants produced flowers outwardly similar to wild flowers, but often with rotting/brittle pedicles. Seeds were obtained from only six 35S::*MhyFIL3* and two 35S::*MhyFIL1* plants. In the T_1 generation, changes in plant morphology increased. Only one bushy plant 35S::*MhyFIL3* formed a wild-type shoot that blossomed and gave seeds. The obtained seeds germinated, but the seedlings were characterized by abnormal development of roots (severe shortening and arrest in development) and shoot meristems (maximum shoot height 1.5–3.0 cm, bushiness, early development stop), which led to the death of the seedlings. In this regard, further analysis of the transgenic phenotype was impossible.

A microscopic analysis of the leaf surface of transgenic plants in comparison with the control confirmed that the cell identity on the adaxial side was partially changed as a

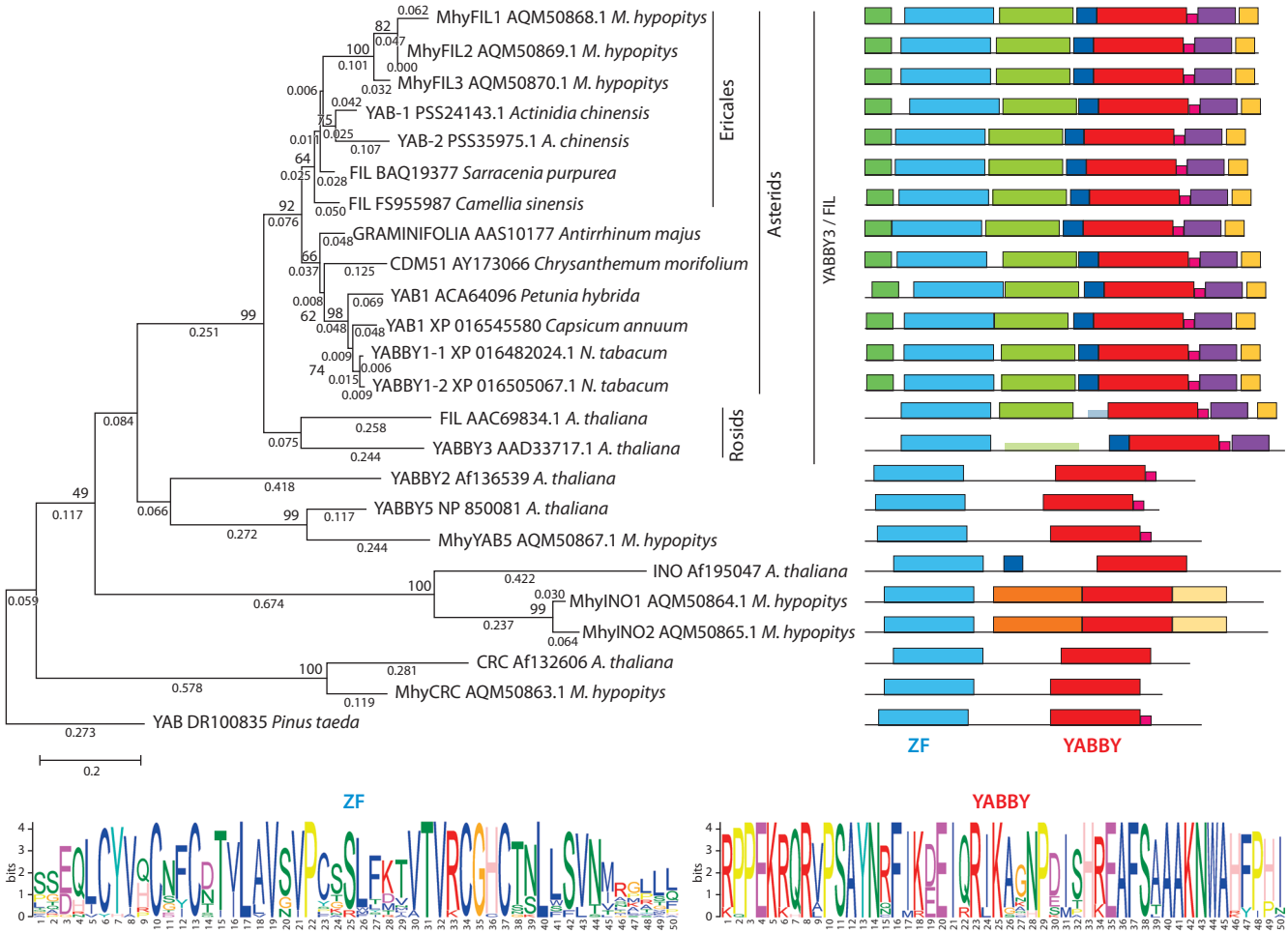

Fig. 1. Phylogenetic tree based on the alignment of 24 amino acid sequences of the YABBY transcription factors of pinesap and other plant species. Analysis was performed in MEGA 6.0 using the maximum likelihood method based on the JTT model (Tamura et al., 2013).

The *Pinus taeda* YAB sequence was used as an outgroup. The lengths of the branches estimated as the genetic distance (the number of substitutions per site), and the essential bootstrap values for 1000 replicates are shown at the base of the branches. The NCBI accession numbers are given against the names of proteins. To the right of the dendrogram – a scheme of conserved motifs of the analyzed proteins obtained as a result of the MEME 5.0.1 (http://meme-suite.org/tools/meme) analysis is represented. Below are the sequences of two motifs corresponding to the zinc finger (ZF) and YABBY domains.

result of heterologous transgene expression. There appeared stomata-like structures, which normally should not be on the upper surface of the leaf. Probably, they were the cause of leaf twisting.

Discussion

It is believed that mycoheterotrophic plants adaptively evolved from photosynthetic mycorrhiza lines, and the growth of such plants at poor insolation led to the inactivation and loss of the photosynthesis apparatus (Bidartondo, 2005; Buchanan-Wollaston et al., 2005; Zhang, Zhou, 2013; Ravin et al., 2016). In pinesap *M. hypopitys*, this was probably the cause of the subsequent disappearance of the unnecessary aboveground vegetative structures, including leaves (Wallace et al., 1975; Merckx et al., 2013). The achlorophyllous pinesap reproductive axis is often mistaken for a stem with leaves. However, the presence of MADS-box gene transcripts homologous to *APETALA3*, *TM6* and *SEPALLATA3* in sterile bracts ("leaves"), whereas in higher plants these genes are expressed only in flowers, is one of the signatures of the reproductive nature of the *M. hypopitys* aerial part (Shulga et al., 2018).

The origin of asymmetric leaves and their further transformations, including the emergence of asymmetric flowering organs, as mentioned above, are associated, in part, with the evolutionary duplication and diversification of plant-specific *YABBY* genes (Eckardt, 2010; Mathews, Kramer, 2012). The structure and function of these genes are described in detail in a photosynthetic plants, model and other species (Bowman, 2000; Bowman et al., 2002; Finet et al., 2016; Strable et al., 2017). In complete mycoheterotrophs, *YABBY* genes are also present and transcribed (Shchennikova et al., 2018). It is not clear, however, whether the functions of the vegetative *YABBY* genes are preserved in these leafless plants.

In this study, we investigated possible functions of two "vegetative" *YABBY* genes of pinesap, *MhyFIL1* and *MhyFIL3*, by obtaining and characterizing two types of transgenic tobacco plants with overexpression of each of the analyzed genes.

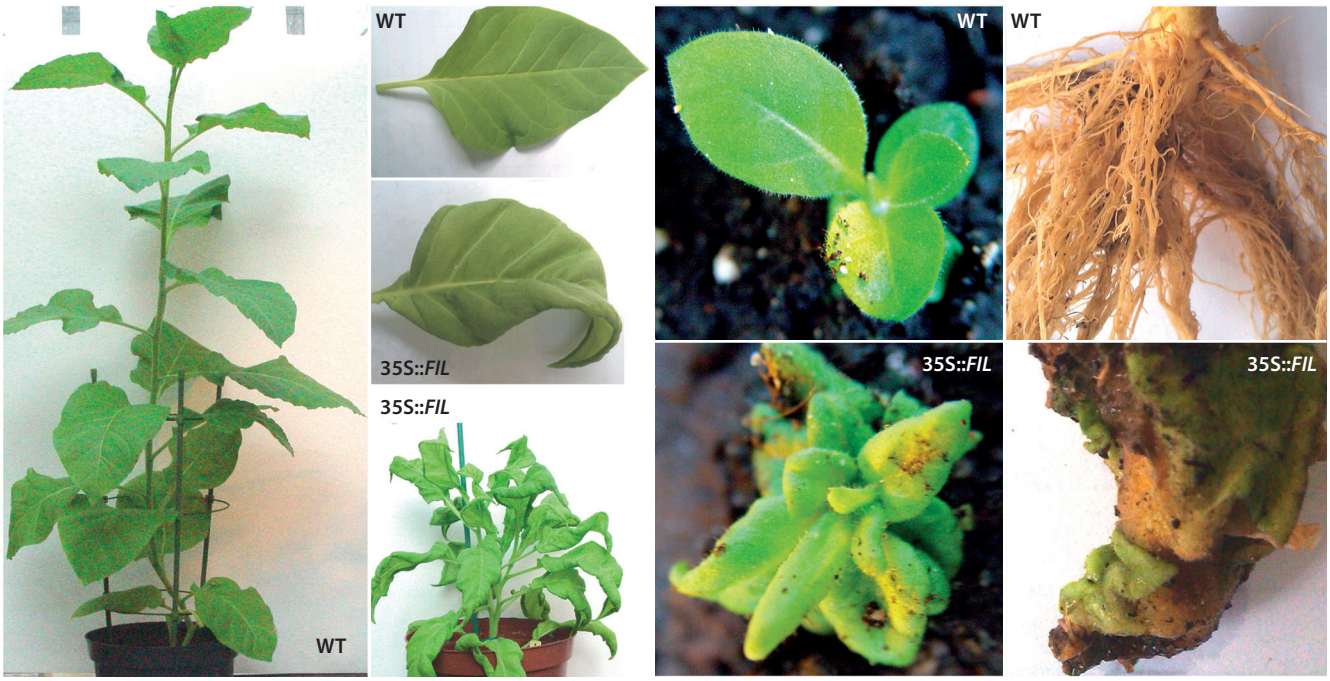

Fig. 2. Transgenic tobacco plants 35S::*FIL1/3* (indicated as 35S::*FIL*) in comparison with the control nontransgenic plant *N. tabacum* (WT).

It is known that the simultaneous knockout of all "vegetative" *YABBY* genes in an *A. thaliana* plant leads to the formation of narrow twisted or radially symmetric leaves, since all leaf cells become adaxial (Stahle et al., 2009). Theoretically, in case of overexpression of such genes, the formation of radially symmetric leaves should also be expected, the only difference being their abaxial identity. The observed narrowing and curling of leaves in 35S::*MhyFIL1/3* plants confirm this assumption.

Interestingly, the effects described above also occurred with the heterologous overexpression of the *FIL* genes *BraYAB1-702* (*Brassica rapa*) and *TaYAB1* (*Triticum aestivum*) in transgenic *A. thaliana* plants (Zhao et al., 2006; Zhang et al., 2013). Both species (*B. rapa* and *T. aestivum*) are photosynthetic autotrophs; therefore, the similarity of the effect of constitutive expression of the *BraYAB1-702* and *TaYAB1* genes in *A. thaliana* with the effect of the overexpression of *MhyFIL1/3* in transgenic tobacco plants indicates the preservation of the ancestral role of the *FIL* genes in determining the identity of cells of the abaxial leaf surface.

It is also known that the correct morphogenesis of the meristem depends on the correct activity of the *FIL* genes (Bartholmes et al., 2012). For instance, *A. thaliana* with a double mutation, *fil yab3*, among other defects, demonstrates aberrant phyllotaxis (Goldshmidt et al., 2008). It is shown that transcription factor FIL nonautonomously and consistently affects the phyllotaxis and growth of lateral organs, coordinating the expression of markers (WUSCHEL, CLAVATA3 (CLV3)) of the central zone of the shoot apical meristem (Goldshmidt et al., 2008). The ectopic expression of *SrGRAM* (*FIL*-like gene in *Streptocarpus rexii*) completely suppressed the development of the *A. thaliana* shoot meristem (Tononi et al., 2010). The disturbance of the aboveground architecture of the 35S::*MhyFIL1/3* transgenic plants and the resulting protracted vegetation may thus be caused by aberrant phyllotaxis up to the arrest of the shoot apical meristem development caused by ectopic *MhyFIL1/3* overexpression.

It is worth highlighting the dramatic changes in the root structure of 35S::*MhyFIL1/3* plants. In previously published papers, there was no information about what happens to the roots of such plants. The researchers may have omitted this aspect, since normally *YABBY* genes are expressed only in leaves and flowers, and therefore their functions are associated exclusively with these organs (Siegfried et al., 1999; Sarojam et al., 2010). Indeed, various combinations of *yabby*-mutations in *A. thaliana* do not affect root development (Boter et al., 2015). It is known that the apical meristems of the root and shoot are supported in a similar way, and *CLV3* and *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*) genes are markers of the quiescent center of the root meristem (Fiers et al., 2005; Stahl et al., 2009; Chu et al., 2013). Hence, it is reasonable to assume that the root phenotype in 35S::*MhyFIL1/3* plants is a result of suppression of the apical root meristem development due to the interference of the MhyFIL1/3 transcription factor in the regulation of the expression of *N. tabacum* genes homologous to *CLV3* and *WOX5*.

Conclusion

The obtained results may indicate that, despite the absence of aboveground vegetative organs from pinesap, the function of the *MhyFIL1/3* genes as "vegetative" *YABBY* genes is preserved. In *M. hypopitys*, transcription factors FIL1 and FIL3 still determine the asymmetric development of the lateral organs of the plant aerial part, which follows from the normal structure of pinesap floral organs, as well as the characteristics of the influence of heterologous *MhyFIL1/3* gene expression on the development of tobacco, in particular, its leaves. Thus, the activity of the *MhyFIL1/3* genes is not

directly related to the loss of the pinesap ability to produce leaves during the evolutionary transition from autotrophic nutrition to heterotrophy.

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Acknowledgements. This work was financially supported by the Russian Science Foundation, project No. 14-24-00175, and the Ministry of Science and Higher Education of the Russian Federation (A.V. Nezhdanova, K.S. Gavrilova), and was performed using the experimental climate control facility in the Institute of Bioengineering, Research Center of Biotechnology, Russian Academy of Sciences.

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

Received September 4, 2018. Revised November 30, 2018. Accepted December 5, 2018.

Вавиловский журнал генетики и селекции. 2019;23(4):412-421
DOI 10.18699/VJ19.510

The sporophytic type of fertility restoration in the A_3 CMS-inducing cytoplasm of sorghum and its modification by plant water availability conditions

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The A_3 type of CMS in sorghum is one of the most difficult to restore fertility because of the low frequency of fertility-restoring genes among sorghum accessions, the complex mechanism of fertility restoration that occurs with the complementary interaction of two gametophytic genes *Rf3* and *Rf4*, and the sensitivity of their expression to air and soil drought. In order to test the hypothesis of the sporophytic type of fertility restoration in CMS lines with A_3 type cytoplasm developed in our laboratory, we analyzed segregation in the self-pollinated progeny of fertile F_1 hybrids grown under different water availability conditions (in a dryland plot, in plots with additional irrigation, in a growth chamber, and in an experimental field with a natural precipitation regime) and in their backcrosses to the maternal CMS-line. The presence of sterile plants in the F_2 and BC_1 families with the maternal CMS line grown in all tested water availability conditions argues for the sporophytic mechanism of fertility restoration. Cytological analysis of fertile F_1 hybrids revealed a significant amount of degenerating pollen grains (PGs) with impaired starch accumulation and detachment of the PG contents from the cell wall. It is assumed that the expression of the fertility-restoring genes *Rf3* and *Rf4* in the hybrids with studied CMS lines starts already in the sporophyte tissues, normalizing the development of a certain part of the PGs carrying the recessive alleles of these genes (*rf3* and *rf4*), which are involved in fertilization and give rise to sterile genotypes found in F_2 and BC_1 families. For the first time, the transgenerational effect of water availability conditions of growing a fertility-restoring line on male fertility of the F_2 generation was detected: a pollinator grown in a plot with additional irrigation produced more fertile and less sterile individuals compared to the same pollinator grown under a rainfall shelter ($p < 0.01$), and the segregation pattern changed from digenic to monogenic, indicating heritable inhibition of the expression of one of the fertility-restoring genes (kind of "grandfather effect"). The possibility of selection for the stability of the fertility restoration system of the A_3 cytoplasm to functioning under conditions of high vapor pressure deficit during the flowering period was shown. These data may contribute to the creation of effective fertility restoring lines for this type of CMS in sorghum.

Key words: *Sorghum bicolor* (L.) Moench; cytoplasmic male sterility; A_3 cytoplasm; fertility-restoring genes; epigenetics; transgenerational inheritance; drought; vapor pressure deficit.

For citation: Elkonin L.A., Kozhemyakin V.V., Tsvetova M.I. The sporophytic type of fertility restoration in the A_3 CMS-inducing cytoplasm of sorghum and its modification by plant water availability conditions. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):412-421. DOI 10.18699/VJ19.510

Спорофитный тип восстановления фертильности в ЦМС-индуцирующей цитоплазме сорго типа A_3 и его модификация условиями влагообеспеченности растений

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A_3-тип ЦМС сорго – один из самых трудных для восстановления фертильности вследствие низкой частоты встречаемости генов-восстановителей, сложного механизма восстановления фертильности, происходящего при комплементарном взаимодействии двух гаметофитных генов, *Rf3* и *Rf4*, чувствительности их экспрессии к воздушной и почвенной засухе. С целью проверки гипотезы о спорофитном типе восстановления фертильности у созданных нами ЦМС-линий на основе цитоплазмы типа A_3 анализировали расщепление в самоопыленном потомстве фертильных гибридов F_1, выращенных при разных режимах влагообеспеченности (на делянках с засушливым фоном, с влагообеспеченным фоном, в климатической камере и на опытном поле при естественном режиме влагообеспеченности). Присутствие стерильных растений в семьях F_2 и BC_1 с материнской ЦМС-линией, выращенных при всех испытанных режимах влагообеспеченности, свидетельствует в пользу спорофитного механизма восстановления фертильности. Цитологический анализ фертильных ги-

бридов F_1 выявил значительное число дегенерирующих пыльцевых зерен (ПЗ) с нарушением накопления крахмала, отрывом содержимого ПЗ от клеточной стенки. Предполагается, что у гибридов с изученными ЦМС-линиями гены-восстановители *Rf3* и *Rf4* начинают функционировать уже в тканях спорофита, нормализуя развитие некоторой части ПЗ, несущих рецессивные аллели генов *rf3* и *rf4*, которые участвуют в оплодотворении и дают начало стерильным генотипам в семьях F_2 и в BC_1. Впервые обнаружено трансгенерационное влияние условий влагообеспеченности растений линии-восстановителя на характер расщепления по мужской фертильности в поколении F_2: опылитель, выращенный в грядке с дополнительным поливом, давал больше фертильных и меньше стерильных индивидуумов по сравнению с опылителем, выращенным в «засушнике» ($p < 0.01$). При этом характер расщепления изменялся с дигенного на моногенный, свидетельствуя о наследуемом ингибировании экспрессии одного из генов-восстановителей (своеобразный «эффект дедушки»). Показана возможность отбора на устойчивость системы восстановления фертильности в цитоплазме A_3 к функционированию в условиях дефицита влажности воздуха в период цветения, что может способствовать созданию новых восстановителей фертильности этого типа ЦМС.
Ключевые слова: *Sorghum bicolor* (L.) Moench; цитоплазматическая мужская стерильность; цитоплазма A_3; гены-восстановители фертильности; эпигенетика; трансгенерационное наследование; засуха; дефицит влажности воздуха.

Introduction

The development of the reproductive structures is the stage of plant ontogeny most sensitive to environmental stresses. One of the causes for this sensitivity is the high energy intensity in microspore- and gametogenesis (Dolferus et al., 2013). It is known that the energy demand of plant is covered by the mitochondriome of the plant cell. Besides, mitochondria are the primary targets of environmental stresses, which disrupt their functioning and information exchange between the mitochondrial and nuclear genomes (Atkin, Macherel, 2009; Jacoby et al., 2012; Ng et al., 2014; Liberatore et al., 2016). This fact is of particular importance for hybrids with cytoplasmic male sterility (CMS), resulting from remote hybridization, in which the information exchange between the nuclear and mitochondrial genome, established during coevolution, is disturbed (Touzet, Meyer, 2014) and the resistance to environmental stresses is debilitated (Li et al., 2012).

Among the different types of sterile cytoplasm found in sorghum (Reddy et al., 2005), A_3 cytoplasm, the source of which is IS1112C accession, is one of the most difficult to restore fertility. This difficulty arises from the low frequency of fertility-restoring genes among sorghum accessions (Worstell et al., 1984; Torres-Cardona et al., 1990; Dahlberg, Madera-Torres, 1997) and the sensitivity of their expression to air and soil drought (Kozhemyakin et al., 2017). Genetic analysis of fertility restoration in hybrid combinations between CMS lines with A_3 cytoplasm of the Texas Agricultural Experimental Station (USA) and IS1112C indicated the functioning of a digenic gametophytic fertility restoration system, in which the complementary interaction of two genes (*Rf3* and *Rf4*) is necessary for the formation of viable pollen (Tang et al., 1998; Pring et al., 1999). F_1 hybrids with restored male fertility, heterozygous for the *Rf3* and *Rf4* genes, yield 25 % of the fertile pollen that reduces the seed set and limits the practical use of A_3 cytoplasm in sorghum breeding, compared to other types of sterile cytoplasms (A_1 and A_2), providing the 100 % seed set in F_1 hybrids.

Later, a sporophytic fertility restoration system for the A_3 CMS type was found in Sudan grass accessions (Tang et al., 2007). It is known that gametophytic and sporophytic fertility restoration systems have fundamental differences (Chase, Gabay-Laughnan, 2004). In a sporophytic fertility restoration system, a fertility-restoring gene, functioning in the anther tissues (sporophyte), prevents degeneration of pollen grains (PGs) after meiosis, which is typical of most types of CMS (Kaul, 1988). As a result, hybrids that are heterozygous for fertility-restoring genes (*Rf*/*rf*) produce PGs carrying the fertility-restoring allele (*Rf*) and PGs carrying the recessive allele (*rf*), unable to restore fertility. Therefore, sterile plants are present in the self-pollinated progeny of such hybrids (F_2), as well as in backcrosses with the maternal CMS line (*rf*/*rf*). With the gametophytic fertility restoration system, the fertility-restoring gene functions in male gametophytes (developing PGs). As a result, hybrids that are heterozygous for fertility-restoring genes (*Rf*/*rf*) contain both fertile PGs, carrying the *Rf* allele, and sterile PGs, which degenerate because of the lack of the restoring allele. Therefore, sterile plants are absent from the self-pollinated offspring of such hybrids, as well as from their backcrosses with the maternal CMS line, and only plants with full restoration of male fertility (*Rf*/*Rf*) and semi-sterile individuals (*Rf*/*rf*), arising from the transmission of the *rf* allele through egg cells, are present.

We showed in our previous study that severe vapor pressure deficit during flowering inversely correlated to the level of fertility of F_1 hybrids in the A_3 cytoplasm; also, the cultivation of hybrid populations with artificial irrigation favored the selection of lines restoring fertility for this type of CMS (Kozhemyakin et al., 2017). The presence of a significant number of sterile plants in F_2 families formally supported the sporophytic type of male fertility restoration in F_1 hybrids, but careful analysis showed that sterile plants flowered on dates with severe vapor pressure deficit, which could be the cause of their sterile phenotype.

The purpose of the experiments presented in this paper was to test the hypotheses of the sporophytic type of fertility restoration of CMS A_3 by the *Rf3* and *Rf4* genes, known as gametophytic fertility-restoring genes, and of the transgeneration effect of plant water availability conditions on the expression of male fertility in the offspring of F_1 hybrids. In addition, this paper presents the first data on the efficiency of selection for the sustainable functioning of the male fertility restoration system in the A_3 cytoplasm under conditions of air drought during the flowering phase.

Materials and methods

Experiments were conducted with F_1–F_3 hybrid populations obtained by crossing the CMS lines A_3 KP-70 and A_3 Topaz, created earlier by us, to the A_3-type CMS fertility restorer KVV-96. These CMS lines were developed using a nonrecurrent backcrossing program with A_3 Tx398 as the nonrecurrent parent. The genotype of A_3 Tx398 is *rf3rf3rf4rf4* (Tang et al., 1998). We created the KVV-96 line by selection of fertile plants from the hybrid combination A_3 Karlikovoe beloe × IS1112C (Kozhemyakin et al., 2017). The IS1112C line has the A_3 type of cytoplasm and is a donor of the *Rf3* and *Rf4* genes (Pring et al., 1999; Kulhman et al., 2006).

The CMS lines, fertility restorers, F_1, F_2, and testcross hybrids were grown in experimental fields of the Agricultural Research Institute for the South-East Region (Saratov, Russia) (51° 32' N, 46° 02' E) in 2016–2018. Sowing was made in the last third of May. Plants were grown in 4-meter rows with row spacing 70 cm and a distance between plants 12–15 cm. Standard agricultural techniques for grain sorghum growing were applied.

Agricultural climatic conditions of vegetation periods varied from year to year (Table 1). Year 2016 was characterized by dry conditions: the average daily temperatures in each calendar month during the growing season (June-August) were 1.6–4.8 °C higher than the average annual value, and the amount of precipitation was three times less (hydrothermal coefficient 0.02). Year 2017 was characterized by a significant excess of precipitation in the first half of the growing season (from germination to flowering), while in June, during the booting phase, the average daily temperature was lower than June temperatures averaged over years. In 2018, the first half of the vegetation, before flowering, proceeded under dry conditions, whereas in the second half of the growing season, from mid-July (during the flowering period), a significant amount of precipitation was recorded.

To study the effect of plant water availability on the expression of male fertility and the inheritance of ability for fertility restoration, plots with different irrigation regimes were used: with additional watering (two times per week, 7–8 L/m^2, starting from the booting phase to the end of flowering) and without watering (dryland environment). To obtain a dryland environment, the plot was covered with a translucent polycarbonate rainfall shelter before the beginning of the booting phase.

The F_1 hybrids used in this study were obtained by individual crosses of plants of CMS line A_3 KP-70 grown at natural water availability to KVV-96 plants grown in dryland and irrigated plots (two pollinators from each environment). The F_1 hybrids obtained from individual crosses were grown in 2016–2018 in dryland and irrigated plots (four hybrid combinations in each environment). The offspring of four paternal plants were also grown in such plots as a control.

In 2017–2018, F_2 families obtained from individual self-pollinated F_1 hybrids from different selective environments and BC_1 obtained from crossing these F_1 hybrids to the CMS line A_3 KP-70 were grown in the experimental field with the natural plant water availability regime. Some F_2 families were also grown in irrigated plots. One of the hybrid populations of F_2 (A_3 Topaz/KVV-96) was grown in an LGC-4203 growth chamber (Daihan, Korea) at the 14L : 10D light/dark schedule, 70 % relative humidity, and temperatures 28 ºC in the daytime/24 ºC at night.

To determine the level of male fertility, all plants were isolated with parchment bags before flowering. Depending on the level of seed set, plants were classified as sterile (s) (0 % seed set), partially sterile (ps) (< 40 %; most often, 10–20 %), fertile (f) (> 40 %; most often, 80–100 %).

For the cytological analysis of pollen, branches from different parts of panicles of the F_1 hybrids and the offspring of paternal plants were fixed in acetoalcohol (1 : 3), washed twice, and stored in 75 % alcohol at 4–6 °C. Slides were prepared from a mixture of anthers isolated from 10–15 branches of individual plants. Pollen was stained with 1 % iodine solution in potassium iodide. In each plant, pollen from hermaphrodite and male flowers was analyzed separately. In each slide, 100 PGs were counted in four replicates. In each selective environment, 15–17 F_1 plants and the same numbers of individuals from the offspring of paternal plants were studied.

Indicators of vapor pressure deficit (VPD) during the flowering phase were provided by the Laboratory of Hydrometeorology of the Agricultural Research Institute for the South-East Region. For each plant an individual indicator was calculated, which was the average of indicators within five days before the flowering of the top of the panicle and five days after the beginning of flowering.

Statistical analysis of segregations in the F_2 and BC_1 populations was performed using the χ^2 test with Yates' correction and the exact binomial test (McDonald, 2014), checking the

Table 1. Agroclimatic conditions during the experiment[1]

Month	Indices											
	Average daily air temperature, °C				Average daily relative humidity, %				Precipitation, mm			
	Perennial	2016	2017	2018	Perennial	2016	2017	2018	Average perennial	2016	2017	2018
May	15.0	16.1	14.0	18.3	52	62.9	57.5	50.0	43	77.2	99.3	28.1
June	19.4	21.0	18.0	19.9	54	52.8	60.7	46.9	45	9.0	66.7	14.1
July	21.4	23.6	21.8	22.8	56	53.9	62.1	60.2	51	28.8	51.3	88.7
August	19.9	24.7	22.8	23.7	58	48.6	58.0	51.2	44	8.3	9.1	4.4

[1] According to the Laboratory of Hydrometeorology of the Agricultural Research Institute for South-East Region (Saratov, Russia).

hypotheses of mono- and digenic control. The significance of differences in the level of fertility in different experimental variants was assessed by variance analysis using the AGROS software package, version 2.09 (S.P. Martynov, Institute of General Genetics, RAS). The proportions of fertile and sterile plants in different progenies were also compared by Fisher's tests (Zaitsev, 1984).

Results

Effect of drought stress on restoration of fertility in the F_1 hybrids. Analysis of seed sets in F_1 ♀ A_3 KP-70 × ♂ KVV-96 hybrids grown under different water availability conditions shows that the KVV-96 line can restore the fertility of A_3 type CMS (Table 2). However, the proportion of fertile plants among F_1 hybrids decreases significantly in dryland plots, and the proportion of partially sterile plants increases as compared to irrigated plots. These data indicate that plant water availability conditions regulate the expression of the fertility-restoring genes of the KVV-96 line, and this fact should be taken into consideration when analyzing segregation in the F_2 and BC_1 families.

Table 2. Characterization of male fertility of F_1 sorghum hybrids ♀A_3 KP-70 × ♂KVV-96 grown under different plant water availability regimes (2016)

Water availability conditions	Percent of plants with fertility level (%)[a]		
	f	ps	s
Irrigated plots	98.0 a	1.3	0.7
Dryland plots	76.9 b	16.9	6.2
F	36.05**	8.80	4.18

Note: [a] – average over four replications; f – fertile (seed set > 40 %, usually 80–100 %); ps – partially sterile (< 40 %, usually, 10–20 %); s – sterile (0 % seed set); Data within the columns marked with different letters differ significantly at $p < 0.05$; **$p < 0.01$.

Sporophytic type of male fertility restoration in CMS A_3. In accordance with the hypothesis of the gametophytic nature of male fertility restoration in the CMS-inducing cytoplasm A_3 by two fertility-restoring genes (Tang et al., 1998; Pring et al., 1999), F_1 hybrids obtained by crossing CMS lines (*rf3rf3rf4rf4*) with the line IS1112c (*Rf3Rf3Rf4Rf4*), have the genotype *Rf3rf3Rf4rf4*. As a result of meiosis, four classes of gametes are produced in such hybrids (*Rf3Rf4*, *Rf3rf4*, *rf3Rf4*, *rf3rf4*), while only PGs with the genotype *Rf3Rf4* turn out to be fertile and PGs with other genotypes degenerate. In this regard, only plants with restored male fertility should be present in the F_2 generation, since only pollen grains carrying both fertility-restoring genes are involved in fertilization. Similarly, only plants with restored male fertility (*Rf3rf3Rf4rf4*) should be expected in the backcross of F_1 hybrids to the maternal CMS line.

However, in our experiments in the F_2 families obtained by crossings A_3-CMS lines to the KVV-96 line, created on the basis of the IS1112C line, grown in different environmental conditions (in irrigated plots, in field plots with the natural water availability regime, and in the growth chamber), male-sterile plants were regularly observed. In this regard, data from the irrigated plots and the growth chamber with high relative humidity (70 %) are the most impressive, because in such conditions the drought effects, which prevent the restoration of fertility in A_3 type CMS (Kozhemyakin et al., 2017), were absent.

In 2016 (see Table 3), in the irrigated plot, the ratio of plants with restored fertility (f+ps) and sterile plants (s) corresponded to digenic segregation 15 : 1, or 12f : 3ps : 1s, when considering partly sterile plants a separate class. Such segregation testified that the restoration of fertility in the conditions of water abundance is controlled by two fertility-restoring genes. When growing the same F_2 family in a dryland plot, the ratio of plants with fully or partially restored fertility and sterile plants differed from the digenic segregation, but corresponded to the monogenic segregation 3 : 1 (Table 3), which indicated inhibition of the expression of one of the fertility-restoring genes in drought conditions. The causes of the segregation 3 : 1 in the growth chamber are uncertain. Perhaps, with a larger sample size, the segregation pattern in the growth chamber would have been different.

In the 2017 season, the segregation in the progeny of F_1 hybrids ♀A_3 KP-70 × ♀KVV-96 was studied, while the size of the F_2 populations grown in the irrigated plot was increased. Again, as in the progeny of F_1 ♂A_3 Topaz × ♂KVV-96 hybrids, sterile plants were present in the F_2 families, which favored the sporophytic type of fertility restoration. The ratio of fertile, partially sterile, and sterile individuals corresponded to the digenic segregation 12 : 3 : 1 (Table 4). It is noteworthy that sterile plants from the F_2 families, transferred from the plots with additional irrigation to the growth chamber with 70 % relative humidity, formed male-sterile panicles. This fact testified that the male sterility of such plants was caused by their genotype rather than environmental factors (severe vapor pressure deficit) at flowering. However, sterile plants were absent from the BC_1F_1 families obtained by pollinating the maternal CMS-line with the pollen of F_1 hybrids (see Table 4).

The causes of the discrepancy between the segregations in F_2 and BC_1 are not clear. They may be related to differences in the functioning of fertility restoration systems in BC_1 and F_2 or with gamete selection in F_1 plants, since the progeny of panicles from the main stem was used to obtain F_2, whereas the pollen from the second and third panicles was used to obtain BC_1. Also, the average daily vapor pressure deficit during the flowering of the main panicles was 24.9 hPa, whereas during the flowering of the second and third panicles, 15.8 hPa. It is possible that the lack of air humidity was a factor affecting the functioning of gametophytic fertility-restoring genes, and thus contributing to the selection of certain classes of pollen grains.

In 2018, F_2 progeny obtained from F_1 hybrids grown in the dryland and high water availability environments was analyzed. The studied progenies were grown concomitantly under conditions of the experimental field and irrigated plots. All the analyzed families included significant numbers of sterile and semi-sterile plants (Table 5); however, when analyzing the observed segregations, only those sterile plants that bloomed on the same dates as the fertile plants were taken into account, because their sterility was caused by the genotype but not by the severe vapor pressure deficit.

Table 3. Segregation of the hybrid ♀A_3Topaz × ♂KVV-96 in the F_2 generation under different plant water availability conditions (2016)

Water availability conditions	Number of plants			Ratio	χ^2	*p*
	f	ps	s			
Irrigated plot	35	9	4	15(f+ps):1s	0.154	0.694
				12f:3ps:1s	0.157	0.924
Dryland plot	27	18	18	3(f+ps):1s	0.161	0.688
Growth chamber	11	9	4	3(f+ps):1s	0.505	0.477

Table 4. Segregation for male fertility in the F_2 generation of the ♀A_3KP-70 × ♂KVV-96 hybrids and in BC_1F_1 with the maternal CMS line grown in irrigated plots (2017)

Hybrid combination	Number of plants			Ratio	χ^2	*p*
	f	ps	s			
F_2 (♀A_3 KP-70 × ♂KVV-96-1)	71	10	4	12f:3ps:1s	1.859	0.395
F_2 (♀A_3 KP-70 × ♂KVV-96-5)	74	21	3	12f:3ps:1s	1.231	0.540
BC_1 [♀A_3 KP-70 × (♀A_3 KP-70 × ♂KVV-96-1)]	7	23	1[b]			
BC_1 [♀A_3 KP-70 × (♀A_3 KP-70 × ♂KVV-96-5)]	6	26	3[c]			

Note: [a] – average indicators of vapor pressure deficit (VPD) at flowering: average daily VPD = 15.1 hPa; maximum VPD = 31.2 hPa; [b] – when transferred to the growth chamber, panicles with 30 % seed set developed; [c] – 1 % seed set.

In the F_2 families grown in the irrigated plots, the ratio of fertile, partially sterile, and sterile plants corresponded to the digenic segregation 9f : 6ps : 1s. It is plausible that under conditions of 2018, only those plants whose genotype contained both fertility-restoring genes (*Rf3–Rf4–*) were fertile, whereas plants whose genotype contained only one dominant gene (*Rf3* or *Rf4*), had the partially sterile phenotype. In BC_1, the ratio of plants with partial restoration of fertility and sterile plants corresponded to the segregation 3ps : 1s (see Table 5), which should have been observed when there are four classes of male gametes (*Rf3Rf4*; *rf3Rf4*; *Rf3rf4*; *rf3rf4*) in diheterozygous paternal plants (F_1 hybrids) crossed to plants of the CMS line (*rf3rf3rf4rf4*). These data confirm the sporophytic type of fertility restoration in F_1 hybrids.

It is noteworthy that in the F_2 families derived from the F_1 hybrids obtained with the participation of the paternal parents grown in the dryland environment (KVV-96-♂1dr), the segregation pattern in the field conditions deviated from the digenic segregation (9 : 6 : 1) and corresponded to the monogenic segregation 3 : 1, indicating that one of the fertility-restoring genes of the paternal parent did not function in these families (see Table 5).

A detailed analysis of the influence of growing conditions of fertility-restoring lines and F_1 hybrids on the expression of male fertility in the F_2 generation showed that water availability conditions of F_1 hybrids did not affect the segregation pattern in F_2. However, the conditions of growing of the fertility-restoring line (donor of the *Rf* genes) influenced the segregation pattern in the progeny of F_1 hybrids (see Table 5). The proportion of sterile plants in the F_2 families derived from the donor of the *Rf*-genes from a dryland environment (KVV-96-♂1dr) was significantly higher ($p < 0.01$), and the proportion of fertile plants was lower ($p < 0.01$) than in the F_2 families derived from the F_1 hybrids obtained by using the *Rf* donor grown under high water availability conditions (KVV-96-♂2ir). As a result, the F_2 families derived from the donor of the *Rf* genes from the high water availability conditions showed the digenic segregation 9 : 6 : 1, whereas in F_2 families derived from the *Rf* donor from the dryland environment (KVV-96-♂1dr), the proportion of sterile plants was significantly higher ($p < 0.01$), and of fertile plants, lower ($p < 0.01$) than with sterile individuals, and the segregation was monogenic (3 : 1). This result presumably indicates that the inhibition of one of the *Rf* genes in the fertility restorer grown under a rainfall shelter had a transgenerational effect. This effect was inherited over two generations. However, when growing the F_2 family derived from the F_1 hybrid obtained with the *Rf* donor from the dryland plot (KVV-96-1dr) in the irrigated plot (see Table 5, first row), the segregation corresponded to the ratio 9 : 6 : 1. This fact testifies to the reversibility of drought-induced inhibition of the fertility-restoring gene under a high level of plant water availability. In addition, this fact provides an example of the transgenerational effect of growing conditions of the donor of *Rf* genes on the expression pattern of these genes in the F_2 generation.

Cytological analysis of pollen of F_1 hybrids. Assuming that with the sporophytic type of fertility restoration, pollen in F_1 hybrids contains mostly fertile PGs, whereas under gametophytic fertility restoration, segregation for different types of PGs (fertile:sterile) takes place in the anthers of F_1 hybrids, we performed a cytological analysis of the pollen of F_1 ♀A3 KP-70 × ♂KVV-96. We noted a significant number of sterile PGs in anthers of fertile hybrids; the proportion of fertile PGs with no signs of degeneration was low (3–7 %),

Table 5. Characterization of the male fertility of hybrid combinations in the A_3 cytoplasm (2018)[a]

Hybrid combination	Gene-ration	Water availability conditions[b]			Number of plants			Ratio	χ^2	*p*
		♂	F_1	F_2 (BC_1)	f	ps	s			
♀A_3 KP-70 × ♂ KVV-96-1 dr	F_2	dr	dr	ir	42	25	7	9f:6ps:1s	0.943	0.624
♀A_3 KP-70 × ♂ KVV-96-2 ir	F_2	ir	ir	ir	44	27	2	9f:6ps:1s	–	0.707
♀A_3 KP-70 × ♂ (A_3 KP-70/KVV-96-2 ir)	BC_1	ir	ir	ir	–	23	8	3ps:1s	0.011	0.916
♀A_3 KP-70 × ♂ KVV-96-1 dr	F_2	dr	ir	Field	29 (42.0 %)	23	17 (24.6 %)	9f:6ps:1s	40.13	0.0
								3(f+ps):1s	0.005	0.944
♀A_3 KP-70 × ♂ KVV-96-2 ir	F_2	ir	ir	Field	51 (54.3 %)	34	9 (9.6 %)*	9ϕ:6ps:1s	1.225	0.542
♀A_3 KP-70 × ♂ KVV-96-1 dr	F_2	dr	dr	Field	18 (26.5 %)	35	15 (22.1 %)	9f:6ps:1s	41.451	0.0
								3(f+ps):1s	0.176	0.675
♀A_3 KP-70 × ♂ KVV-96-2 ir	F_2	ir	dr	Field	49 (51.6 %)**	35	11 (11.6 %)	9f:6ps:1s	3.796	0.150
Total for (♀A_3 KP-70 × ♂ KVV-96-1 dr)	F_2	dr		Field	47 (34.3 %)	58	32 (23.4 %)			
Total for (♀A_3 KP-70 × ♂ KVV-96-2 ir)	F_2	ir		Field	93 (53.1 %)**	62	20 (11.4 %)**			

Note: [a] – the average indicators of the vapor pressure deficit during the flowering period: the average daily = 10.1 hPa; the maximum = 19.5 hPa; [b] – dr – dryland plot; ir – irrigated plot; *, ** – differs from the proportion of plants of a similar fertility class in the offspring obtained using a pollinator grown in a dryland plot at $p < 0.05$ and $p < 0.01$, respectively.

and it did not match the 25 % expected from the literature data (Pring et al., 1999) (Table 6). A significant part of the PGs that looked fertile at low magnification showed disorders of starch accumulation and detachment of the PG contents from the cell wall under a detailed study at high magnifications (Figure). In addition, starch color in some PGs was changed, apparently due to amylose replacement by amylopectin. However, if these violations did not affect the ability of the PGs to fertilize, then the ratio of all the "fertile" PGs and "sterile" PGs did correspond to the 1:3 segregation in some plants (No. 161-1 ($\chi^2 = 1.08$, $p = 0.299$) and No. 165-2 ($\chi^2 = 0.013$, $p = 0.909$) from the irrigated plots), while one of the plants from the dryland plot had a higher proportion of fertile pollen, and the segregation of fertile and sterile PGs did not fit the 1 : 3 ratio (No. 165-9: $\chi^2 = 9.72$, $p < 0.01$).

These data indicate that PGs containing both dominant genes *Rf3* and *Rf4* and the recessive alleles of these genes could develop in the F_1 hybrids. It is noteworthy that PGs with the aberrations described above were also observed in plants of paternal *Rf* donor lines; however, the proportion of fertile pollen was significantly higher than in the hybrids.

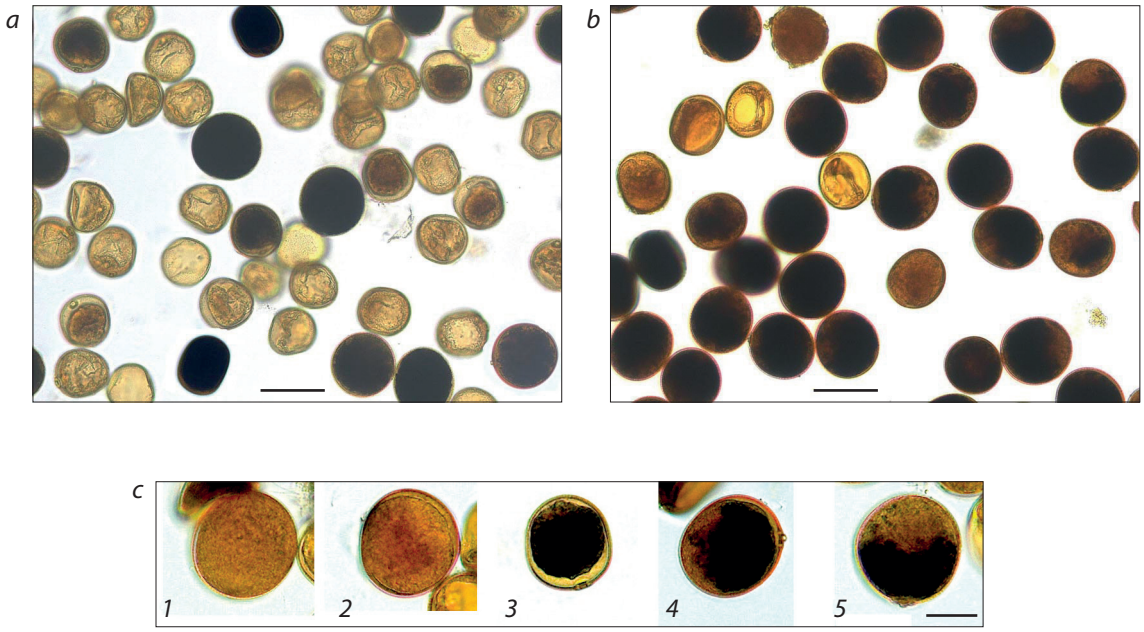
Thus, the results of cytological analysis indicate that pollen grains bearing recessive alleles of these genes could develop in the F_1 hybrids heterozygous for the fertility-restoring genes *Rf3* and *Rf4*. It is possible that in the CMS-lines created by us the fertility-restoring genes *Rf3* and *Rf4* begin to function in the tissues of the sporophyte, normalizing the development of part of the PGs carrying the recessive alleles of these genes (*rf3* and *rf4*), which participate in the fertilization and give rise to sterile genotypes detected in F_2 or BC_1. However, such "precocious" functioning of these genes contributes to the development of only part of the PGs, while a significant portion of pollen grains without restoring alleles still degenerates. The presence of numerous "intermediate" PGs phenotypes with signs of partial degeneration (see Figure), which were not described in earlier studies on A_3 type CMS of sorghum, argues for this hypothesis.

Selection for severe VPD tolerance. Water and its movement inside the anther play important roles in pollen development and anther dehiscence (Wilson et al., 2011). An important factor regulating anther dehiscence is air humidity. As is known, an indicator characterizing air humidity is vapor pressure deficit: the difference between the maximum possible and actual water vapor tension at specific temperature and pressure.

Assuming that vapor pressure deficit at the flowering phase suppresses fertility restoration in A_3 CMS of sorghum (Kozhemyakin et al., 2017), we set up an experiment to study the possibility of selection for tolerance for a high level of this factor (Table 7). In the F_2 ♀A3 Topaz × ♂ KVV-96 population, two groups of fully fertile plants were selected: flowering at VPD = 13.8 hPa and at VPD = 23.0 hPa, respectively. In the F_3 generation, under conditions of 16.4–16.6 hPa VPD at flowering, the levels of fertility in these groups differed significantly: 61.0 and 80.7 %, respectively ($p < 0.01$) (see Table 7). These differences were due to the presence of plants with low seed sets (< 50 %) in the first group, whereas such plants were absent at all from the second group. These data indicate the presence of a genetic factor contributing to tolerance for severe VPD, which acts at the flowering stage and affects the expression of the fertility-restoring genes. Apparently, under conditions of high VPD level (23.0 hPa), fertility restoration was possible only under homozygosity for this genetic factor; as a result, there was no segregation in the progeny of these plants, whereas during selection under the conditions of low VPD level (13.8 hPa) there could be heterozygotes, which segregated in the F_3 generation (see Table 7). These data indicate the efficiency of the selection for tolerance of the fertility restoration system of A_3-type CMS for severe VPD.

Table 6. Results of the cytological analysis of pollen formed in hermaphrodite flowers of the F_1 sorghum hybrids with A_3-type CMS and the paternal line with the same type of cytoplasm grown in dryland and irrigated plots (2017)

Plant No., seed set	Percentages of pollen grains		
	Fertile		Sterile
	Total	Without signs of degeneration	
Irrigated plots			
KVV-96 (100 %)	75.2 ± 2.4	42.3 ± 7.5	24.5 ± 4.0
161-1 (F_1 A_3 KP-70/KVV-96), 70 %	30.2 ± 3.6	2.7 ± 1.1	69.8 ± 3.6
165-2 (F_1 A_3 KP-70/KVV-96), 100 %	26.0 ± 1.1	5.0 ± 0.5	74.0 ± 1.1
Dryland plots			
KVV-96 (60 %)	80.7 ± 3.7	31.3 ± 3.3	20.2 ± 4.0
KVV-96 (40 %)	75.4 ± 2.4	18.5 ± 2.6	24.5 ± 4.0
161-9 (F_1 A_3 KP-70/KVV-96), 100 %	29.0 ± 3.4	10.0 ± 2.1	71.0 ± 3.4
165-9 (F_1 A_3 KP-70/KVV-96), 90 %	39.2 ± 2.3	4.0 ± 0.4	60.8 ± 2.3
167-7 (F_1 A_3 KP-70/KVV-96), 100 %	32.2 ± 1.4	3.2 ± 1.2	67.8 ± 1.3



Pollen of fertile F_1 hybrid ♀A_3 KP-70 × ♂KVV-96 (*a*); pollen of fertility-restoring line KVV-96 (*b*), scale bar 50 μm; *c* – types of pollen grains (PGs): *1*, *2* – change of starch coloration, *2–4* – detachment of PG content from the pollen cell wall, *4*, *5* – abnormal starch accumulation; scale bar 20 μm.

Discussion

The current notion of the genetic control of CMS is that the sterilization of the male reproductive system results from the operation of mitochondrial CMS-inducing genes, whereas the nuclear genes restoring fertility generally arrest the expression of the former at the transcriptional or posttranscriptional level (Chase, Gabay-Laughnan, 2004; Horn et al., 2014; Bohra et al., 2016). If the expression of the restorer gene starts in sporophyte (anther) tissue, the fertility restoration follows the sporophytic mode. Both fertile PGs, carrying the dominant fertility-restoring gene, and PGs carrying its recessive allele, unable to restore fertility, develop in the anthers. If the restorer expression starts in gametogeny, only PGs carrying the restorer gene are fertile, whereas those not carrying it degenerate and fail to participate in fertilization (gametophytic type of fertility restoration) (Kaul, 1988).

Our experimental results indicate that this classification is somewhat conventional. The appearance of sterile plants in F_2 and BC_1 families with the maternal CMS line grown at different water availability conditions (in an irrigated plot, in

Table 7. Effect of selection under contrasting air humidity conditions during the flowering period on the manifestation of male fertility in the self-pollinated progeny (F_3) of the F_2 hybrids ♀A_3Topaz × ♂KVV-96

Family	Number of plants with seed set levels			Mean seed set level, %	Vapor pressure deficit during the flowering period, hPa
	100–71 %	70–51 %	< 50 %		
F_3 families from the F_2-plants that bloom at the vapor pressure deficit 13.8 hPa					
8/2	4	5	6	57.7	16.4
9/2	7	3	5	63.0	16.4
10/1	5	3	7	60.7	16.2
10/2	6	3	6	62.7	16.5
Total	22	14	24	61.0 a	16.4
F_3 families from F_2 plants that bloom at the vapor pressure deficit 23.0 hPa					
11/1	9	5	0	76.0	16.9
11/2	10	4	0	81.0	16.6
13/1	11	4	0	83.7	16.7
13/2	10	4	0	81.9	16.3
Total	40	17	0	80.7 b	16.6
F				300.3^{**}	
LSD_{05}				3.6	

Note: Data denoted by different letters differ at $p < 0.05$, in accordance with the Duncan Multiple Range test; $^{**}p < 0.01$.

a growth chamber at 70 % humidity, under a rainfall shelter, and in an experimental field at natural water supply) argues for the sporophytic control of fertility restoration by two restorer genes in hybrid combinations studied. Taking into account that the source of the fertility-restoring genes was IS1112C, carrying the fertility-restoring genes *Rf3* and *Rf4*, which, according to literature data (Tang et al., 1998; Pring et al., 1999), are gametophytic fertility-restorers, we consider this fact exceptionally interesting and unusual, since the gametophytic and sporophytic types of fertility restoration are controlled by different genetic systems (Kaul, 1988).

However, the cytological analysis of pollen from fertile F_1 hybrids revealed a significant percentage of degenerating pollen grains to argue for the gametophytic type of fertility restoration. It is conceivable that in the genotype of the CMS-lines created by us, these fertility-restoring genes *Rf3* and *Rf4* act differently than in the genotype of CMS lines created in the USA at the Texas Experimental Station. Indian researchers also showed the sporophytic type of inheritance of fertility restoration in CMS A_3, controlled by three complementary fertility-restoring genes (Reddy et al., 2010).

Probably, the expression of the fertility-restoring genes *Rf3* and *Rf4* in the hybrids with studied CMS lines starts already in the sporophyte tissues, normalizing the development of a certain part of the PGs carrying the recessive alleles of these genes (*rf3* and *rf4*), which are involved in fertilization and give rise to sterile genotypes found in F_2 and BC_1 families. A similar hypothesis was put forward to explain the appearance of sterile plants in the progeny of some maize hybrids with the S type of CMS, having the gametophytic type of fertility restoration (Duvick, 1965).

This is the first finding of the transgenerational effect of water availability conditions of growing a fertility-restoring line on male fertility in the F_2 generation: a pollinator grown in a plot with additional irrigation formed much more fertile and less sterile individuals compared to the same pollinator line grown under dryland conditions. The segregation pattern changed from digenic to monogenic, indicating a heritable inhibition of the expression of one fertility-restoring gene under drought conditions (kind of "grandfather effect"). It is known that such transgenerational effects arise from a diversity of epigenetic changes (Hauser et al., 2011; Paszkowski, Grossniklaus, 2011; Kumar et al., 2015; Tricker, 2015; Alsdurf et al., 2016). They are based on changes in the methylation pattern, induced, among all, by environmental factors, in particular, drought stress (Lukens, Zhan, 2007; Wang et al., 2011; Tricker et al., 2012; Zheng et al., 2013).

Perhaps, just the methylation changes cause the loss of the function by one of the A_3 CMS restorer genes under drought conditions. By the MSAP analysis of F_1 hybrids A_3 KP-70/KVV-96, we found DNA fragments whose methylation patters under contrasting water availability conditions correlated with the manifestation of male fertility (Elkonin et al., 2019). Apparently, methylation pattern changes are an important mechanism controlling fertility restoration in the A_3 sorghum cytoplasm.

The possibility of selection for the stability of functioning of the fertility restoration system for the A_3 cytoplasm in

conditions of severe VPD during the flowering period was demonstrated in our work (see Table 7) also deserves attention. Such selection may contribute to the creation of new fertility-restoring lines for this CMS type.

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Acknowledgements. This work was partially supported by the Russian Foundation for Basic Research (project No. 16-04-01131).
The authors are grateful to Dr. O.P. Kibalnik (Russian Research and Development Institute of Sorghum and Maize, Saratov, Russia) for providing seeds of the grain sorghum lines KP-70 and Topaz; Dr. K.F. Schertz (Texas Agricultural Experimental Station, USA) for providing seeds of the A_3Tx398 line; Dr. H.D. Upadhyaya, (ICRISAT, Patancheru, India) for providing seeds of the IS1112C line. The authors thank the anonymous reviewer for critical analysis of the manuscript and valuable comments.

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

Received December 17, 2018. Revised March 3, 2019. Accepted March 6, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):422-429
DOI 10.18699/VJ19.505

Модифицированный метод дроплет-витрификации для криоконсервации апексов *in vitro* растений картофеля

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Коллекции возделываемого картофеля *Solanum tuberosum*, сохраняемые в полевых генбанках, несут значительные потери из-за воздействия экстремальных факторов внешней среды, заболеваний и вредителей. Практическое решение проблемы надежного хранения генофонда возделываемого картофеля состоит в создании дублетных криоколлекций, сохраняемых при сверхнизких температурах в криобанках. Известно несколько методов криоконсервации картофеля, из них в настоящее время в мировых генбанках наиболее широко используется метод дроплет-витрификации, разработанный Б. Панисом с коллегами в 2005 г. В нашей статье представлено подробное описание модифицированного метода дроплет-витрификации, который применяется для криоконсервации апексов *in vitro* растений картофеля во Всероссийском институте генетических ресурсов растений им. Н.И. Вавилова (ВИР). Модифицированный в ВИР метод включает основные этапы оригинального метода дроплет-витрификации, разработанного Б. Панисом с коллегами: 1) подготовка растительного материала; 2) изоляция апексов микрорастений; 3) обработка эксплантов растворами с криопротекторами; 4) криоконсервация/погружение в жидкий азот; 5) оттаивание; 6) посткриогенное восстановление и учет регенерационной способности. Предложенные нами модификации этапов 1, 2 и 6 позволяют существенно сократить продолжительность экспериментов по криоконсервации в сравнении с оригинальным методом. В работе представлены результаты экспериментов по криоконсервации аборигенных южноамериканских сортов и современных селекционных сортов картофеля, которые были выполнены с использованием модифицированного в ВИР метода дроплет-витрификации. Большая часть (76.7 %) изученных образцов культурного картофеля характеризовалась высокими показателями посткриогенной регенерации (40–95 %), и у 23.3 % изученных образцов частота регенерации после замораживания–оттаивания варьировала от 20 до 39 %, что соответствует предельно допустимым минимальным значениям для закладки на длительное хранение в криобанк. В настоящее время модифицированный метод дроплет-витрификации используется для расширения криоколлекции культурных видов картофеля в ВИР.
Ключевые слова: картофель; криоконсервация; дроплет-витрификация; криобанк.

Для цитирования: Гавриленко Т.А., Швачко Н.А., Волкова Н.Н., Ухатова Ю.В. Модифицированный метод дроплет-витрификации для криоконсервации апексов *in vitro* растений картофеля. Вавиловский журнал генетики и селекции. 2019;23(4):422-429. DOI 10.18699/VJ19.505

A modified droplet vitrification method for cryopreservation of shoot tips from *in vitro* potato plants

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Collections of common potato maintained in the field genebanks suffer significant losses due to the impact of extreme environmental factors, diseases and pests. The solution of the problem of safe long-term preservation of common potato accessions is to create doublet *in vitro* and *cryo*-collections. Cryogenic collections are stored at ultra-low temperatures in cryobanks. Several methods of potato cryoconservation are known, of which the droplet vitrification method developed by B. Panis with colleagues in 2005 is the most widely used in genebanks. This paper provides a detailed description of the modified method of droplet vitrification, which is used for cryopreservation of apexes (shoot tips) of potato *in vitro* plants at the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR). The method modified at VIR includes the main steps of the original droplet-vitrification method developed by B. Panis and colleagues: 1) preparation of plant material, 2) isolation of shoot tips, 3) treatment of explants with cryoprotector solutions, 4) freezing/immersion in liquid nitrogen, 5) thawing, 6) post-cryogenic recovery and evaluation of viability and regeneration capacity. The modifications of stages 1, 2 and 6 proposed at VIR lead to a significant reduction in the duration of cryopreservation experiments in comparison with the original method of B. Panis. This paper presents the results of cryopreservation of modern potato cultivars and South American landraces which were obtained using the method of droplet vitrification as modified at VIR. The majority (76.7 %) of

the studied accessions of cultivated potato were characterized by high rates of postcryogenic recovery (40–95 %) and 23.3 % of the samples had the values of postcryogenic regeneration from 20 to 39 %, which corresponds to the minimal permissible values for long-term storage in a cryobank. Currently the modified droplet-vitrification method is used for further expanding of the VIR potato cryocollection.
Key words: potato; cryopreservation; droplet-vitrification; cryobank.

For citation: Gavrilenko T.A., Shvachko N.A., Volkova N.N., Ukhatova Yu.V. A modified droplet vitrification method for cryopreservation of shoot tips from *in vitro* potato plants. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):422-429. DOI 10.18699/VJ19.505 (in Russian)

Введение

Коллекции возделываемого картофеля *Solanum tuberosum*, сохраняемые в полевых генбанках, несут значительные потери из-за воздействия экстремальных факторов внешней среды, заболеваний и вредителей. Практическое решение проблемы надежного хранения генофонда возделываемого картофеля состоит в формировании дублетных *in vitro* коллекций и создании на их основе криоколлекций, которые долгосрочно сохраняют в криобанках при сверхнизких температурах (Keller et al., 2006; Гавриленко и др., 2007; Engelman, 2014; Niino, Valle Arizaga, 2015; Panis et al., 2016; Vollmer et al., 2016).

В настоящее время разработаны международные стандарты генбанков для длительного хранения семян в условиях низких температур (FAO, 2014), согласно которым сохраняется и коллекция образцов семян в ВИР (Филипенко, 2007). Что касается стандартов хранения криоколлекций образцов вегетативно размножаемых культур, в частности картофеля, то до последнего времени они находятся на стадии активного обсуждения (Kaczmarczyk et al., 2011; Niino, Valle Arizaga, 2015; Vollmer et al., 2016, 2017; Ухатова, Гавриленко, 2018). Данная ситуация связана с тем, что ни один из способов криоконсервации не является унифицированным для определенного растительного объекта. Например, в мировой практике для криоконсервации апексов *in vitro* растений возделываемого картофеля *S. tuberosum* используют методы быстрого замораживания: дроплет-замораживания (Kaczmarczyk et al., 2011), витрификации (Kushnarenko et al., 2017), дроплет-витрификации (Kim et al., 2006; Bamberg et al., 2016; Vollmer et al., 2017). Перечисленные методы различаются техникой исполнения отдельных этапов и составом растворов с криопротекторами (Ухатова, Гавриленко, 2018). Так, при использовании метода витрификации экспланты обрабатывают раствором с криопротектором (чаще всего – PVS2) непосредственно в криопробирке, а в варианте метода дроплет-замораживания экспланты погружают в капли жидкой среды MS с криопротектором (10 % раствор DMSO), нанесенные на полоски фольги. Метод дроплет-витрификации сочетает особенности двух предыдущих методов: экспланты помещают в капли с раствором криопротектора (чаще всего – PVS2), нанесенные на полоски фольги.

В настоящее время для криоконсервации картофеля широко используется метод дроплет-витрификации, как наиболее воспроизводимый и простой в исполнении. Изначально метод был разработан Б. Панисом с коллегами для криоконсервации образцов банана (Panis et al., 2005). В дальнейшем оригинальный протокол Б. Паниса был многократно модифицирован для различных растительных объектов, в том числе картофеля (Kim et al., 2006; Bamberg et al., 2016; Vollmer et al., 2017) (Приложение 1)[1]. В ВИР криоконсервация апексов микрорастений картофеля с использованием метода дроплет-витрификации была начата в 2010 г., при этом протокол Б. Паниса также был существенно модифицирован (Дунаева и др., 2011; Shvachko, Gavrilenko, 2011; Ухатова и др., 2017). В Приложении 1 приведены четыре модификации оригинального метода дроплет-витрификации Б. Паниса (Panis et al., 2005), которые сегодня используют для криоконсервации образцов картофеля в ведущих мировых генбанках: CIP (Перу); USPG (США); NAC, NAAS, RDA (Южная Корея); ВИР (РФ) (протокол IPK, Германия не приводится, поскольку в этом генбанке до сих пор используется дроплет-метод). Все модификации включают шесть общих этапов: 1) подготовка растительного материала; 2) изоляция апексов микрорастений; 3) обработка эксплантов раствором PVS2 с криопротекторами; 4) криоконсервация/погружение в жидкий азот полосок фольги с каплями раствора PVS2, в которых находятся экспланты; 5) оттаивание; 6) посткриогенное восстановление и учет регенерационной способности (см. Прил. 1 и рисунок). Сравнение оригинального и четырех модифицированных методов дроплет-витрификации позволяет заключить, что фактически каждая модификация отличается по продолжительности отдельных этапов, составу культуральных сред и условиям культивирования. По сравнению с оригинальным методом Б. Паниса предложенные в ВИР модификации этапов 1, 2 и 6 позволили существенно сократить продолжительность всего цикла криоконсервации (см. Прил. 1).

В данной статье приведено подробное описание модифицированного в ВИР метода дроплет-витрификации, а также представлены результаты его использования для криоконсервации апексов *in vitro* растений 43 образцов трех культурных видов картофеля.

Материал исследования

В качестве материала исследования использованы 44 образца из *in vitro* коллекции ВИР, включая 43 образца культурного картофеля и один образец дикого вида *S. spegazzinii* (см. таблицу). Образцы культурного картофеля были представлены: 5 образцами тетраплоидных андийских аборигенных сортов – *S. tuberosum* ssp. *andigenum*; 7 образцами чилийских аборигенных сортов – *S. tuberosum* ssp. *tuberosum*; 20 образцами андийских диплоидных культурных видов – *S. phureja* (8 образцов) и *S. stenotomum* (12 образцов); 10 селекционными сортами возделываемо-

[1] Приложения 1 и 2 см. по адресу:
http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx7.pdf

го картофеля *S. tuberosum* и одним селекционным клоном NZ-32. Все образцы ранее были детально охарактеризованы по числу хромосом, генотипированы с использованием SSR-маркеров и маркеров разных типов цитоплазм (Gavrilenko et al., 2010; Antonova et al., 2017).

Методы

Питательные среды и растворы для криоконсервации

Безгормональная среда Мурасиге и Скуга (MS) твердая: макроэлементы, микроэлементы, витамины (Murashige, Skoog, 1962), сахароза 30 г/л, агар 7 г/л, pH 5.8. После приготовления среду автоклавируют.

Безгормональная среда MS жидкая: макросоли, микросоли, витамины (Murashige, Skoog, 1962), сахароза 30 г/л, pH 5.8. Среду необходимо проавтоклавировать.

Раствор LS (Loading solution): осмо- и криопротектор на основе MS с добавлением сахарозы 136.8 г/л, глицерола 184 г/л, pH 5.8. Приготовленную среду не автоклавируют и хранят в морозильнике при –20 °C. Перед применением необходимое количество среды стерилизуют через стерильный мембранный фильтр (диаметр пор 0.45 мкм) (Panis et al., 2005).

Раствор PVS2 (Plant Vitrification Solution – витрифицирующий раствор): макросоли, микросоли, витамины и глицин по MS (Murashige, Skoog, 1962), сахароза 136.8 г/л (0.4 M), глицерол 300 г/л, этиленгликоль 150 г/л, DMSO 150 г/л, pH 5.8. Среда не подлежит автоклавированию и стерилизуется с помощью стерильных мембранных фильтров с размером пор 0.45 мкм (Sakai et al., 1990). Хранится в морозильнике при –20 °C.

Раствор RS (среда для размораживания): жидкая среда MS с добавлением 410.4 г/л сахарозы, pH в пределах 5.6–5.8. Среду можно автоклавировать.

Среда MSTo для посткриогенной регенерации: макросоли, микросоли, витамины по MS, сахароза 20 г/л, агар 7 г/л, зеатин рибозид 0.5 мг/л, ИУК 0.5 мг/л, ГК 0.2 мг/л (фитогормоны добавляют после автоклавирования), pH 5.8 (Towill, 1983). Для стерилизации безгормональную среду MS автоклавируют. Раствор фитогормонов стерилизуют через стерильный мембранный фильтр (размер пор 0.22 мкм) и добавляют в охлажденную до 50 °C среду MS.

Модифицированный в ВИР метод дроплет-витрификации для криоконсервации апексов *in vitro* растений картофеля

Первый этап – подготовка микрорастений. Основная задача первого этапа – микроразмножение и получение достаточного количества апексов для проведения трех повторностей эксперимента. С этой целью используют одноузловые микрочеренки *in vitro* растений картофеля, которые высаживают по 15–20 штук в стеклянные сосуды объемом 0.5 л, заполненные 45–50 мл питательной агаризованной среды MS без гормонов с 30 г/л сахарозы на 3–4 недели (см. рисунок, *а*). Культивирование проводят на светоустановке с освещенностью 3–4 клк при 20–25 °C с 16-часовым фотопериодом.

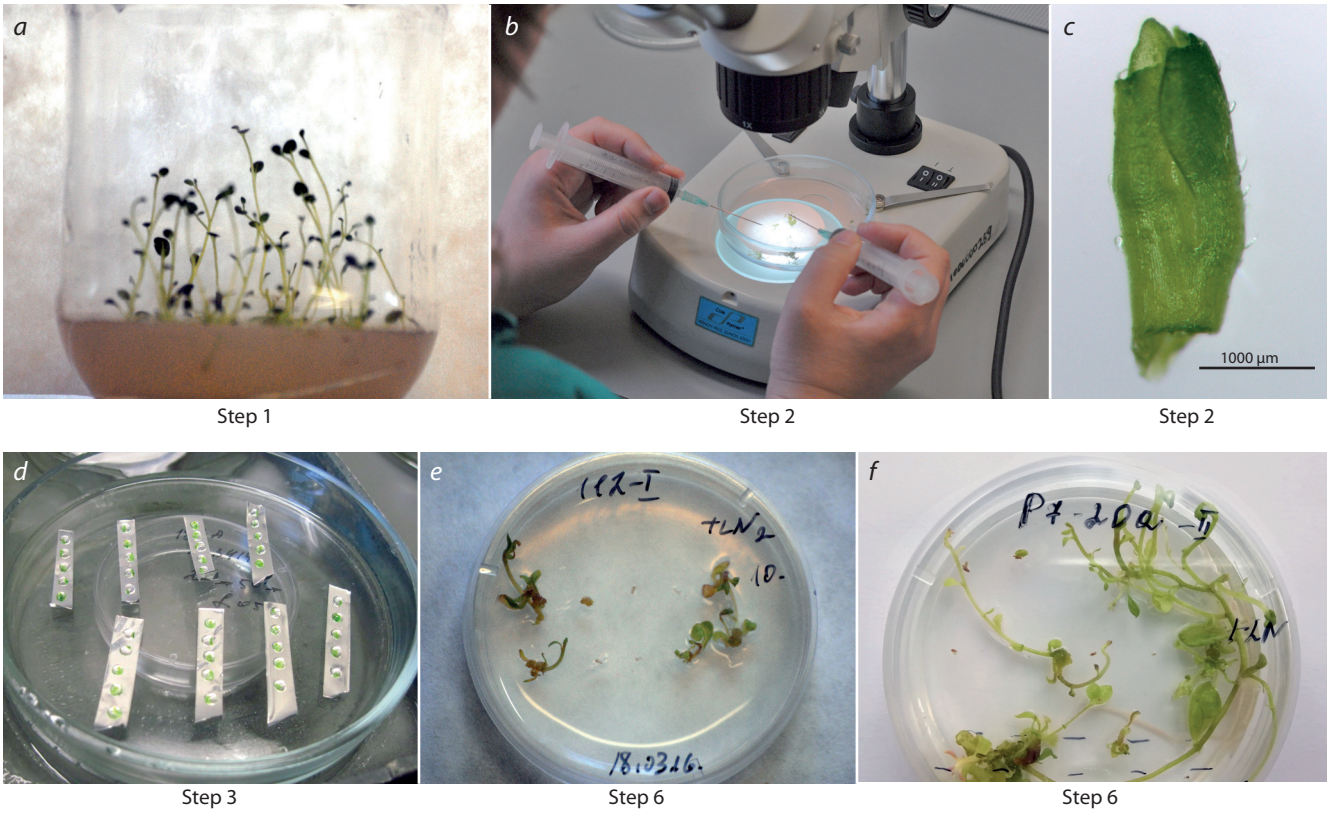
Второй этап – изоляция эксплантов. Направлен на получение достаточного числа эксплантов для криоконсервации. В качестве эксплантов используют апексы побегов микрорастений. Для изоляции апексов выбирают хорошо развитые микрорастения картофеля. Все работы на данном этапе проводят со стереомикроскопом и со стерильными медицинскими иглами, надетыми на два одноразовых шприца – по одному для каждой руки. Левой рукой оператор прижимает часть микропобега (микрочеренок), а правой рукой отсекает листья и стебель, оставляя апекс неповрежденным (см. рисунок, *б*, *в*). Верхушечные почки микрорастений размером 1.8–2.5 мм помещают в жидкую среду MS на время изоляции остальных эксплантов.

Отдельно остановимся на числе эксплантов, необходимом для надежного криохранения образца. Для каждого образца криоконсервация выполняется в трех независимых повторностях. В каждой повторности опыта изолируют 60 эксплантов: 10 из них используют в качестве контроля для проверки влияния питательных сред, осмо- и криопротекторов на жизнеспособность эксплантов (без обработки жидким азотом); 20 – погружают в жидкий азот на 1 ч для учета регенерационной способности после оттаивания; 30 эксплантов (без оттаивания) передают на длительное криохранение в биокриокомплекс ВИР. Согласно литературным данным, одного часа экспозиции эксплантов в жидком азоте достаточно для адекватной оценки эффективности посткриогенной регенерации (Panis et al., 2005; Kim et al., 2006; Yoon et al., 2006; Kaczmarczyk et al., 2011; Bamberg et al., 2016).

Третий этап – обработка эксплантов криопротектором, проводят для осмо- и криопротекции эксплантов. На этом этапе применяют двухступенчатую инкубацию изолированных эксплантов: сразу после изоляции апексы в чашке Петри помещают на 20 мин при комнатной температуре в раствор LS, после чего их переносят на 30 мин на лед в заранее охлажденный раствор PVS2. Растворы LS и PVS2 содержат смесь осмо- и криопротекторов (LS – сахарозы и глицерола, PVS2 – сахарозы, глицерола, этиленгликоля и DMSO).

Четвертый этап – замораживание эксплантов в жидком азоте, заключается в непосредственном воздействии жидкого азота на апексы побегов. Перед погружением в жидкий азот подготавливают полоски алюминиевой фольги размером 25 × 5 мм. Стерильным наконечником наносят по 5 капель среды PVS2 на каждую полоску. Переносят по одному апексу в каждую каплю (см. рисунок, *г*). Затем полоски фольги с эксплантами погружают в криопробирки, заполненные жидким азотом (по 2 полоски на пробирку), плотно их закрывают и опускают в переносной сосуд Дьюара с жидким азотом. Криопробирки перед началом эксперимента маркируют, обозначив название сорта, номер в каталоге ВИР и год закладки на длительное криохранение. Сосуд Дьюара с маркированными криопробирками, содержащими апексы побегов, передают в биокриокомплекс на длительное криохранение. Для оценки частоты посткриогенной регенерации образцов достаточно погрузить криопробирку в жидкий азот на один час. Одного часа экспозиции в жидком азоте достаточно для адекватной оценки эффективности регенерации (Panis et al., 2005; Kaczmarczyk et al., 2011).

Пятый этап – оттаивание. Этот этап необходим для оценки частоты посткриогенной регенерации образцов.



Stages of cryopreservation of potato accessions by the droplet vitrification method modified at VIR:

(*a*) *In vitro* micropropagation of plants (step 1); (*b*) Isolation of shoot tips of *in vitro* of plants; (*c*) An isolated explant (step 2); (*d*) Incubation of the explants in drops of the PVS2 cryoprotectant solution immediately before immersion into liquid nitrogen (step 3); (*e*) Post-cryogenic regeneration of a sample of *S. stenotomum*, k-11053 (112) on the 6th week of cultivation after freezing–thawing; (*f*) Post-cryogenic regeneration of a sample of *S. phureja* k-99 (P7-20a) on the 8th week after freezing–thawing (step 6).

Полоски фольги с эксплантами извлекают пинцетом из жидкого азота и быстро погружают их в раствор RS на 15 мин. Затем экспланты переносят в чашки Петри со средой MSTo.

Шестой этап – изучение способности образцов к посткриогенному восстановлению (оценка частоты посткриогенной регенерации). Чашки Петри с эксплантами на среде MSTo помещают на светоустановку с освещенностью 3–4 клк, температурой 20–25 °С и 16-часовым фотопериодом. Эффективность восстановления после криоконсервации для каждого образца оценивают на 8-й неделе беспересадочного культивирования по показателям: 1) жизнеспособности эксплантов (число, % зеленых почек на питательной среде MSTo; параллельно учитывают и процентное содержание некротизировавшихся эксплантов) и 2) регенерационной способности (число, % эксплантов, сформировавших микропобеги) (см. рисунок, *e*). Эти показатели вносят в таблицу рекомендуемого образца (Прил. 2), вычисляя средние значения по трем независимым повторностям опыта с подсчетом ошибки среднего. Статистическую обработку данных проводят с использованием методов описательной статистики. Влияние фактора «генотип» оценивали методом однофакторного анализа.

Минимальные значения частоты посткриогенной регенерации образца, передаваемого на длительное хранение в криобанк, четко не регламентированы; этот вопрос, с подробным анализом подходов к расчетам значений данного показателя в зависимости от числа сохраняемых эксплантов, подробно обсуждается в обзоре (Ухатова, Гавриленко, 2018). Ранее международные организации, занимающиеся вопросами сохранения биоразнообразия, рекомендовали включать в криоколлекции образцы, частота посткриогенной регенерации которых составляет не менее 20 % (IPGRI, 2000). В настоящее время в отдельных генбанках принят более жесткий регламент: для закладки образца в криобанк рекомендуется минимально допустимая частота посткриогенной регенерации эксплантов не ниже 39 % (Panis et al., 2016).

В ВИР криоколлекция картофеля формируется согласно следующим требованиям: для определения частоты посткриогенной регенерации каждого образца необходимо проведение трех независимых повторностей криоконсервации. В соответствии с полученными результатами посткриогенного восстановления образцы дифференцируются на три группы: А) образцы с высокой частотой посткриогенной регенерации – выше 40 %; Б) образцы со средними показателями частоты регенерации – от 21 до 39 %; В) образцы с низкой регенерационной способностью – ниже 20 %. Образцы из первых двух групп (А и Б) считаются надежно сохраняемыми в криобанке, образцы из группы В требуют индивидуального подхода –

либо увеличения числа повторностей, либо дальнейшей модификации метода дроплет-витрификации (Ухатова, Гавриленко, 2018).

Пошаговый протокол

1. Одноузловые микрочеренки *in vitro* растений картофеля высадить по 15–20 штук в стеклянные сосуды объемом 0.5 л, заполненные 45–50 мл питательной агаризованной среды MS без гормонов с 30 г/л сахарозы, и культивировать в течение 3–4 недель при 22 ± 2 °C, фотопериоде 16/8 (см. рисунок, *а*).
2. В ламинаре с использованием стереомикроскопа для каждой повторности изолировать стерильными иглами, закрепленными на шприцах, 60 апексов микрорастений картофеля размером 1.8–2.5 мм (см. рисунок, *б*, *в*).
3. Изолированные апексы поместить в стерильную одноразовую пластиковую чашку Петри (диаметром 6 см) с 5 мл жидкой среды MS до окончания изоляции всех остальных эксплантов.
4. Дозатором со стерильным наконечником объемом 1 мл или стерильной пипеткой Пастера объемом 2–3 мл удалить жидкую среду MS из чашки Петри. Следить за тем, чтобы апексы не попали внутрь наконечника. Наконечник сменить.
5. Дозатором со стерильным наконечником объемом 1 мл или стерильной пипеткой Пастера объемом 2–3 мл добавить в чашку Петри 5–10 мл раствора LS так, чтобы все апексы были им полностью покрыты. Оставить в ламинаре на 20 мин при комнатной температуре.
6. Дозатором со стерильным наконечником объемом 1 мл или стерильной пипеткой Пастера объемом 2–3 мл удалить раствор LS из чашки Петри. Следить за тем, чтобы апексы не попали внутрь наконечника. Наконечник сменить.
7. Дозатором со стерильным наконечником объемом 1 мл или стерильной пипеткой Пастера объемом 2–3 мл внести в чашку Петри 5–10 мл заранее охлажденного раствора PVS2 так, чтобы все апексы были им полностью покрыты. Оставить чашку Петри с апексами в ламинаре на 30 мин на хладоэлементе.
8. Спустя 20 мин инкубации апексов в растворе PVS2 начать подготовку полосок алюминиевой фольги: в стеклянную чашку Петри диаметром 10 см пинцетом поместить 10 полосок стерильной фольги.
9. Дозатором со стерильным наконечником объемом 10 мкл или диспенсером нанести на каждую полоску по 5 капель раствора PVS2 объемом 2.5 мкл каждая.
10. Глазным пинцетом переместить по одному апексу из пластиковой чашки Петри в каждую каплю на полоске фольги. Десять апексов оставить в исходной чашке Петри (см. рисунок, *г*).
11. Провести маркировку пяти криопробирок, указав название и номер каталога образца, а также год проведения криоконсервации.
12. Поставить в ламинар пенопластовый штатив для криопробирок.
13. Налить в штатив жидкий азот.
14. Открытые маркированные криопробирки по одной ставить в штатив, заполненный жидким азотом; крышки складывать в пустую стеклянную чашку Петри.
15. Все криопробирки полностью залить жидким азотом.
16. После 30-минутной инкубации в растворе PVS2 полоски фольги с апексами быстро погрузить по одной в каждую криопробирку, долить жидкий азот до краев и поместить в каждую криопробирку вторую полоску фольги с апексами.
17. Криопробирки закрыть, перенести их из штатива в заполненный жидким азотом сосуд Дьюара и поставить в морозильник на один час.
18. Поставить в ламинар стерильную одноразовую пластиковую чашку Петри (диаметром 6 см).
19. Налить в чашку Петри 15–25 мл раствора RS при помощи стерильной пипетки Пастера объемом 3–5 мл.
20. Апексы, оставленные в исходной чашке Петри с раствором PVS2, глазным пинцетом перенести в чашку Петри с раствором RS и оставить на 15 мин при комнатной температуре (вариант контроля «– LN»).
21. Через 15 мин контрольные апексы извлечь пинцетом из раствора RS и поместить их в чашку Петри со средой MSTo для посткриогенного восстановления.
22. Чашку Петри закрыть парафилмом, подписать название образца, номер повторности опыта, дату и слово «контроль» или «–LN», обозначая отсутствие этапа погружения эксплантов в жидкий азот.
23. Чашку Петри поставить на светоустановку.
24. По прошествии одного часа криоконсервации в жидком азоте достать из морозильника сосуд Дьюара с криопробирками.
25. Поставить в ламинар пенопластовый штатив для криопробирок.
26. Налить в штатив жидкий азот.
27. Пинцетом достать две криопробирки из сосуда Дьюара и поместить их в штатив, аккуратно открыть криопробирки и долить в них жидкий азот.
28. Быстрым и точным движением пинцета перенести полоски фольги с эксплантами из криопробирки в чашку Петри с раствором RS и оставить на 15 мин при комнатной температуре (вариант «опыт» или «+LN»).
29. Через 15 мин «криоконсервированные» апексы извлечь пинцетом из раствора RS и поместить их в чашку Петри со средой MSTo для посткриогенного восстановления.
30. Чашки Петри закрыть парафилмом, подписать название образца, номер повторности опыта, дату и слово «опыт» или «+LN», обозначая этап погружения эксплантов в жидкий азот.
31. Чашки Петри поставить на светоустановку.

Необходимые материалы и оборудование

- Весы аналитические с точностью до 0.01 г;
- pH-метр;
- электроплитка с водяной баней или микроволновая печь для растворения агара;
- холодильник для хранения рабочих растворов и реактивов;
- автоклав;
- сухожаровые шкафы для сушки и стерилизации посуды;

Indicators of post-cryogenic recovery of potato accessions cryopreserved by the droplet vitrification method modified at VIR

No.	Potato variety	Ploidy level*	Accession no. in the VIR catalogue	Control (without freezing) (–LN)		Indicators of post-cryogenic recovery after freezing–thawing (+LN)	
				Viability, %	Regeneration rate, %	Viability after thawing, %	Regeneration rate after thawing, %,
	Group A. Regeneration rate after thawing above 40 %						
1	*S. tuberosum* ssp. *andigenum*	4x	1741	100 ± 0.0	100 ± 0.0	96.7 ± 0.8	94.9 ± 0.1
2	*S. phureja*	2x	15845	86.7 ± 8.9	86.7 ± 8.9	80.1 ± 10.0	75.0 ± 13.3
3	*S. phureja*	2x	1678	100 ± 0.0	100 ± 0.0	75.0 ± 1.6	73.3 ± 1.6
4	*S. tuberosum* ssp. *tuberosum*	4x	2117	71.1 ± 1.5	60.3 ± 8.4	71.1 ± 1.9	71.1 ± 1.9
5	*S. stenotomum*	2x	9303	100 ± 0.0	100 ± 0.0	81.7 ± 7.5	71.7 ± 1.4
6	*S. phureja*	2x	9393	80.0 ± 5.8	80.0 ± 5.8	70.0 ± 10.0	70.0 ± 10.0
7	*S. stenotomum*	2x	10194	83.3 ± 3.3	83.3 ± 3.3	73.9 ± 2.6	69.0 ± 5.5
8	*S. tuberosum* ssp. *andigenum*	4x	8931	83.3 ± 8.8	83.3 ± 8.8	68.3 ± 3.6	66.7 ± 2.8
9	*S. phureja*	2x	16530	93.3 ± 3.3	93.3 ± 3.3	68.3 ± 2.0	63.3 ± 1.0
10	*S. phureja*	2x	15843	100 ± 0.0	100 ± 0.0	61.7 ± 1.0	61.7 ± 1.0
11	*S. tuberosum* ssp. *andigenum*	4x	3041	100 ± 0.0	100 ± 0.0	63.3 ± 2.1	61.7 ± 2.1
12	*S. tuberosum* ssp. *andigenum*	4x	12892	100 ± 0.0	100 ± 0.0	60.0 ± 2.0	60.0 ± 2.0
13	*S. phureja*	2x	22210	90.0 ± 5.8	90.0 ± 5.8	60.0 ± 10.0	60.0 ± 10.0
14	*S. stenotomum*	2x	9889	80.0 ± 0.0	80.0 ± 0.0	60.2 ± 13.3	60.0 ± 13.3
15	*S. tuberosum*, cv. Sudarynya			93.3 ± 3.3	93.3 ± 3.3	60.0 ± 15.3	60.0 ± 15.3
16	*S. tuberosum* ssp. *tuberosum*	4x	7523	96.7 ± 3.3	96.7 ± 3.3	60.0 ± 15.5	60.0 ± 15.5
17	*S. stenotomum*	2x	9039	86.7 ± 3.3	86.7 ± 3.3	60.0 ± 2.9	58.0 ± 5.8
18	*S. tuberosum* ssp. *tuberosum*	4x	7530	70.0 ± 5.8	70.0 ± 5.8	56.7 ± 2.1	56.7 ± 2.1
19	*S. stenotomum*	2x	7037	73.3 ± 3.3	73.3 ± 3.3	59.7 ± 4.6	56.5 ± 2.3
20	*S. phureja*	2x	99	86.7 ± 13.3	86.7 ± 13.3	62.2 ± 12.2	53.3 ± 3.3
21	*S. tuberosum* ssp. *tuberosum*	4x	7586	90.0 ± 0.0	90.0 ± 0.0	55.0 ± 5.8	53.3 ± 3.3
22	*S. tuberosum*, cv. Amado			73.3 ± 3.3	73.3 ± 3.3	50.0 ± 10.0	50.0 ± 10.0
23	*S. phureja*	2x	16530	96.7 ± 3.3	96.7 ± 3.3	50.0 ± 12.3	50.0 ± 12.3
24	*S. stenotomum*	2x	8880	80.0 ± 0.0	80.0 ± 0.0	48.5 ± 5.5	48.5 ± 5.5
25	*S. tuberosum* ssp. *tuberosum*	4x	7580	83.3 ± 3.3	83.3 ± 3.3	50.0 ± 3.8	48.0 ± 3.3
26	NZ-32, elite clone			83.3 ± 3.3	83.3 ± 3.3	52.0 ± 5.8	47.5 ± 2.4
27	*S. stenotomum*	2x	8865	73.3 ± 3.3	73.3 ± 3.3	48.1 ± 3.8	46.7 ± 3.3
28	*S. stenotomum*	2x	11053	73.3 ± 3.3	73.3 ± 3.3	48.0 ± 10.8	46.7 ± 12.0
29	*S. tuberosum*, cv. Delikat			81.7 ± 4.4	81.7 ± 4.4	50.0 ± 5.8	45.0 ± 5.0
30	*S. tuberosum*, cv. Forelle			76.7 ± 3.3	76.7 ± 3.3	45.1 ± 13.8	42.8 ± 12.8
31	*S. tuberosum*, cv. Baltica			80.0 ± 5.8	80.0 ± 5.8	50.0 ± 5.8	42.7 ± 2.7
32	*S. tuberosum*, cv. Atlantic			73.3 ± 3.3	73.3 ± 3.3	45.0 ± 5.5	42.5 ± 2.5
33	*S. tuberosum* ssp. *tuberosum*	4x	7529	80.0 ± 5.8	80.0 ± 5.8	40.0 ± 16.0	40.0 ± 16.0
	Group B. Regeneration rate after thawing within 21–39 %						
1	*S. tuberosum*, сорт Rasant			80.0 ± 5.8	80.0 ± 5.8	35.5 ± 5.0	35.5 ± 5.0
2	*S. stenotomum*	2x	8935	73.3 ± 3.3	73.3 ± 3.3	33.3 ± 1.0	33.3 ± 1.0
3	*S. stenotomum*	2x	7126	73.3 ± 3.3	73.3 ± 3.3	40.0 ± 5.5	33.3 ± 13.3
4	*S. stenotomum* (*S. yabari*)	2x	3640	80.0 ± 0.0	80.0 ± 0.0	61.7 ± 6.4	30.9 ± 10.9
5	*S. tuberosum*, cv. Solara			78.3 ± 1.7	78.3 ± 1.7	54.0 ± 5.4	30.0 ± 0.1
6	*S. stenotomum*	2x	10433	80.0 ± 5.8	80.0 ± 5.8	40.0 ± 15.5	30.0 ± 15.3
7	*S. tuberosum*, cv. Sonate			73.3 ± 3.3	73.3 ± 3.3	35.0 ± 5.3	29.7 ± 0.7
8	*S. tuberosum*, cv. Maxi			76.7 ± 8.8	76.7 ± 8.8	35.0 ± 5.8	25.0 ± 5.0
9	*S. tuberosum* ssp. *tuberosum*	4x	3385	86.7 ± 6.7	86.7 ± 6.7	36.7 ± 3.3	21.1 ± 6.7
10	*S. tuberosum* ssp. *andigenum*	4x	1763	80.0 ± 11.5	80.0 ± 11.5	52.3 ± 2.3	20.0 ± 5.7
	Group C. Regeneration rate after thawing below 20 %						
1	*S. spegazzinii*	2x	23061	53.3 ± 6.7	53.3 ± 6.7	36.7 ± 8.8	13.3 ± 3.3

* (Gavrilenko et al., 2010)

- ламинарный бокс с горизонтальным потоком воздуха;
- стереомикроскоп;
- светоустановки в комнатах с терморегуляцией или климатические камеры;
- сосуд Дьюара объемом 25 л для хранения жидкого азота;
- сосуд Дьюара или термос объемом 0.5–1 л для проведения криоконсервации и переноса криоконсервированного материала в криобанк;
- инструменты и расходные материалы: пинцеты различного размера, препаравальные иглы, скальпели, ножницы, криопробирки объемом 1.8 мл, стерильные мембранные фильтры, жидкий азот.

Результаты

Результаты криоконсервации 44 образцов картофеля, выполненной с использованием модифицированного в ВИР метода дроплет-витрификации, представлены в таблице и на рисунке.

В варианте контроля «–LN» (без погружения апексов в жидкий азот) число жизнеспособных и регенерировавших эксплантов совпадало: фактически все выжившие экспланты были способны к регенерации. Частота регенерации в контроле варьировала от 60.3 до 100 % и существенно не зависела от генотипа. Только у одного образца *S. spegazzinii* отмечен сравнительно низкий уровень регенерации в контроле (53.3 %) (см. таблицу).

Большая часть (76.7 %) изученных образцов культурного картофеля проявила высокую частоту посткриогенной регенерации (40.0–94.9 %) и вошла в группу А (см. таблицу). Группу Б составили 23.3 % образцов культурного картофеля, у которых средняя частота посткриогенного восстановления варьировала от 20 до 35.5 %, что соответствует предельно допустимым минимальным значениям закладки на длительное хранение в криобанк. Наиболее низкая частота регенерации после оттаивания (13.3 %) зафиксирована у образца дикого вида *S. spegazzinii*. Вероятно, для данного образца необходимы дополнительные эксперименты по подбору оптимальных условий криоконсервации. Отметим, что модифицированный в ВИР метод дроплет-витрификации разрабатывался для криоконсервации культурных видов картофеля – аборигенных и селекционных сортов.

Отмечена достоверная положительная корреляция (0.99; $p < 0.05$) между показателями жизнеспособности и регенерационной способности эксплантов в контроле (–LN), а также статистически значимая положительная корреляция (0.88) между уровнями посткриогенной жизнеспособности и регенерационной способности эксплантов после замораживания–оттаивания (+LN). Показатели жизнеспособности эксплантов в контроле (–LN) и в варианте криоконсервации (+LN) не коррелировали (0.55; $p > 0.05$), как и показатели регенерационной способности (0.53; $p > 0.05$).

Уровень плоидности и видовая принадлежность образцов культурных видов картофеля не оказывали значимого влияния на частоту посткриогенной регенерации. Существенное ($p < 0.05$) влияние на частоту посткриогенной регенерации после оттаивания оказывал генотип. Группы А и Б сформировались вне зависимости от видовой принадлежности образцов и их уровня плоидности.

Заключение

Существующие методы криоконсервации сортов картофеля не являются универсальными и требуют постоянного совершенствования, что особенно актуально при работе с большими коллекциями, включающими широкое генетическое разнообразие. На сегодняшний день в мировых генбанках для криоконсервации картофеля наиболее широко используется метод дроплет-витрификации, разработанный Б. Панисом (Panis et al., 2005). По сравнению с оригинальным методом предложенные в ВИР модификации позволяют существенно сократить продолжительность экспериментов по криоконсервации, при этом большая часть (76.7 %) изученных образцов культурного картофеля имела высокие показатели посткриогенного восстановления (40–95 %) и у 23.3 % образцов средняя частота посткриогенного восстановления варьировала от 20 до 39 %. В настоящее время модифицированный метод дроплет-витрификации используется в ВИР для расширения криоколлекции культурных видов картофеля.

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Acknowledgements. The test of the capability for postcryogenic repair in 44 potato accessions was supported by the Russian Science Foundation, project 16-16-04125. The maintenance of the *in vitro* potato collection whose accessions were chosen for cryopreservation was supported by budgeted project 0662-2019-0004. The photograph of the isolated explant (Figure, *c*) was taken with the equipment of the Shared Access Center "Cellular and Molecular Technologies for Studying Plants and Fungi", Komarov Botanical Institute, St. Petersburg.

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

Received December 13, 2018. Revised February 24, 2019. Accepted February 24, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):430-438
DOI 10.18699/VJ19.511

Использование метода путевых коэффициентов S. Wright для статистического анализа системы взаимосвязанных признаков риса

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В настоящее время актуален путевой анализ S. Wright продуктивности растений. Целью исследований было определить парные коэффициенты корреляций и путевые коэффициенты S. Wright признаков сортов риса и на их основе выявить вклад каждого из них в продуктивность растения. Исходным материалом были 10 сортов риса. Опыты проведены в 2013, 2014 и 2016 гг. в условиях орошения на опытном поле Института риса Национальной академии аграрных наук Украины. Посев осуществляли сеялкой СКС-6А с нормой высева 7.0 млн всхожих семян на 1 га. Предшественник – люцерна. Площадь делянки – 5 м^2, междурядья – 15 см. Анализировали растения по признакам: продуктивность (масса зерна) растения, масса всей метелки, масса зерна с боковых стеблей, продуктивная кустистость, количество зерен в метелке, количество колосков в метелке, масса 1000 зерен, масса зерна с метелки, высота растения, длина и плотность метелки, количество пустых колосков в метелке, пустозерность. Парные коэффициенты корреляций определяли по методике Б.А. Доспехова, путевой анализ – по методике S. Wright по работе А.И. Седловского, С.П. Мартынова и Л.К. Мамонова (1982). Определена корреляция продуктивности с 12 количественными признаками риса: тесная – с массой зерна с боковых стеблей; средняя – с массой всей метелки и с массой зерна с метелки. Согласно путевому анализу продуктивности растений, корреляция признаков растений с продуктивностью зависит как от прямых, так и от косвенных эффектов влияния каждого признака на продуктивность. Установлен относительный вклад влияния каждого из 12 исследуемых признаков на продуктивность риса, как прямой (непосредственный), так и косвенный (побочный) эффекты их при взаимодействии с другими признаками. Это дало возможность раскрыть причины и следствия взаимозависимостей между признаками и выделить селекционно-ценные для отбора признаки, такие как масса всей метелки и продуктивная кустистость, которые имели наибольший прямой эффект влияния на продуктивность и достоверную корреляцию с ней.
Ключевые слова: рис; сорт; признак; продуктивность растения; корреляция; путевой анализ; вклад количественного признака в продуктивность; селекционное значение признака.

Для цитирования: Паламарчук Д.П., Козаченко М.Р., Святченко С.И. Использование метода путевых коэффициентов S. Wright для статистического анализа системы взаимосвязанных признаков риса. Вавиловский журнал генетики и селекции. 2019;23(4):430-438. DOI 10.18699/VJ19.511

Use of S. Wright's path coefficient method for statistical analysis of interrelated traits in rice

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S. Wright's analysis of plant productivity is of great current interest. The research objective was to determine the pair correlation coefficients and S. Wright's path coefficients for rice varieties and, on their basis, to identify the contribution of each of them to the plant productivity. Ten rice varieties were taken as the test material. The experiments were conducted in the irrigated experimental field of the Institute of Rice of the National Academy of Agrarian Sciences of Ukraine in 2013, 2014 and 2016. Seeds were sown with an SKS-6A manual seeder; the seeding rate was 7.0 mln germinable seeds per hectare. The predecessor was alfalfa. The plot area was 5 m^2; the sowing distance was 15 cm. The plants were analyzed for the following traits: plant productivity (grain weight), panicle weight, grain weight from side stems, productive tillering capacity, grain number per panicle, spikelet number per panicle, 1000-grain weight, grain weight per panicle, plant height, panicle length and density, empty spikelet number per panicle, and incidence of blind seed disease. Pair correlation coefficients were determined by B.A. Dospekhov's method; path analysis, by S. Wright's method. The correlations of productivity with 12 quantitative traits of rice were determined: the correlation was close with the grain weight from side stems and medium with the panicle weight and with the grain weight per panicle. Path analysis of the plant productivity established that the correlations of plant traits with

the productivity depended both on direct and indirect effects of each trait on the productivity. The relative contribution of each of the studied 12 traits to the rice productivity was determined; both direct and indirect effects of their interactions with other traits were evaluated. This made it possible to discover causes and consequences of interrelations between the traits and, as a result, to choose valuable-for-selection traits, such as panicle weight and productive tillering capacity, which had the greatest direct effects on the productivity and significant correlations with it.
Key words: rice; variety; trait; plant productivity; correlation; path analysis; contribution of a quantitative trait to the productivity; selection value of the trait.

For citation: Palamarchuk D.P., Kozachenko M.R., Sviatchenko S.I. Use of S. Wright's path coefficient method for statistical analysis of interrelated traits in rice. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):430-438. DOI 10.18699/VJ19.511 (in Russian)

Введение

Эффективность использования исходного материала в селекции зависит от возможности установить особенности количественных признаков растений, основным из которых является продуктивность, или масса зерна с растения. На уровень продуктивности влияют как условия внешней среды, так и генотипические особенности характера проявления отдельных признаков исследуемых объектов. В связи с этим селекционную оценку сортов целесообразно проводить не по отдельным признакам, а с учетом их взаимозависимости, определяемой методом корреляционного анализа. Это позволяет узнать, в какой степени изменяется основной признак, в частности продуктивность растения, в зависимости от значений других признаков, в результате чего возможен прогноз эффективности отбора уже по ним.

На важность установления корреляций различных признаков растений для определения эффективности отбора по отдельным из них в разных условиях, на целесообразность отбора по их комплексу, выделения отдельных признаков (характеристик) с наибольшим вкладом в изменчивость основного признака указывали многие авторы на примере таких культур, как рис (Samonte et al., 1998; Sürek, Beşer, 2003; Орлюк и др., 2008, 2011; Khan et al., 2009; Akinwale et al., 2011; Шпак, 2013; Hossain et al., 2015; Ratna et al., 2015) и ячмень (Bhutta et al., 2005; Ataei, 2006; Ilker, 2006; Emine, Necmettin, 2012; Akdeniz et al., 2014).

При определении простой парной корреляции не всегда можно выявить влияние отдельных признаков. Существенное значение могут иметь косвенные эффекты других признаков. То есть парные коэффициенты корреляции хотя и важны для определения основных компонентов влияния на показатели основного признака, однако не дают полного представления о значении прямых и косвенных воздействий отдельных признаков.

Поэтому для определения вклада каждого из количественных признаков в основной, например продуктивность растения, непосредственно (прямые эффекты) и при взаимодействии с другими признаками (непрямые, косвенные, побочные эффекты) используют так называемый путевой анализ S. Wright. Это эффективный метод статистического анализа причин и следствий в системе взаимозависимых признаков. Таким образом определяют детерминантные признаки как критерии отбора, по которым он будет эффективным, на что указано в некоторых работах по рису (Samonte et al., 1998; Ekka et al., 2011), ячменю (Sinebo, 2002) и пшенице (Finne et al., 2000).

Путевой метод в селекции растений для анализа продуктивности в зависимости от других признаков растений впервые был использован в 1959 г. D.R. Dewey и K.H. Lu (Седловский и др., 1982). Ряд исследователей использовали анализ коэффициентов путей S. Wright и коэффициентов корреляции для объяснения взаимосвязей между количественными признаками различных растений, в частности риса (Samonte et al., 1998; Sürek, Beşer, 2003; Akhtar et al., 2011; Bagheri et al., 2011; Basavaraja et al., 2011; Bhadru et al., 2011; Ekka et al., 2011; Mugemangango Cyprien, Vinod Cumar, 2011; Haider et al., 2012; Hossain et al., 2015), ячменя (Ataei, 2006; Мухордова, 2011) и пшеницы (Мухордова, Калашник, 2010; Мухордова, 2014).

Были установлены неодинаковые уровни прямых эффектов признаков растений на продуктивность сортов различных культур: для индекса продуктивности риса (Sürek, Beşer, 2003), количества зерен в колосе ячменя (Ataei, 2006) и пшеницы (Shahid et al., 2002), высоты растений пшеницы (Aycicek, Yildirim, 2006). Необходимость изучения взаимосвязей структурных элементов растений методом путевого анализа отмечена также в исследованиях на бобах (Türk et al., 2008) и сое (Сичкарь, 1998). Таким образом, в разных исследованиях говорится о целесообразности определения взаимосвязи между основным и другими признаками растений с использованием не только парных коэффициентов корреляции, но и путевых коэффициентов для нахождения прямых и косвенных эффектов взаимосвязанных признаков.

Целью настоящей работы было установить особенности взаимосвязей между количественными признаками растений риса путем определения парных коэффициентов корреляции и путевых коэффициентов, по которым выявить прямое и косвенное влияние различных признаков на продуктивность растения, на основе чего определить их селекционное значение.

Материалы и методы

Исследования выполнены в 2013, 2014 и 2016 гг. в отделе селекции и на опытном поле Института риса Национальной академии аграрных наук Украины в различных погодных условиях. Гидротермический коэффициент Селянинова в 2013 г. составил 0.9, в 2014 г. – 0.9, в более благоприятном 2016 г. – 1.2.

Материалом для исследований служили 10 лучших сортов риса: Командор, Южанин, Украина 96, Magic, Lotto, Виконт, Адмирал, Fukushikiri, Giza-177, Sakha 101. Растения были выращены в сортоиспытании в условиях орошения.

Орошение проведено по типу укороченного затопления. После посева риса чеки сразу заливали водой на 5 дней, после чего почву в чеках подсушивали до получения пол-

ных всходов. Второе затопление чеков начинали после получения полных всходов и начала роста растений риса и по мере их развития поднимали слой воды до уровня 15 см, после чего поддерживали его на этой отметке до полной спелости посевов.

Против сорняков в посевах риса применяли гербицид Цитадель 25 ЕД м.д. нормой 1.6 л/га с использованием опрыскивателя ОВМ-630. Против пирикуляриоза посевы обрабатывали фунгицидом Импакт К в количестве 1.6 л/га.

Перед дискованием пласта люцерны весной, в первой декаде апреля, вносили удобрения N90 (сульфат аммония) и P20 (суперфосфат простой). Подкормку растений риса проводили карбамидом (мочевина) в фазу кущения нормой N50 действующего вещества на 1 га. Предшественником была люцерна.

Посев осуществляли сеялкой СКС-6А с нормой высева 4.5 млн всхожих семян на 1 га. В 2013 г. к уборке сохранилось 101–105 растений на 1 га, в 2014 г. – 96–100, в 2016 г. – 93–98 растений на 1 га.

Площадь делянки – 5 м^2, количество рядков – 6, междурядья – 12.5 см. Количество повторений – 4. Урожай собирали селекционным комбайном Yanmar.

Структурный анализ по 50 растениям (с корнями) делали по 13 признакам для всех 10 сортов без исключения: продуктивность (масса зерна) растения, масса всей метелки, масса зерна с боковых стеблей, продуктивная кустистость, количество зерен в метелке, количество колосков в метелке, масса 1000 зерен, масса зерна с метелки, высота растения, длина и плотность метелки, количество пустых колосков в метелке, пустозерность.

Парные коэффициенты корреляций (r) определяли между всеми 13 количественными признаками с использованием основ статистической обработки по (Доспехов, 1973). Путевой анализ проводили в соответствии с методикой S. Wright по работе А.Н. Седловского, С.П. Мартынова и Л.К. Мамонова (Седловский и др., 1982). Экспериментальные данные обрабатывали с использованием программного обеспечения Excel персонального компьютера.

Зависимость продуктивности от других признаков можно выразить через уравнение множественной регрессии

$$Y = Yc + b_1 \cdot (X_1 - X_{1c}) + b_2 \cdot (X_2 - X_{2c}) + \ldots$$
$$\ldots + b_n \cdot (X_n - X_{nc}) + E, \quad (1)$$

где Y – продуктивность; Yc – средняя статистическая продуктивность; X_1, X_2, X_n – значения 1-го, 2-го и n-го признаков соответственно; X_{1c}, X_{2c}, X_{nc} – среднее статистическое значение 1-го, 2-го и n-го признаков соответственно; b_1 – коэффициент регрессии X_1 на Y; b_2 – коэффициент регрессии X_2 на Y; b_n – коэффициент регрессии X_n на Y; E – общая ошибка уравнения, которая включает и ошибку опыта.

Затем вводятся обозначения:

$$y = Y - Yc;\ x_1 = X_1 - X_{1c};\ x_2 = X_2 - X_{2c};\ \ldots;\ x_n = X_n - X_{nc},$$

а также рекомендованные изменения признаков от деления их на свои стандартные отклонения σ_i:

$$x_i = (X_i - X_{ic})/\sigma_i \quad \text{для } i = 1, 2, \ldots, n, \quad (2)$$

где n – общее количество признаков, влияющих на продуктивность.

Тогда уравнение (1) принимает вид

$$y/\sigma_y = b_1 \cdot (\sigma_1/\sigma_y) \cdot (x_1/\sigma_1) + b_2 \cdot (\sigma_2/\sigma_y) \cdot (x_2/\sigma_2) + \ldots$$
$$\ldots + b_n \cdot (\sigma_n/\sigma_y) \cdot (x_n/\sigma_n) + \sigma_e/\sigma_y) \cdot (E/\sigma_e). \quad (3)$$

Величину $P_i = b_i \cdot (\sigma_i/\sigma_y)$ называют коэффициентом пути признака x_i к зависимой переменной y (в нашем случае – продуктивность). Таким образом, путевой коэффициент S. Wright (Райта) – это стандартизированный коэффициент регрессии независимого признака на зависимую функцию.

В отличие от коэффициента корреляции, путевой коэффициент является векторной величиной, т. е. имеет направление в системе признаков. Для нахождения значений путевых коэффициентов необходимо сначала рассчитать парные коэффициенты корреляций между признаками.

Результаты

Установлены парные коэффициенты корреляций количественных признаков риса и путевые коэффициенты продуктивности растений.

Корреляция количественных признаков риса

Установлен уровень взаимосвязей между 13 количественными признаками всех 10 сортов риса в 2013, 2014 и 2016 гг. по парным коэффициентам корреляции (табл. 1).

Основной признак растения – продуктивность (масса зерна) – достоверно положительно и очень тесно коррелировал во все три года с массой зерна с боковых стеблей (r = 0.99*; r = 0.99*; r = 0.99*; здесь и далее значения коэффициента корреляции r приведены для 2013, 2014 и 2016 гг. соответственно), т. е. отбор по этому признаку будет наиболее эффективным, средне – с массой зерна с метелки (r = 0.40*; r = 0.60*; r = 0.56*), массой всей метелки (r = 0.49*; r = 0.60*; r = 0.56*) и кустистостью (r = 0.55*; r = 0.32; r = 0.34), однако отрицательно коррелировал с количеством пустых колосков (r = –0.12; r = –0.25; r = –0.44*) и пустозерностью (r = –0.14; r = –0.22; r = –0.45*).

Продуктивность (масса зерна) метелки как один из структурных элементов продуктивности растения имела очень тесную положительную корреляцию с массой всей метелки (r = 0.99*; r = 0.99*; r = 0.99*) и массой зерна с боковых стеблей (r = 0.90*; r = 0.87*; r = 0.89*), по которым отбор будет наиболее эффективным, среднюю – с продуктивностью растения (r = 0.40*; r = 0.60*; r = 0.56*), количеством зерен в метелке (r = 0.53*; r = 0.61*; r = 0.78*) и количеством колосков в метелке (r = 0.53*; r = 0.61*; r = 0.78*), однако отрицательную – с продуктивной кустистостью (r = –0.36; r = –0.50*; r = –0.22) и пустозерностью (r = –0.33; r = –0.32; r = –0.41*).

Продуктивная кустистость средне коррелировала с массой зерна с боковых стеблей (r = 0.66*; r = 0.56*; r = 0.46*) и продуктивностью растений (r = 0.55*; r = 0.32; r = 0.34), а отрицательно (достоверно в 2014 г.) – с массой всей метелки (r = –0.25; r = –0.50*; r = –0.22), количеством колосков в метелке (r = –0.24; r = –0.41*; r = –0.26), количеством зерен в метелке (r = –0.24; r = –0.41*; r = 0.25) и массой зерна с метелки (r = –0.36; r = –0.50*; r = –0.22).

Длина метелки имела положительную корреляцию с массой зерна с боковых стеблей (r = 0.94*; r = 0.89*;

Table 1. Correlation coefficients of quantitative traits in 10 rice varieties

No.	Year	2	3	4	5	6	7	8	9	10	11	12	13
1	2013	0.13	0.16	0.27	0.31	0.39	–0.07	–0.06	0.01	0.14	0.21	–0.04	–0.23
	2014	–0.12	0.04	–0.05	–0.03	0.31	–0.06	–0.06	0.01	0.04	0.01	–0.02	–0.20
	2016	–0.09	0.47*	–0.06	0.08	0.54*	0.10	0.12	–0.06	0.47*	0.53*	–0.07	–0.26
2	2013		–0.25	0.66	0.55*	0.18	–0.24	–0.24	0.23	–0.36	–0.01	0.23	–0.31
	2014		–0.50*	0.56*	0.32	–0.15	–0.41*	–0.41*	0.42*	–0.50*	0.08	0.39	–0.32
	2016		–0.22	0.46*	0.34	0.03	–0.26	–0.25	0.12	–0.22	0.08	0.18	–0.21
3	2013			0.15	0.49*	–0.24	0.52*	0.52*	–0.55*	0.99*	0.22	–0.54*	0.64*
	2014			0.36	0.60*	0.04	0.61*	0.61*	–0.57*	1.00*	0.03	–0.51*	0.55*
	2016			0.32	0.56*	0.22	0.64*	0.78*	–0.63*	1.00*	0.34	–0.68*	0.38
4	2013				0.99*	0.94*	0.96*	0.96*	0.94*	0.90*	0.95*	0.92*	0.97*
	2014				0.99*	0.89*	0.93*	0.92*	0.90*	0.87*	0.87*	0.86*	0.92*
	2016				0.99*	0.96*	0.98*	0.98*	0.97*	0.89*	0.98*	0.97*	0.95*
5	2013					–0.04	0.07	0.07	–0.12	0.40*	0.22	–0.14	0.14
	2014					–0.07	0.27	0.27	–0.25	0.60*	0.11	–0.22	0.28
	2016					–0.01	0.39	0.50*	–0.44*	0.56*	0.14	–0.45*	0.32
6	2013						0.11	0.12	–0.16	–0.25	–0.34	–0.17	–0.40*
	2014						0.15	0.15	–0.13	0.04	–0.13	–0.11	–0.33
	2016						0.14	0.06	0.09	0.22	0.13	0.05	–0.56*
7	2013							0.99*	–0.94*	0.53*	–0.66*	–0.87*	0.84*
	2014							0.99*	–0.91*	0.61*	–0.59*	–0.81*	0.87*
	2016							0.90*	–0.28	0.64*	–0.38	–0.42*	0.73*
8	2013								–0.96*	0.53*	–0.66*	–0.89*	0.84*
	2014								–0.93*	0.61*	–0.62*	–0.84*	0.87*
	2016								–0.68*	0.78*	–0.31	–0.78*	0.70*
9	2013									–0.34	0.39	0.61*	–0.48*
	2014									–0.36	0.47*	0.60*	–0.52*
	2016									–0.37	0.03	0.57*	–0.19
10	2013										0.12	–0.33	0.39
	2014										0.02	–0.32	0.35
	2016										0.20	–0.41*	0.24
11	2013											0.37	–0.23
	2014											0.49*	–0.34
	2016											0.08	–0.24
12	2013												–0.44*
	2014												–0.46*
	2016												–0.24

1, Plant height; 2, tillering capacity; 3, panicle weight; 4, grain weight from side stems; 5, productivity (grain weight per plant); 6, panicle length; 7, spikelet number per panicle; 8, grain number per panicle; 9, number of empty spikelets per panicle; 10, grain weight per panicle; 11, 1000-grain weight; 12, incidence of blind seed disease; 13, panicle density. *The correlation coefficient is significant at 5 %.

$r = 0.96$*), однако отрицательную – с плотностью метелки ($r = –0.40$*; $r = –0.33$; $r = –0.56$*).

Количество зерен в метелке положительно коррелировало с плотностью метелки ($r = 0.84$*; $r = 0.87$*; $r = 0.70$*), массой зерна с боковых стеблей ($r = 0.96$*; $r = 0.92$*; $r = 0.98$*) и количеством колосков в метелке ($r = 0.99$*; $r = 0.99$*; $r = 0.90$*), средне – с продуктивностью метелки ($r = 0.53$*; $r = 0.61$*; $r = 0.78$*), а отрицательно – с количеством пустых колосков в метелке ($r = –0.96$*; $r = –0.93$*; $r = –0.68$*), пустозерностью ($r = –0.89$*; $r = 0.84$*; $r = 0.78$*) и массой 1000 зерен ($r = –0.66$*; $r = –0.62$*; $r = –0.31$).

Масса 1000 зерен имела положительную корреляцию с массой зерна с боковых стеблей ($r = 0.95$*; $r = 0.87$*; $r = 0.98$*), однако отрицательную – с количеством колосков в метелке ($r = –0.66$*; $r = –0.59$*; $r = –0.38$) и количеством зерен в метелке ($r = –0.66$*; $r = –0.62$*; $r = –0.31$).

В результате определения парных коэффициентов корреляции установлено, что продуктивность растения, масса зерна с метелки, масса зерна с боковых стеблей положительно взаимосвязаны между собой.

Положительная связь получена также между массой метелки, количеством зерен в метелке и плотностью метелки, между массой зерна с метелки, количеством зерен и количеством колосков в метелке, между массой зерна с боковых стеблей и количеством колосков в метелке и плотностью метелки. Пустозерность отрицательно влияет на массу, количество зерен и колосков в метелке и через эти признаки – на плотность метелки.

Путевой анализ продуктивности растений риса

Для определения путевых коэффициентов (P_j) мы воспользовались линейным уравнением

$$\sum r_{ij} \cdot P_j = r_{yi}, \quad (4)$$

где P_j – путевой коэфициент от j-го признака к y; r_{ij} – коэффициент корреляции между i-м и j-м признаками; r_{yi} – коэффициент корреляции между y и i-м признаком.

Затем с использованием значений коэффициентов корреляции за 2013 г. была составлена система из двенадцати уравнений (4) с двенадцатью неизвестными путевыми коэффициентами (прямыми эффектами признаков): P_1 (высоты растения), P_2 (кустистости), P_3 (массы метелки), P_4 (массы зерна с боковых стеблей), P_5 (длины метелки), P_6 (количества колосков в метелке), P_7 (количества зерен в метелке), P_8 (количества пустых колосков в метелке), P_9 (массы зерна с метелки), P_{10} (массы 1000 зерен), P_{11} (пустозерности), P_{12} (плотности метелки) (см. рисунок).

С помощью доступного в интернете метода Гаусса (https://planetcalc.ru/3571) было найдено решение этой системы линейных алгебраических уравнений, заданных в виде матрицы. Решением является вектор путевых коэффициентов за 2013 г. Аналогично мы получили вектор путевых коэффициентов за 2014 и 2016 гг. (табл. 2).

Подставив значения прямых эффектов P_j (j = 1, 2, …, 12), с помощью табличного компьютерного процессора Excel получили разложение корреляции между продуктивностью и каждым количественным признаком на прямой его эффект и косвенные эффекты других признаков всех 10 сортов (табл. 3).

Результаты были сведены в таблицу – матрицу путевых коэффициентов каждого признака к продуктивности растения: прямых эффектов каждого из них на продуктивность и побочных эффектов других признаков, которые составляют причинно-следственную систему. Структура матрицы соответствует системе двенадцати уравнений с 12 прямыми эффектами.

В результате путевого анализа продуктивности установлены прямые эффекты 12 признаков в их вкладе в уровень продуктивности (по центральной диагонали табл. 3), их косвенный эффект в связях продуктивности с другими признаками (по вертикали каждого из признаков, см. табл. 3), а также косвенный эффект других признаков в связях каждого из 12 признаков в отдельности с продуктивностью (по горизонтали каждого из признаков, см. табл. 3).

Наибольший прямой эффект на продуктивность (см. табл. 3) за 2013, 2014 и 2016 гг. наблюдался у признака «масса всей метелки» (0.51; 0.95; 0.75 соответственно по годам). Его косвенный эффект (по вертикали табл. 3) был положительным в связях продуктивности с высотой растения в 2016 г., массой зерна с боковых стеблей (2014, 2016 гг.), количеством колосков в метелке, количеством зе-

$$\begin{cases}
1.00 \cdot P_1 + 0.13 \cdot P_2 + 0.16 \cdot P_3 + 0.27 \cdot P_4 + 0.39 \cdot P_5 - 0.07 \cdot P_6 - 0.06 \cdot P_7 + 0.01 \cdot P_8 + 0.14 \cdot P_9 + 0.21 \cdot P_{10} - 0.04 \cdot P_{11} - 0.23 \cdot P_{12} = 0.31 \\
0.13 \cdot P_1 + 1.00 \cdot P_2 - 0.25 \cdot P_3 + 0.66 \cdot P_4 + 0.18 \cdot P_5 - 0.24 \cdot P_6 - 0.24 \cdot P_7 + 0.23 \cdot P_8 - 0.36 \cdot P_9 - 0.01 \cdot P_{10} + 0.23 \cdot P_{11} - 0.31 \cdot P_{12} = 0.55 \\
0.16 \cdot P_1 - 0.25 \cdot P_2 + 1.00 \cdot P_3 + 0.15 \cdot P_4 - 0.24 \cdot P_5 + 0.52 \cdot P_6 + 0.52 \cdot P_7 - 0.55 \cdot P_8 + 0.99 \cdot P_9 + 0.22 \cdot P_{10} - 0.54 \cdot P_{11} + 0.64 \cdot P_{12} = 0.49 \\
0.27 \cdot P_1 + 0.66 \cdot P_2 + 0.15 \cdot P_3 + 1.00 \cdot P_4 - 0.94 \cdot P_5 + 0.96 \cdot P_6 + 0.96 \cdot P_7 + 0.94 \cdot P_8 + 0.90 \cdot P_9 + 0.95 \cdot P_{10} + 0.92 \cdot P_{11} + 0.97 \cdot P_{12} = 0.99 \\
0.39 \cdot P_1 + 0.18 \cdot P_2 - 0.24 \cdot P_3 + 0.94 \cdot P_4 + 1.00 \cdot P_5 + 0.11 \cdot P_6 + 0.12 \cdot P_7 - 0.16 \cdot P_8 - 0.25 \cdot P_9 - 0.34 \cdot P_{10} - 0.17 \cdot P_{11} + 0.40 \cdot P_{12} = -0.04 \\
-0.07 \cdot P_1 - 0.24 \cdot P_2 + 0.52 \cdot P_3 + 0.96 \cdot P_4 + 0.11 \cdot P_5 + 1.00 \cdot P_6 + 0.99 \cdot P_7 - 0.94 \cdot P_8 + 0.53 \cdot P_9 - 0.66 \cdot P_{10} - 0.87 \cdot P_{11} + 0.84 \cdot P_{12} = 0.07 \\
-0.06 \cdot P_1 - 0.24 \cdot P_2 + 0.52 \cdot P_3 + 0.96 \cdot P_4 + 0.12 \cdot P_5 + 0.99 \cdot P_6 + 1.00 \cdot P_7 - 0.96 \cdot P_8 + 0.53 \cdot P_9 - 0.66 \cdot P_{10} - 0.89 \cdot P_{11} + 0.84 \cdot P_{12} = 0.07 \\
0.01 \cdot P_1 + 0.23 \cdot P_2 - 0.55 \cdot P_3 + 0.94 \cdot P_4 - 0.16 \cdot P_5 - 0.94 \cdot P_6 - 0.96 \cdot P_7 + 1.00 \cdot P_8 - 0.34 \cdot P_9 + 0.39 \cdot P_{10} + 0.61 \cdot P_{11} - 0.48 \cdot P_{12} = -0.12 \\
0.14 \cdot P_1 - 0.36 \cdot P_2 + 0.99 \cdot P_3 + 0.90 \cdot P_4 - 0.25 \cdot P_5 + 0.53 \cdot P_6 + 0.53 \cdot P_7 - 0.34 \cdot P_8 + 1.00 \cdot P_9 + 0.12 \cdot P_{10} - 0.33 \cdot P_{11} + 0.39 \cdot P_{12} = 0.40 \\
0.21 \cdot P_1 - 0.01 \cdot P_2 + 0.22 \cdot P_3 + 0.95 \cdot P_4 - 0.25 \cdot P_5 - 0.66 \cdot P_6 - 0.66 \cdot P_7 + 0.39 \cdot P_8 + 0.12 \cdot P_9 + 1.00 \cdot P_{10} + 0.37 \cdot P_{11} - 0.23 \cdot P_{12} = 0.22 \\
-0.04 \cdot P_1 + 0.23 \cdot P_2 - 0.54 \cdot P_3 + 0.92 \cdot P_4 - 0.17 \cdot P_5 - 0.87 \cdot P_6 - 0.89 \cdot P_7 + 0.61 \cdot P_8 - 0.33 \cdot P_9 + 0.37 \cdot P_{10} + 1.00 \cdot P_{11} - 0.44 \cdot P_{12} = -0.14 \\
-0.23 \cdot P_1 - 0.31 \cdot P_2 + 0.64 \cdot P_3 + 0.97 \cdot P_4 - 0.40 \cdot P_5 + 0.84 \cdot P_6 + 0.84 \cdot P_7 - 0.48 \cdot P_8 + 0.39 \cdot P_9 - 0.23 \cdot P_{10} - 0.44 \cdot P_{11} + 1.00 \cdot P_{12} = 0.14
\end{cases}$$

The system of 12 equations with 12 variables, 2013.

Table 2. Path coefficients (P_j) of the j^{th} trait on y (productivity of all 10 varieties)

Year	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8	P_9	P_{10}	P_{11}	P_{12}
2013	0.12	0.72	0.51	–0.01	–0.02	–0.01	0.09	0.08	0.11	0.11	0.02	0.02
2014	0.03	0.85	0.95	–0.01	0.01	–0.06	0.16	0.01	0.03	0.08	–0.01	–0.01
2016	–0.17	0.52	0.75	–0.03	0.25	–0.73	0.42	0.13	0.12	–0.17	0.09	0.47

Table 3. Results of the path analysis of the productivity of all 10 rice varieties

No.	Year	1	2	3	4	5	6	7	8	9	10	11	12	P_0	13
1	2013	**0.1200**	0.0936	0.0816	–0.0027	–0.0078	0.0007	–0.0054	0.0008	0.0154	0.0231	–0.0008	–0.0046	–0.0039	**0.31**
	2014	**0.0300**	–0.1020	0.0380	0.0005	0.0031	0.0036	–0.0096	0.0001	0.0012	0.0008	0.0002	0.0020	0.0021	**–0.03**
	2016	**–0.1700**	–0.0468	0.3525	0.0018	0.1350	–0.0730	0.0504	–0.0078	0.0564	–0.0901	–0.0063	–0.1222	0.0001	**0.08**
2	2013	0.0156	**0.7200**	–0.1275	–0.0066	–0.0036	0.0024	–0.0216	0.0184	–0.0396	–0.0011	0.0046	–0.0062	–0.0048	**0.55***
	2014	–0.0036	**0.8500**	–0.4750	–0.0056	–0.0015	0.0246	–0.0656	0.0042	–0.0150	0.0064	–0.0039	0.0032	0.0018	**0.32**
	2016	0.0153	**0.5200**	–0.1650	–0.0138	0.0075	0.1898	–0.1050	0.0156	–0.0264	–0.0136	0.0162	–0.0987	–0.0019	**0.34**
3	2013	0.0192	–0.1800	**0.5100**	–0.0015	0.0048	–0.0052	0.0468	–0.0440	0.1089	0.0242	–0.0108	0.0128	0.0048	**0.49***
	2014	0.0012	–0.4250	**0.9500**	–0.0036	0.0004	–0.0366	0.0976	–0.0057	0.0300	0.0024	0.0051	–0.0055	–0.0103	**0.60***
	2016	–0.0799	–0.1144	**0.7500**	–0.0096	0.0550	–0.4672	0.3276	–0.0819	0.1200	–0.0578	–0.0612	0.1786	0.0008	**0.56***
4	2013	0.0324	0.4752	0.0765	**–0.0100**	–0.0188	–0.0096	0.0864	0.0752	0.0990	0.1045	0.0184	0.0194	0.0414	0.99*
	2014	–0.0015	0.4760	0.3420	**–0.0100**	0.0089	–0.0558	0.1472	0.0090	0.0261	0.0696	–0.0086	–0.0092	–0.0037	**0.99***
	2016	0.0102	0.2392	0.2400	**–0.0300**	0.2400	–0.7154	0.4116	0.1261	0.1068	–0.1666	0.0873	0.4465	–0.0057	**0.99***
5	2013	0.0468	0.1296	–0.1224	–0.0094	**–0.0200**	–0.0011	0.0108	–0.0128	–0.0275	–0.0374	–0.0034	–0.0080	0.0148	**–0.04**
	2014	0.0093	–0.1275	0.0380	–0.0089	**0.0100**	–0.0090	0.0240	–0.0013	0.0012	–0.0104	0.0011	0.0033	0.0002	**–0.07**
	2016	–0.0918	0.0156	0.1650	–0.0288	**0.2500**	–0.1022	0.0252	0.0117	0.0264	–0.0221	0.0045	–0.2632	–0.0003	**–0.01**
6	2013	–0.0084	–0.1728	0.2652	–0.0096	–0.0022	**–0.0100**	0.0891	–0.0752	0.0583	–0.0726	–0.0174	0.0168	0.0088	**0.07**
	2014	–0.0018	–0.3485	0.5795	–0.0093	0.0015	**–0.0600**	0.1584	–0.0091	0.0183	–0.0472	0.0081	–0.0087	–0.0112	**0.27**
	2016	–0.0170	–0.1352	0.4800	–0.0294	0.0350	**–0.7300**	0.3780	–0.0364	0.0768	0.0646	–0.0378	0.3431	–0.0017	**0.39**
7	2013	–0.0072	–0.1728	0.2652	–0.0096	–0.0024	–0.0099	**0.0900**	–0.0768	0.0583	–0.0726	–0.0178	0.0168	0.0088	**0.07**
	2014	–0.0018	–0.3485	0.5795	–0.0092	0.0015	–0.0594	**0.1600**	–0.0093	0.0183	–0.0496	0.0084	–0.0087	–0.0112	**0.27**
	2016	–0.0204	–0.1300	0.5850	–0.0294	0.0150	–0.6570	**0.4200**	–0.0884	0.0936	0.0527	–0.0702	0.3290	0.0001	**0.50***
8	2013	0.0012	0.1656	–0.2805	–0.0094	0.0032	0.0094	–0.0864	**0.0800**	–0.0374	0.0429	0.0122	–0.0096	–0.0112	**–0.12**
	2014	0.0003	0.3570	–0.5415	–0.0090	–0.0013	0.0546	–0.1488	**0.0100**	–0.0108	0.0376	–0.0060	0.0052	0.0027	**–0.25**
	2016	0.0102	0.0624	–0.4725	–0.0291	0.0225	0.2044	–0.2856	**0.1300**	–0.0444	–0.0051	0.0513	–0.0893	0.0052	**–0.44***
9	2013	0.0168	–0.2592	0.5049	–0.0090	0.0050	–0.0053	0.0477	–0.0272	**0.1100**	0.0132	–0.0066	0.0078	0.0019	**0.40**
	2014	0.0012	–0.4250	0.9500	–0.0087	0.0004	–0.0366	0.0976	–0.0036	**0.0300**	0.0016	0.0032	–0.0035	–0.0066	**0.60***
	2016	–0.0799	–0.1144	0.7500	–0.0267	0.0550	–0.4672	0.3276	–0.0481	**0.1200**	–0.0340	–0.0369	0.1128	0.0018	**0.56***
10	2013	0.0252	–0.0072	0.1122	–0.0095	0.0050	0.0066	–0.0594	0.0312	0.0132	**0.1100**	0.0074	–0.0046	–0.0101	**0.22**
	2014	0.0003	0.0680	0.0285	–0.0087	0.0004	0.0354	–0.0992	0.0047	0.0006	**0.0800**	–0.0049	0.0034	0.0015	**0.11**
	2016	–0.0901	0.0416	0.2550	–0.0294	0.0550	0.2774	–0.1302	0.0039	0.0240	**–0.1700**	0.0072	–0.1128	0.0084	**0.14**
11	2013	–0.0048	0.1656	–0.2754	–0.0092	0.0034	0.0087	–0.0801	0.0488	–0.0363	0.0407	**0.0200**	–0.0088	–0.0126	**–0.14**
	2014	–0.0006	0.3315	–0.4845	–0.0086	–0.0011	0.0486	–0.1344	0.0060	–0.0096	0.0392	**–0.0100**	0.0046	–0.0011	**–0.22**
	2016	0.0119	0.0936	–0.5100	–0.0291	0.0125	0.3066	–0.3276	0.0741	–0.0492	–0.0136	**0.0900**	–0.1128	0.0036	**–0.45***
12	2013	–0.0276	–0.2232	0.3264	–0.0097	0.0080	–0.0084	0.0756	–0.0384	0.0429	–0.0253	–0.0088	**0.0200**	0.0085	**0.14**
	2014	–0.0060	–0.2720	0.5225	–0.0092	–0.0033	–0.0522	0.1392	–0.0052	0.0105	–0.0272	0.0046	**–0.0100**	–0.0117	**0.28**
	2016	0.0442	–0.1092	0.2850	–0.0285	–0.1400	–0.5329	0.3066	–0.0247	0.0288	0.0408	–0.0216	**0.4700**	0.0015	**0.32**

1, Plant height; 2, tillering capacity; 3, panicle weight; 4, grain weight from side stems; 5, panicle length; 6, spikelet number per panicle; 7, grain number per panicle; 8, empty spikelet number per panicle; 9, grain weight per panicle; 10, 1000-grain weight; 11, incidence of blind seed disease; 12, panicle density; 13, productivity (grain weight per plant); P_0, unaccounted (residual) factors (2013: min = –0.0126, max = 0.0414; 2014: min = –0.0117; max = 0.0027; 2016: min = –0.0057, max = 0.0084).

* The correlation coefficient is significant at 5 %.

рен в метелке, массой зерна с метелки, массой 1 000 зерен и плотностью метелки, однако отрицательным – в связях продуктивности с кустистостью, количеством пустых колосков в метелке и пустозерностью. В связях признака «масса всей метелки» с продуктивностью (по горизонтали табл. 3) положительным был косвенный эффект признаков «масса зерна с метелки» и «количество зерен в метелке», а отрицательным – кустистости, что с учетом большого прямого эффекта массы метелки выразилось в достоверной средней корреляции последней с продуктивностью (r = 0.49; 0.60; 0.56). Прямой (–0.01; –0.01; –0.03) и косвенные эффекты признака «масса зерна с боковых

побегов» незначимы. А очень тесная положительная корреляция этого признака с продуктивностью (0.99; 0.99; 0.99) была обусловлена во все три года косвенным эффектом кустистости, в 2013 г. – еще и массой 1000 зерен, в 2014 г. – и массой метелки, и количеством зерен с метелки, в 2016 г. – массой метелки, длиной метелки, количеством зерен с метелки, массой зерна с метелки и плотностью метелки. Слабый прямой эффект влияния на продуктивность оказывал признак «масса зерна с метелки» (0.11; 0.03; 0.12), несущественным был также и его косвенный эффект в связях продуктивности с другими признаками. Однако в связях признака «масса зерна с метелки» с продуктивностью положительным был большой косвенный эффект признака «масса всей метелки» при небольшом отрицательном косвенном эффекте кустистости, что в итоге выразилось в среднем положительном достоверном парном коэффициенте корреляции массы зерна с метелки с продуктивностью (0.40; 0.60; 0.56).

Большим был прямой эффект признака «кустистость» (0.72; 0.85; 0.52), положительными оказались также его косвенные эффекты в связях продуктивности с массой зерна с боковых побегов, однако отрицательными – с массой всей метелки, массой зерна с метелки и количеством колосков и зерен в метелке. В связях кустистости с продуктивностью положительные косвенные эффекты других признаков были незначительными, а отрицательный эффект массы всей метелки был небольшим, что в итоге сказалось на средней положительной величине парной корреляции между кустистостью и продуктивностью (0.55; 0.32; 0.34).

По признаку «масса 1000 зерен» прямой эффект был слабым (0.11; 0.08; –0.17), побочный – незначительным, а косвенный эффект большинства других признаков – невысоким положительным, в результате чего коэффициент парной корреляции между массой 1000 зерен и продуктивностью оказался низким (0.22; 0.11; 0.14). Неодинаковый уровень прямого эффекта на продуктивность наблюдался по признаку «плотность метелки»: незначительный в 2013 (0.02) и 2014 гг. (–0.01) при незначительном косвенном эффекте и при невысоком косвенном эффекте большинства других признаков, однако в 2016 г. прямой эффект был положительным (0.47) при незначительном косвенном эффекте признака и положительном косвенном эффекте отдельных других признаков (масса всей метелки, количество зерен в метелке), но отрицательным эффекте большинства других признаков, что выразилось в низких, хотя и положительных парных коэффициентах корреляции плотности метелки с продуктивностью (0.14; 0.28; 0.32).

Признак «высота растений» не имел значительного как прямого (к тому же разнонаправленного по годам – 0.12; 0.03; –0.17), так и косвенного влияния на продуктивность. Оно сказалось через небольшие положительные побочные эффекты ряда других признаков: в 2013 г. – кустистости, массы всей метелки, массы зерна с метелки и массы 1000 зерен, что выразилось в среднем парном коэффициенте корреляции между высотой и продуктивностью (0.31), а в 2014 г. – только массы всей метелки при отсутствии корреляции (–0.03), в 2016 г. – массы всей метелки и длины метелки, однако при значении прямого коэффициента пути –0.17 корреляция между высотой и продуктивностью практически отсутствовала (0.08).

Пустозерность тоже практически не имела прямого (0.02; –0.01; 0.09) и косвенного влияния на продуктивность, однако в связи с отрицательным косвенным эффектом других признаков (особенно массы всей метелки и количества зерен в метелке) парные коэффициенты корреляции между пустозерностью и продуктивностью были отрицательными, хотя и незначимыми в 2013 и 2014 гг. (–0.14; –0.22; –0.45).

Длина метелки в 2013 и 2014 гг. практически не оказывала значимого как прямого (–0.02; 0.01), так и косвенного эффекта на продуктивность, а положительный косвенный эффект кустистости был компенсирован отрицательным влиянием массы всей метелки и других признаков, в результате чего парные коэффициенты корреляции между длиной метелки и продуктивностью растения оказались практически нулевыми (–0.04; –0.07). В 2016 г., напротив, прямой эффект длины метелки был на уровне 0.25, а косвенный эффект в связях продуктивности с высотой и массой зерна с боковых побегов – положительным. Однако из-за отрицательного косвенного эффекта других признаков (плотность метелки, высота, количество колосков в метелке, масса 1000 зерен) парный коэффициент корреляции между длиной метелки и продуктивностью оказался практически нулевым (–0.01).

Признак «количество зерен в метелке» в 2013 и 2014 гг. не имел большого прямого (0.09; 0.16) и значимого побочного эффектов на продуктивность, и хотя косвенный эффект массы всей метелки был положительным, однако по большинству других признаков в 2013 г. он получен отрицательным, и в результате корреляция между количеством зерен в метелке и продуктивностью в 2013 г. оказалась незначимой (0.07). В 2014 г. отрицательные эффекты были менее значимы, и соответственно увеличился и коэффициент корреляции (0.27). В 2016 г. прямой эффект признака «количество зерен в метелке» составил 0.42 при положительном косвенном эффекте признака в связях продуктивности с массой всей метелки, массой зерна с боковых побегов, количеством колосков в метелке, массой зерна с метелки и плотностью метелки, а также при значительном косвенном эффекте массы всей метелки и плотности метелки, что выразилось в достоверном среднем парном коэффициенте корреляции между признаком «количество зерен в метелке» и продуктивностью растения (0.50).

Как прямой эффект признака «количество колосков в метелке» (–0.01), так и его побочные эффекты в 2013 г. были незначимыми, и хотя косвенный эффект признака «масса всей метелки» был положительным, однако из-за отрицательных косвенных эффектов большинства других признаков корреляция между количеством колосков в метелке и продуктивностью растения практически отсутствовала (0.07). В 2014 г. его значение составило 0.27, так как были положительными косвенные эффекты признаков «масса всей метелки» и «количество зерен в метелке», хотя прямой (–0.06) и косвенный эффекты признака «количество колосков в метелке» были незначимыми. В 2016 г. во влиянии признака «количество зерен в метелке» на продуктивность наблюдалась совсем иная закономерность:

прямой эффект был очень отрицательным, отрицательным был также побочный эффект его в связях продуктивности с массой всей метелки, массой зерна с боковых колосков, длиной метелки, количеством зерен в метелке, массой зерна с метелки и плотностью метелки. Отмечен положительный эффект с кустистостью, количеством пустых колосков в метелке, пустозерностью и массой 1 000 зерен, однако вследствие положительного косвенного эффекта других признаков (масса всей метелки, количество зерен в метелке, плотность метелки) парный коэффициент корреляции между изучаемым признаком и продуктивностью был средним (0.39).

Признаки «количество пустых колосков в метелке» и «пустозерность» практически не имели ни прямого, ни косвенного влияния на продуктивность растения, однако в связи с отрицательным косвенным эффектом ряда других признаков (особенно массы всей метелки и количества зерен в метелке) парный коэффициент корреляции был отрицательным.

Обсуждение

В исследованиях многих авторов показана целесообразность определения взаимосвязей между признаками растений различных культур, в том числе риса, с помощью как парных коэффициентов корреляции, так и путевых коэффициентов. В частности, установлены неодинаковые прямые и косвенные вклады отдельных признаков в их влияние на продуктивность риса (Samonte et al., 1998; Sürek, Beşer, 2003; Ekka et al., 2011).

В наших исследованиях найдены особенности взаимосвязей между различными признаками растений риса по парным коэффициентам корреляции, а также причинно-следственные зависимости в системе взаимосвязанных признаков в результате путевого анализа продуктивности растений. Установлено, что продуктивность растения, масса зерна с метелки и масса зерна с боковых стеблей положительно связаны между собой. Кроме того, положительная взаимосвязь наблюдалась между массой всей метелки, количеством зерен в метелке и плотностью метелки; между массой зерна с метелки, количеством зерен в метелке и количеством колосков в метелке; а также между массой зерна с боковых стеблей, количеством зерен в метелке, количеством колосков в метелке и плотностью метелки. Пустозерность отрицательно влияет на массу всей метелки, количество зерен в метелке, количество колосков в метелке и посредством этих признаков на плотность метелки. На массу 1 000 зерен отрицательно влияют количество зерен и количество колосков в метелке.

В результате путевого анализа продуктивности растений риса решен комплекс задач в системе причинно-следственной взаимосвязи признаков: установлен относительный вклад каждого из 12 признаков на продуктивность, причем как прямой (непосредственный), так и косвенный (побочный) эффекты при взаимодействии с другими признаками. Это дало возможность раскрыть причины взаимозависимостей между признаками и выделить признаки, с помощью которых целесообразно прогнозировать продуктивность растений.

Установлено, что уровень корреляции между показателями продуктивности и другими признаками растений неодинаково зависит как от прямого и косвенных эффектов влияния отдельного признака на продуктивность в связях продуктивности с другими признаками, так и от косвенных эффектов влияния других признаков в связях продуктивности с изучаемым отдельным признаком.

Уровень прямого эффекта влияния каждого отдельного признака на продуктивность неодинаков и может быть высоким положительным во все годы (масса всей метелки, кустистость) или в отдельный наиболее благоприятный по погодным условиям 2016 г. (плотность метелки, количество зерен в метелке, длина метелки), очень низким в благоприятный год (количество колосков в метелке) или незначимым (по остальным признакам).

Уровни каждого из косвенных (побочных) эффектов влияния каждого отдельного признака в связях продуктивности с другими признаками, а также косвенных эффектов других признаков в связях продуктивности с изученным отдельным признаком могут быть разными, даже противоположного направления, и зависят от погодных условий выращивания растений. К тому же уровень прямого эффекта влияния отдельных признаков на продуктивность растений не всегда одинаково влияет на уровень парных коэффициентов корреляции между ними и продуктивностью, так как в данном случае проявляется зависимость и от соотношения косвенных эффектов влияния других признаков на продуктивность.

Выводы

В результате путевого анализа продуктивности определена наибольшая селекционная ценность отдельных количественных признаков растений риса, которые могут быть детерминантными для отбора и прогноза уровня продуктивности растений:

– масса всей метелки, которая во все три года имела высокие прямой и в некоторой степени косвенный эффекты влияния на продуктивность и для которой получена достоверная положительная парная корреляция с продуктивностью;
– продуктивная кустистость, которая также во все годы имела высокие прямой и косвенный эффекты влияния на продуктивность и положительную парную корреляцию с продуктивностью.

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Conflict of interest. The authors declare no conflict of interest.
Received March 15, 2018. Revised February 28, 2019. Accepted March 2, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):439-447
DOI 10.18699/VJ19.512

Factors to affect inbred beet plants while developing material for linear selection

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Considering its capacities, the generative system of *Beta vulgaris* L. is regarded as highly productive. While inbreeding, the reproductive potential of cross-pollinated beet plants with gametophytic self-incompatibility (SI) changes significantly and is determined by a joint effect of multiple factors including the level of inbred depression. In the present study, original data have been obtained revealing relationships between inbred beet seed productivity, its self-incompatibility and microgametophyte parameters, which is crucial for developing and maintaining constant fertile beet lines. It has been discovered that inbred depression increases the number of sterile microgametes and anomalous pollen grains, reduces pollen fertility and the length of pollen tubes. As a result, the seed yield in inbred beet progeny, including SI ones, reduces significantly just after the third inbreeding. At the same time, highly productive inbred beet is characterized by a lower rate of pollen tube growth *in vitro*. In inbred plants, there is no close relationship between pollen viability and seed productivity, because the elimination of germinated male gametes and degeneration of seed embryos may go over the entire period of fertilization starting its progamic phase. The SI plants have more degenerating embryos than self-fertile ones, but seed vessel outgrowth in the seeds with abortive embryos makes them morphologically similar to fertile seeds. For that reason, when assessing inbred beet plants based on their self-incompatibility/self-fertility, one should consider the qualitative characteristics of the seeds. Using the method of recurrent selection based on such factors as seed productivity, pollen tube length and field germination rate increase the output of plant forms with a potentially high self-compatibility in their progeny. To support such genotypes in the progeny, one has to, starting from the third inbreeding, perform sib crossing to reduce the negative effect of inbred depression and self-incompatibility.

Key words: beetroot; inbreeding; inbreeding depression; seed productivity; incompatibility; microgametophyte; pollen germination; pollen tubes.

For citation: Fedorova M.I., Kozar E.G., Vetrova S.A., Zayachkovskyi V.A., Stepanov V.A. Factors to affect inbred beet plants while developing material for linear selection. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):439-447. DOI 10.18699/VJ19.512

Факторы, влияющие на репродуктивную способность инбредных растений свеклы столовой при создании линейного материала для селекции

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Генеративная система *Beta vulgaris* L. по своему устройству и возможностям относится к высоковоспроизводящей. При самоопылении репродуктивный потенциал перекрестноопыляемых растений свеклы столовой, имеющих гаметофитный тип самонесовместимости, существенно изменяется и определяется совокупным действием разных факторов, в том числе и уровнем инбредной депрессии. Впервые на культуре свеклы столовой получены оригинальные данные о характере взаимосвязей семенной продуктивности инбредных растений с функциональными параметрами микрогаметофита и степенью самонесовместимости, что имеет важное значение при создании и поддержании константных фертильных линий. Установлено, что в результате инбредной депрессии увеличивается число стерильных микрогамет и пыльцевых зерен с аномальным развитием; снижается фертильность пыльцы и длина пыльцевых трубок; семенная продуктивность в потомствах инбредных растений, в том числе склонных к самоопылению, резко снижается уже после третьего инбридинга. При этом для высокопродуктивных инбредных растений свеклы столовой характерна меньшая скорость роста пыльцевых трубок в условиях *in vitro*. Между уровнем жизнеспособности пыльцы и семенной продуктивностью инбредных растений тесная взаимосвязь отсутствует ввиду того, что элиминация проросших мужских гамет и дегенерация зародышей семян могут происходить на всем протяжении прогамной и последующих фаз оплодотворения.

Дегенерирующих зародышей у самонесовместимых форм намного больше, чем у самофертильных, но в результате разрастания околоплодника у семян с недоразвитыми зародышами наблюдается морфологическое сходство со всхожими семенами. Поэтому при оценке инбредных растений свеклы столовой по признаку «самонесовместимость/самофертильность» следует учитывать качественные характеристики семян. Использование метода рекуррентной селекции по признакам «семенная продуктивность», «длина пыльцевых трубок» и «полевая всхожесть» повышает выход форм с потенциально высокой самосовместимостью в потомстве. Для поддержания выделенных в инбредных потомствах ценных генотипов необходимо начиная с третьего инбридинга проводить сибсовые скрещивания, что позволяет снизить негативное влияние инбредной депрессии и самонесовместимости.
Ключевые слова: свекла столовая; инбридинг; инбредная депрессия; семенная продуктивность; самонесовместимость; микрогаметофит; жизнеспособность; пыльцевая трубка.

Introduction

Table beet (*Beta vulgaris* L.) is one of the most common root vegetables and is cultivated all over the world. The presence of heterocyclic nitrogen-containing betacyanin pigments such as betanin and betaine in its roots makes this culture unique and essential in terms of human nutrition (Krasochkin, 1971; Pialetti, 1976; Mabry, 1980; Escribano et al., 1998; Tesoriere et al., 2004; Lapin et al., 2007; Sleptsov et al., 2015).

Table beet outperforms other root vegetables in productivity due to the structure of the generative system and its cross-pollination potential. Table beet is clearly identified as a cross-pollinating plant with the gametophytic self-incompatibility system (Owen, 1942; Maletsky et al., 1970). However, in addition to pollinating by pollen grains from other plants (xenogamy), the plants can be pollinated by pollen grains from other flowers of the same plant (geitonogamy) and from the same flowers (autogamy) (Zhuzhzhalova et al., 2007).

A wide variety of foreign and domestic breeds of table beet is available in the market, however, the producers prefer F_1 hybrids, which are more unified, flexible, and therefore better suited for production. The cultivation technology minimizes the manual labor involved, which in turn reduces money and time costs (Burenin, Pivovarov, 1998; Fedorova, Burenin, 2010). The heterosis effect reaches its peak, when the progeny hybridity is at its maximum that is effectively achieved by various genetic reproductive systems, particularly cytoplasmic male sterility (CMS) potentially presenting in each cross-pollinated population (Maletsky, 2010).

Selection for heterosis includes several consecutive stages as follows: studying a variety of original material preferably among monogerm samples, obtaining inbred progenies, selecting valuable biotypes and using them as the base for obtaining fertile (mf) and sterile (ms) lines with high combining ability, obtaining F_1 hybrids with the set of agriculturally valuable attributes. Obtaining homozygous lines via inbreeding is considered a critical element of a formative process facilitating the identification of valuable biotypes with recessive alleles, when dealing with complex heterogeneous populations of cross-pollinating cultures. Self-pollination makes it possible to decompose heterogeneous populations into series of homozygous lines of target genes, which, in addition to inbreeding depression, possess valuable attributes (Vavilov, 1935).

At the early stages of CMS-based hybrid creation, it is not only genetic and breeding aspects that present problems for breeders, but seed production as well. As opposed to cross-pollinating variety populations, whose genetic stability is ensured by cross-pollination within the intrabreeding population, self-fertilization ability is required for obtaining and maintaining the lines (Balkov, 1978; Fedorova, Stepanov, 2005; Zhuzhzhalova, 2010; Burenin, 2015).

Seed productivity of inbred beet progenies is highly variable as a result of various genes, including the self-incompatibility gene, as well as inbreeding depression. Self-incompatibility during self-pollination occurs, when pollen grains and the pistil have matching S-genes. Depending on the action period of genes and localization of their byproducts, two types of homomorphic self-incompatibility are defined for the eudicot group, namely sporophytic and gametophytic. However, some species may demonstrate a combination of individual attributes of these systems (Zhuzhzhalova et al., 2007).

Growth inhibitor (protein complex) in the SSI system is activated at the stigma, when pollen grains and the pistil have matching monomers, which inhibit pollen germination (Lewis, Crowe, 1958; Knox, Heslop-Harrison, 1970; Foote et al., 1994; Chantha et al., 2013). According to the recent data, the GSI system in a number of species is based on recognizing specific ribonuclease (S-RNase) in the diploid tissue of the pistil splitting the stored rRNA of its own pollen grains (pollen tube growth is terminated) and S-related F-box proteins of the male haploid capable of inactivating a subset of S-RNase alleles, except for their allele relative (Entani et al., 2003; Yamane et al., 2003; Kubo et al., 2010; Ramanauskas, Igić, 2017; Chen et al., 2018). The SI system in beet plants is controlled by four S-loci, while having a gametophytic incompatibility type, but it still remains understudied at the molecular level (Lundqvist et al., 1973; Zhuzhzhalova, 2010).

While the percentage of self-incompatible plants in sugar beet populations may reach 50 % or more, they also include self-compatible plants, whose pollen grains germinate massively as a result of self-pollination (Magassy, 1965; Korneeva, Golichenko, 1989; Logvinov et al., 1993; Darmency et al., 2009). The plants setting up to 50 seeds are considered self-sterile or self-incompatible (SI), 50 to 100 seeds prone to self-fertilization (PSF), 100 seeds and above self-compatible or self-fertile (SF) (Balkov, 1978).

It is shown that compatibility attribute and the percentage of SF plants in inbred sugar beet progenies may become fixated; their seed productivity in a series of generations may increase, though it is not a universally observed phenomenon (Konovalov, Maletsky, 1990; Zhuzhzhalova, 2010; Oshevnev, Gribanova, 2010). Consecutive self-pollination reduces pollen grain fertility in inbred progenies, which in turn depends on the

genotype of the inbred plants (Maletsky, 1995; Zayachkovsky et al., 1999; Korneeva, Vlasyuk, 2003; Darmency et al., 2009). In addition, pollen grain fertility in self-compatible plants following single to triple inbreeding decreases significantly slower than in inbred progenies of self-incompatible plants, demonstrating a sharp increase in pollen grain development anomalies leading to microgametophytic sterilization during gametogenesis.

The further inbred reproduction leads to lower pollen fertility in SF-forms, which in case of deep inbreeding (up to I_{10}) may fall down to 20 % of the initial value (Konovalov, Maletsky, 1990; Fedorova, Stepanov, 2005). As a result, similarly to SI-forms, valuable genotypes may be lost in SF-forms, which occurs most often in the process of creating fertile B-lines responsible for CMS fixation (Lyalko et al., 1997; Zhuzhzhalova, 2010). Thus, when dealing with inbred table beet progenies, the primary goal is to search for the mf-forms not only prone to self-pollination but also capable of overcoming the inbreeding depression. At the same time, it is important that seed productivity threshold under inbreeding depression is not neglected.

The goal of the presented research was to study the relations between seed productivity and functional parameters of male gametophytes in table beet plants during inbreeding in order to develop constant lines and ensure their preservation and reproduction.

Materials and methods

The research was performed at the facilities of the Federal Scientific Vegetable Center (VNIISSOK) from 2007 to 2017 with seed plants of various inbred beet progenies obtained using Nezhnost variety population. The breed was derived via free cross-pollination of foreign hybrids with cylindrical root shape followed by individual selection, inbreeding, and sister crossing. It was the very first inbred generation of this population that included fully fertile and partially sterile plants with the CMS attribute manifested to various degrees (i. e. different ratios of sterile and fertile anthers at flower, inflorescence, or branch scales). CMS in table beet plants is manifested phenotypically as a marker coloring of ms-anthers with individual parts of plants showing a wide spectrum of maroon tints from pink to brown (Fedorova et al., 2011).

The plants were grown in a protected ground in unheated hangar- or block-type hothouses under conditions of spring-summer crop rotation with natural lighting using one-year (stecklings) or two-year development cycles. Enforced self-pollination of the seed plants and sib crossings were performed using two types of pollination bags, namely parchment for isolation of individual branches of the plant, and calico for individual plants and groups of plants. To overcome the inbreeding depression, sib (sister) group crossings (a group of at least three plants under the common pollination cover) were used starting with the third inbreeding.

Biometric and functional microgametophytic parameters in fertile and partially sterile inbred plants were studied *in vitro* using the technique developed at VNIISSOK (Kozar et al., 2017). The sample size for triple analysis calculations and measurements was 300–500 pollen grains. A microphotographic study was carried out using a Micros microscope with DCM 300 digital camera and Canon A560 photographic system with magnifications $\times 10 \times 10$ and $\times 20 \times 10$. Pollen grains from various fractions were calculated and their parameters measured using the Scope Photo software. The viability of the microgametophytic population was estimated as the percentage of germinated pollen grains in the total number of grains in the sample analyzed. Polymorphism was assessed based on the percent ratio between pollen grain fractions, which included fertile, germinated (viable), anomalous, and sterile grains.

The reproductive ability and self-compatibility of inbred plants were assessed based on seed productivity (SP) and field germination rate. Genotypes with seed productivities of 0 to 1 g were classified as SI regardless of the germination rate, as were plants with higher productivity with germination rates below 20 %; a PSF group included the plants with seed productivities from 1 to 4 g; and a SF group – the plants with seed productivities above 4 g with germination rates above 20 %.

Express tests of seed quality were performed by means of microfocus radiography using the PRDU-02 and RM-1 systems. The internal structure of seeds was analyzed using the methods described in Technique of Radiographic Studies for Agriculture (Arkhipov et al., 2001) and Crop Science and Radiographic Analysis of Seed Quality in Vegetable Cultures (Musaev et al., 2012). Mathematical processing of the results was implemented using the Microsoft Excel statistical analysis tools.

Results

Comparative analysis of the obtained SP data showed that inbred progenies (I_1–I_3) included both low productive plants and the ones setting a sufficient number of seeds, and the ratios between them varied significantly within the inbred progeny on the whole and for each sample in particular. The percentage of SI plants in inbred populations showed steady increase of as much as three times on average in each subsequent inbred generation. As a result, the percentage of plants capable of self-pollination in the third inbred populations was 33 %, whereas the same value for the first inbred generation was 90 %, with 56 % of plants having seed productivities of over 5 g. Average seed productivity parameters of the inbred plants from different generations remained approximately the same: I_1 – 11.2; I_2 – 11.9; I_3 – 10.4 g/plant (Suppl. material 1)[1].

The effect of consecutive self-pollination on the variability of microgametophytic parameters was studied and the relation of these parameters with the reproductive ability of the inbred beet plants was analyzed in breed sample 274, whose population was characterized by the lowest amount of SI genotypes (7 %). Here, the plants selected for further inbreeding were primarily the ones prone to self-fertilization, which were typically present in all inbred progenies. The structure of their inbred progenies included fully SI genotypes (SP = 0 g), whose percentage increased gradually with inbreeding depth ($I_1 \rightarrow I_5$) and reached 21 % in the fifth inbred generation (Table 1).

According to the average values, seed productivity in the plants capable of self-pollination remained high up to the third inbred generation. Further inbreeding dramatically reduced

[1] Supplementary Materials 1–3 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2019-23/appx8.pdf

Table 1. Seed yield and pollen fertility for different inbred beet progenies No. 274

Inbreeding	Seed yield, g/plant (average)	Plants (%) with seed productivity			Pollen fertility, % (average)	Portions of plants (%) with different levels of pollen fertility		
		0 g	0.1–4.0 g	>4 g		<70 %	70–90 %	>90 %
I_1	14.5	7	33	60	92	1	26	73
I_2	13.4	8	42	50	84	3	33	64
I_3	9.3	14	55	31	80	10	61	29
I_4	2.5	16	64	20	82	8	44	48
I_5	6.1	21	49	30	76	20	35	45
HCP_{05}	4.2				4			

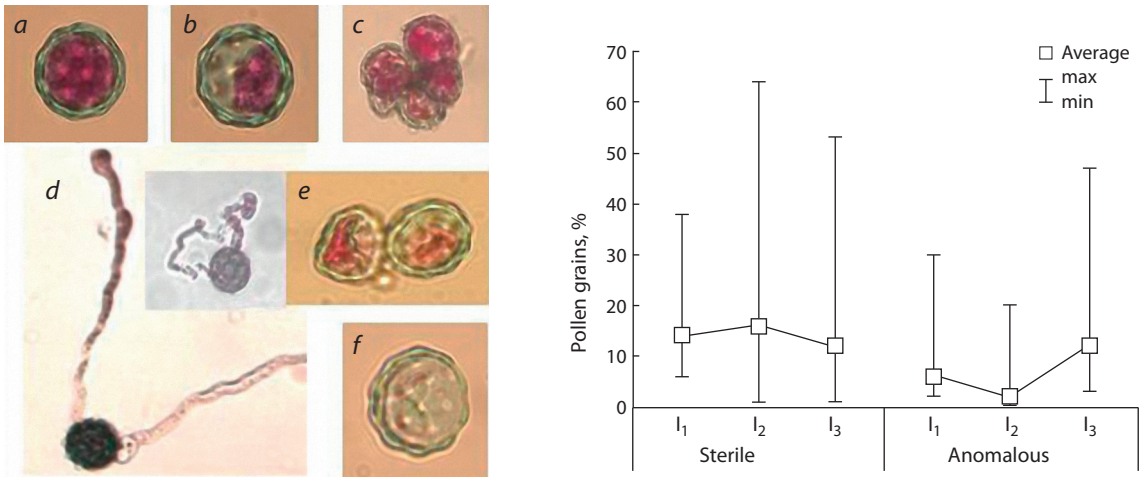

Fig. 1. Sterile and anomalous microgametes in the pollen populations of different inbred beet plants: *a*, fertile pollen grains; *b–e*, anomalous pollen grains; *f*, sterile pollen grains.

seed productivity in plants, including the SF group, which then only accounted for 20–30 %. It may be explained by the effect of growing inbreeding depression on the development of reproductive organs. As a result, pollen grain fertility decreased due to the increasing number of sterile microgametes and anomalous pollen grains (deformation, detachment of contents from the shells, germination of several pollen tubes, etc.); the number of these plants doubled in the fifth inbred progeny (Table 1, Fig. 1).

Inbreeding depression also affected functional parameters of pollen grains, primarily *in vitro* pollen tube growth. The average pollen tube length in plants from the fourth-fifth inbred generations after three-hour germination turned out to be twice as short as in plants from the third inbred generation. In addition, consecutive inbreeding progenies starting from the second inbred generation showed a gradual decrease in the variability of this attribute and an increase in the percentage of plants with low pollen tube growth rate ($L_{pt} < 200$ per unit) (Table 2).

In addition, plants from various inbred generations manifested differences, although less significant, in fertile pollen grain viability, which generally fell within a wide interval (0–55 %). However, no specific regularity was observed to describe variations in population structure regarding ratios of genotypes with different viability values. For instance, genotypes with average pollen grain germination rate (viability of 10–30 %) prevailed in the third inbred generation; low viability (<10 %) prevailed in the fourth inbred generation, but most plants from the fifth inbred generation showed the ability of pollen grains to germinate *in vitro* (with viability of over 30 %). It possibly has to do with phenotypic variability, which is to a significant degree determined by the effect of agricultural climatic cultivation conditions on simultaneous maturation and pollen grain maturity. In other words, the data obtained indicate that the inbreeding depression of microgametophytic development combined with self-incompatibility affects the reproductive ability in inbred plants.

Relation between seed productivity and microgametophytic parameters in beet plants with different inbreeding levels. Structural analysis of individual groups of inbred mf-plants with different SP values showed a certain relation between microgametophytic parameters and seed productivity up to the third inbreeding. For example, in the third inbred generation plants with low fertility (<70 %) and viability (<10 %) were only encountered in the low productive group

Table 2. Variability and structure of inbred beet progenies considering the viability and pollen tube length of the fertile pollen of seed plants

Inbred progeny	Viability, %		Plants with survival capacity, %			Pollen tube length, rel. units		Plants (%) with L_{pt} (rel. units)		
	average	min–max	< 10	10–30	> 30	average	min–max	< 200	200–300	> 300
I_3	20	1–44	23	48	29	446	78–790	19	14	67
I_4	17	0–52	44	32	24	263	94–540	24	44	32
I_5	25	0–55	20	20	60	257	110–430	28	30	42
HCP_{05}	9					84				

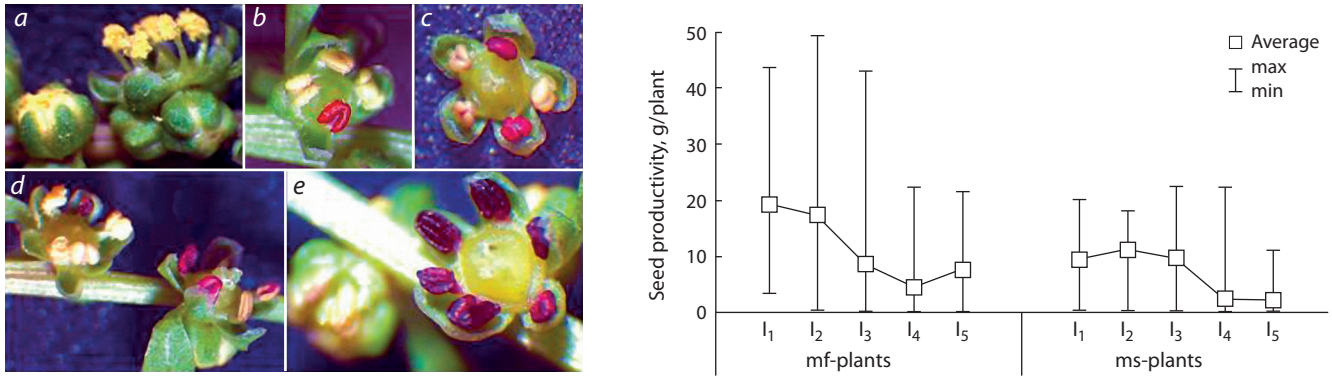

Fig. 2. Variability of the seed productivity in fertile (*a*) and partially sterile (*b, c* – individual sterile anthers at flower; *d, e* – ms-flowers and branches on a plant) plants in different inbred beet progenies.

(SP < 1 g); in the fourth inbred generation these genotypes were found in the group with average SP (1–4 g), and in the fifth inbred generation in the high productivity group (SP > 4 g) as well; in addition, the percentage of inbred plants with low pollen viability almost leveled off in all groups at 20–30 % of the total number of plants in each respective group. Inbreeding depression of pollen tube growth manifested itself in some plants as early as the second inbred generation, and percentages of these genotypes in groups with low and average SP reached 25 and 20 % respectively in the third inbreeding progeny (Suppl. material 2).

From the breeding perspective, the plants from all inbred progenies of high productive groups with high fertility and pollen grain viability of over 30 % are of interest. The percentage of plants with high pollen tube growth rates (L_{pt} > 300 μm/hour) in this group decreased with higher inbreeding levels and reached 20 % in the fifth inbreeding progeny, whereas this value was about 60 % in the low productive group (see Suppl. material 2).

A decrease in seed productivity and its variation range in the plants prone to self-pollination show similar patterns in mf-plants and partially sterile ms-plants (Fig. 2). At the same time, the following differences are observed: average seed productivity of ms-plants in case of self-pollination is significantly lower than in fully fertile plants, whose variation range is almost twice as wide in the first three inbreeding progenies. Sharp decrease in seed productivity was observed in mf-plants from the third inbred generation and in ms-plants from the fourth inbred generation with prevalence of SI forms. Thus, the observed growth in the number of SI plants in the fourth-fifth inbred generations is primarily associated with the presence of partially sterile plants in progenies, which are to be removed starting from the third inbreeding. It is especially important for developing a mf-line responsible for CMS fixation (see Table 1).

In other words, the research showed that reproductive potential of inbred plants subjected to multiple self-pollinations is to a greater extent determined by the degree of self-compatibility since both low and high seed productivities were observed in plants with different CMS manifestations and functional parameter combinations of pollen grains. The further analysis confirmed the absence of constant correlation between the microgametophytic attributes and seed productivity of individual plants from each inbred progeny. However, the mf-plants prone to self-fertilization demonstrated a stable trend in all progenies, namely that of a lower *in vitro* pollen tube growth rates in productive genotypes (Fig. 3, *a*). A similar relation was also observed in individual inbred families, especially in the ones with widely varying seed productivity. For example, the correlation coefficient between pollen tube length and seed productivity in plants from the third inbred generation was $r = –0.64$ for inbred families No. 274-2 and 274-4, while for other families it varied from $r = –0.19$ to $r = –0.59$.

The relation between pollen grain viability and seed productivity of inbred plants is ambiguous. A positive correlation between these parameters ($r = 0.40…0.49$) is only observed in generations with high inbreeding levels $I_{3–5}$ for the mf-plants prone to self-fertilization. Moreover, pollen grain viability

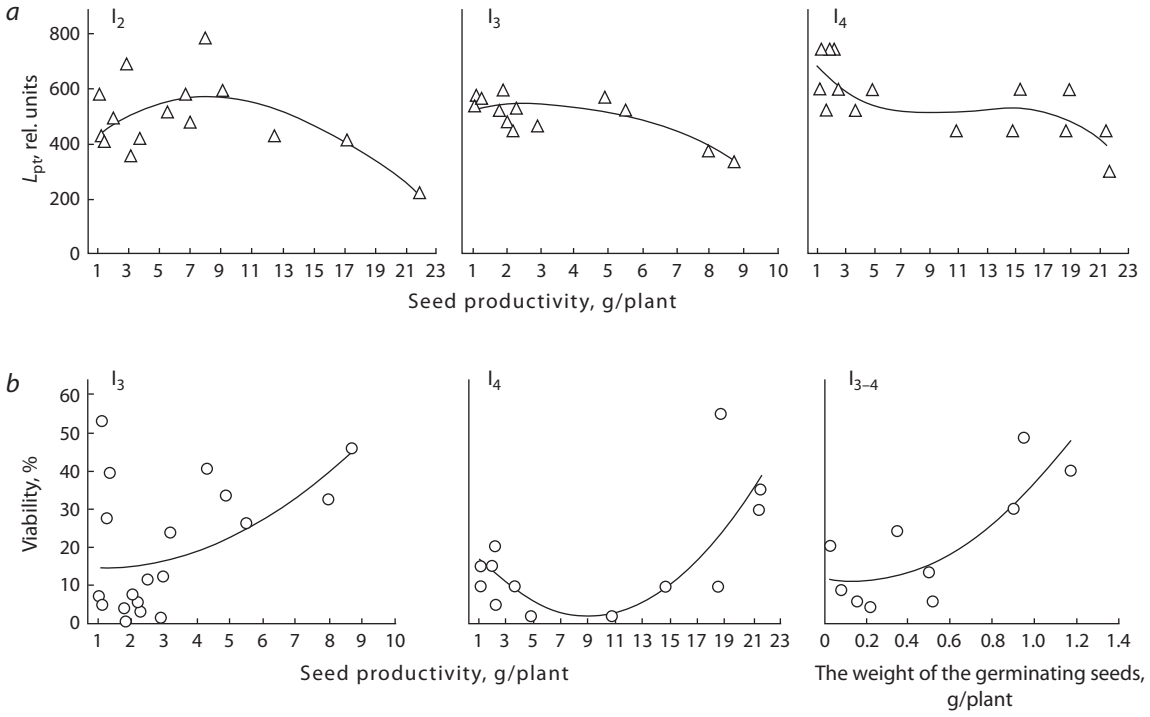

Fig. 3. Relation between seed productivity and pollen tune length (*a*), and the pollen-grain viability (*b*) of inbred mf-beet SI plants.

affected the number of fertile seeds (r = 0.53…0.56) with normal embryos capable of producing adult plants to form roots only in some of these progenies (Fig. 3, *b*). Thus, in case of comparable pollen grain viabilities, the fertilization outcomes may still be different for inbred plants with varying degrees of the SI attribute manifestation.

It is caused by the fact that male gamete elimination occurs throughout the whole duration of the progamic phase, and the more incompatible the plant, the earlier the progamic phase anomalies are observed and the more pronounced they are. The absence of fertilization is the main cause of embryo deaths. However, embryo development may continue up until the sphere stage even without fertilization, but as soon as the central cell does not function properly and does not form an endosperm, the embryo dies. A study of subsequent embryogenesis stages during seed formation has shown that embryo and seed degeneration in case of self-pollination of beet plants is observed at various development stages, while the number of degenerating embryos is much higher in self-sterile forms, than in self-fertile ones. The seeds with underdeveloped embryos bear superficial resemblance to fertile seeds as a result of seed vessel outgrowth (Zhuzhzhalova et al., 2007).

In this context, the fertile seed weight per plant calculated as $M_g = M_o \times B/100$, where M_o is the total seed weight (g) per plant, and B is the field germination rate (%), should be considered a more adequate criterion for ranking plants by self-incompatibility/fertility. Genotypes with $M_g < 0.5$ g/plant are attributed to the SI group; the ones with $0.5 \leq M < 1.0$ g/plant to the PSF group; and the ones with $M_g \geq 1.0$ g/plant to the SF group (Fig. 4, *a*). It can be seen from the figure that both SI and SF groups include plants with comparable total seed productivities, but field germination rate and therefore fertile seed weight is higher in SF plants with low productivity than in SI plants.

Microfocus radiography, which makes it possible to identify the presence of a developed embryo in each seed even before germination, may be used to obtain a preliminary estimate of the manifestation degree of self-incompatibility in the given inbred beet progeny (Musaev et al., 2017a, b). It can be seen from the radiographic image that internal areas in most seeds from inbred progenies 274/5-6-1-9a and 274/2-3-15-4a were either fully shaded or only show rudiments of non-viable embryos (light areas), which did not germinate when sown (Fig. 4, *b*).

Taking seed productivity and field germination rates into account, these progenies were attributed to the SI group (see Fig. 4, *a*); the progenies of families 274/5-5-3-3a and 274/5-5-3-1a were attributed to the SF group, since despite low total seed productivity (about 2 g), they were characterized by high filed germination rates (76 and 53 % respectively), which is also confirmed by radiography results. In addition, radiographic images may be used to identify individual plants and progenies as polygerm or monogerm, which is important for choosing a breeding type (see Fig. 4, *b*).

Sib crossing for preserving valuable inbred lines. Sib crossings within individual progenies of mf-forms reduce the inbreeding depression effect on microgametophytic functional parameters. For instance, pollen grain viability in plants from progenies of the initial inbred form 274-5-6-1 increased by 4–32 % depending on the progeny (apart from 274-5-6-1-11a). Pollen tube growth rate also increased in most samples, the tube length being 1.2–2 times as high as in inbred plants. Progenies of 274-5-6-1-11a and 274-5-6-1-12a were the only

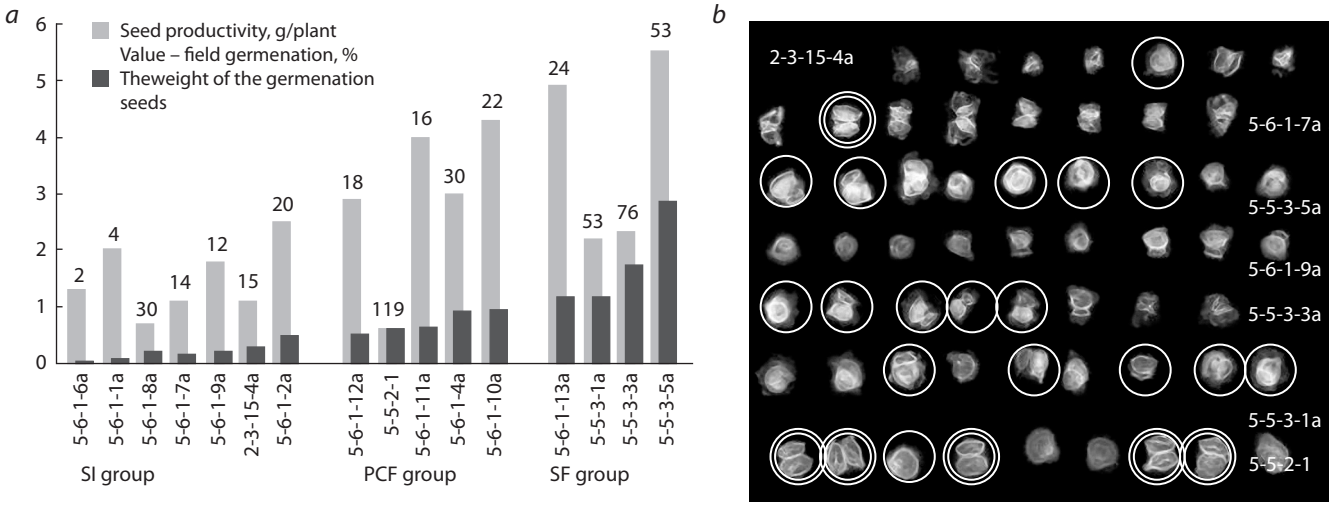

Fig. 4. Fertile-seed mass distribution for inbred beet progenies (*a*), and X-ray image fragments of the seeds (*b*).
O – Fertile one-seeded fruit; ⓪ – Fertile two-seeded fruit.

exceptions, where the value considered varied within the experimental error (Suppl. material 3).

A close relation was observed between pollen grain viability and pollen tube length in plants from inbred and sib progenies ($r = 0.88$ and $r = 0.64$ respectively), as opposed to seed productivity since its increase during sib crossings was caused not only by reduced inbreeding depression but the absence of negative self-incompatibility effects as well. Nevertheless, an inverse relation was observed between tube lengths and seed productivities ($r = –0.47$), similarly to inbred progenies.

In most progenies resulting from sib crossings, productivity either showed statistically significant increases (1.4–6.6 times and more) or remained at the level typical for inbred plants. The quality of the obtained seeds improved significantly, and their field germination rates per seed unit (glome) were over 40 % in most progenies. Here, 42 % of sib progenies had polygerm glomes (with the field germination rate of over 100 %), whereas the percentage of polygerm samples among inbred progenies did not exceed 10 % of all plants studied. This should be taken into account while creating fertile lines, especially the ones responsible for sterility fixation since it requires monogerm plants.

Conclusions

Obtaining self-fertilizing constant lines for table beet heterosis breeding is a complex process, because the self-incompatibility attribute is under gametophytic control, and its manifestation degrees vary significantly in plants from inbred progenies. In case of self-pollination, the reproductive ability of table beet plants also depends on the inbreeding depression level, which affects microgametophytic functional parameters and seed embryo development.

The use of recurrent breeding based on seed productivity, pollen tube length, and field germination rate increases the yield of forms with potentially high self-compatibility in the progeny. To ensure reproduction and preservation of the valuable linear material, one should perform sib crossings not later than the third or earlier inbred generations from promising inbred progenies by selecting the mf-forms most prone to self-fertilization.

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

Received September 21, 2018. Revised March 21, 2019. Accepted March 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):448-455
DOI 10.18699/VJ19.513

Genetic approaches to the investigation of serotonergic neuron functions in animals

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The serotonergic system is one of the most important neurotransmitter systems that take part in the regulation of vital CNS functions. The understanding of its mechanisms will help scientists create new therapeutic approaches to the treatment of mental and neurodegenerative diseases and find out how this neurotransmitter system interacts with other parts of the brain and regulates their activity. Since the serotonergic system anatomy and functionality are heterogeneous and complex, the best tools for studying them are based on manipulation of individual types of neurons without affecting neurons of other neurotransmitter systems. The selective cell control is possible due to the genetic determinism of their functions. Proteins that determine the uniqueness of the cell type are expressed under the regulation of cell-specific promoters. By using promoters that are specific for genes of the serotonin system, one can control the expression of a gene of interest in serotonergic neurons. Here we review approaches based on such promoters. The genetic models to be discussed in the article have already shed the light on the role of the serotonergic system in modulating behavior and processing sensory information. In particular, genetic knockouts of serotonin genes *sert*, *pet1*, and *tph2* promoted the determination of their contribution to the development and functioning of the brain. In addition, the review describes inducible models that allow gene expression to be controlled at various developmental stages. Finally, the application of these genetic approaches in optogenetics and chemogenetics provided a new resource for studying the functions, discharge activity, and signal transduction of serotonergic neurons. Nevertheless, the advantages and limitations of the discussed genetic approaches should be taken into consideration in the course of creating models of pathological conditions and developing pharmacological treatments for their correction.

Key words: serotonergic neurons; genetic models; viral transduction; optogenetics; chemogenetics.

For citation: Drozd U.S., Shaburova E.V., Dygalo N.N. Genetic approaches to the investigation of serotonergic neuron functions in animals. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4): 448-455. DOI 10.18699/VJ19.513

Генетические подходы к изучению функций серотонинергических нейронов у животных

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Серотонинергическая система, которая принимает участие в регуляции большинства функций ЦНС, является одной из важнейших нейротрансмиттерных систем. Патогенез многих психических и нейродегенеративных заболеваний включает нарушения в функционировании этой системы. Понимание механизмов ее работы поможет не только разработать новые терапевтические подходы к лечению, но и установить, как эта нейротрансмиттерная система взаимодействует с другими отделами мозга, регулируя их деятельность. Ввиду сложности и гетерогенности анатомо-функционального устройства серотонинергической системы, в настоящее время лучшими инструментами для ее изучения являются методы, основанные на манипулировании отдельными типами нейронов и не затрагивающие нейроны других нейротрансмиттерных систем. Такое избирательное управление клетками возможно за счет генетической детерминированности их функций. Белки, обусловливающие уникальность клеточного типа, экспрессируются в нем под регуляцией клеточно-специфичных промоторов. С использованием промоторов, специфичных для генов серотониновой системы, возможно управление экспрессией гена интереса в серотонинергических нейронах. В обзоре рассмотрены подходы с применением таких промоторов. Генетические модели, созданные при помощи описанных подходов, используются для установления роли серотонинергической системы в модулировании поведения и обработке сенсорной информации. В частности, генетические нокауты по серотониновым генам *sert*, *pet1* и *tph2* помогли выяснить вклад

этих генов в формирование и функционирование головного мозга. Кроме того, описываются индуцибельные модели, которые позволили управлять экспрессией генов на различных стадиях онтогенеза. И наконец, приведены примеры достижений в применении этих генетических подходов в оптогенетике и хемогенетике, которые предоставили новый ресурс для изучения функций, разрядной активности и сигнальной трансдукции серотонинергических нейронов. При создании моделей патологических состояний и разработке фармакологических средств их коррекции на основе рассмотренных генетических подходов необходимо учитывать, что каждый из них имеет свои достоинства и ограничения, и выбирать наиболее подходящий из них.
Ключевые слова: серотонинергические нейроны; генетические модели; вирусная трансдукция; оптогенетика; хемогенетика.

Introduction

The serotonergic neurotransmitter system is involved in the regulation of many physiological functions, such as pain sensitivity, the sleep/wake cycle (Whitney et al., 2016), nutritional and reproductive behavior, cognitive functions, and mood (Wong-Lin et al., 2017). It also modulates the functions of the olfactory system (Carlson et al., 2016). Many clinical and preclinical studies emphasize the role of serotonin in the pathogenesis of various mental disorders (Vadodaria et al., 2018).

Serotonin synthesis in the CNS is performed by serotonergic neurons located in brainstem nuclei, the ventromedial part of the medulla oblongata, and the reticular formation of the pons (Baker et al., 1991). The projections of these neurons are widely distributed in the brain and spinal cord. The relatively small number of serotonin neurons and the scattered distribution of their axons complicate the task of studying their functions. In addition, although serotonin neurons are anatomically clustered, their groups are not homogeneous in their electrophysiological properties and usually consist of several subpopulations, which are extensively studied (Ren et al., 2018). Thus, electrophysiological studies carried out by introducing an electrode into raphe nuclei do not provide an adequate understanding of the functions of individual cell populations (Calizo et al., 2011). Although works conducted using pharmacological approaches have made a significant contribution to understanding serotonin neurotransmission, they also have limitations: peripheral effects, the inability to control certain types of neurons, and, in some cases, unclear mechanisms of action (Choi et al., 2004). Therefore, nowadays the attention of researchers is focused on genetic tools that make it possible to manipulate certain types of neurons without affecting neurons of other neurotransmitter systems.

Gene promoters used for transgene expression in serotonergic neurons

Expression of a gene of interest in cells of a certain type is supported by tissue-specific promoters. For expression of target genes in serotonergic neurons, the *tph2*, *pet-1*, and *sert* promoters are used (Hainer et al., 2016). The advantage of the *tph2* gene (neuronal tryptophan hydroxylase), the key enzyme in serotonin biosynthesis, is that it is highly expressed exclusively in serotonergic neurons within the brain, unlike the *tph1* gene, which is hardly expressed in the CNS (except for pineal gland cells, in which it serves as an intermediate in melatonin synthesis) (Patel et al., 2004). The *tph2* promoter contains a NRSE silencer, which prevents transcription of this gene in non-neuronal cells (Patel et al., 2007), and binding sites for the Pet-1 transcription factor (Liu et al., 2010), which directs the development of serotonergic neurons during ontogeny and plays an important role in maintaining the serotonergic phenotype in adult neurons (Liu et al., 2010; Fernandez et al., 2017). Due to the fact that Pet-1 itself is a marker of serotonergic neurons, its gene promoter (or, specifically, its enhancer (Scott et al., 2005)) is also considered tissue-specific for them (Deneris, 2011). Among others, Pet-1 controls the expression of *sert* (or *slc6a4*), a serotonin transporter gene, whose promoter is also used in studies of serotonergic neurons (Hainer et al., 2016). However, unlike Pet-1 or Tph2, *sert* is expressed not only in serotonin neurons but also in astrocytic glia (Blakely, 2001) and can be synthesized in neurons of the prefrontal cortex, thalamus, and retina during the development of the nervous system (Gaspar et al., 2003).

In addition to aforementioned genes, those for aromatic L-amino acid decarboxylase (*aadc* or *ddc*), vesicular transporter of monoamines (*slc18a2* or *vmat2*), monoamine oxidase A and B (*maoa* and *maob*), 5-HT1a/b-autoreceptors (*htr1a* and *htr1b*) are vigorously involved in the functioning of the serotonergic neuron, and so are genes encoding enzymes for the synthesis and reduction of tetrahydrobiopterin (BH4) (Müller, Jacobs, 2010). However, the endogenous promoters of these genes are either weak or not specific enough to be used for gene manipulation in serotonergic neurons (Deneris, Wyler, 2012).

Transgenic animal models for examining serotonergic neurotransmission

Transgenic animals are widely used for studying the serotonergic system. Animal models are used to clarify the role of specific genes in the development and functioning at different levels of organization: from a single cell to the whole body. The most common genetic models are knockout, knock-in, and BAC-based animals. A bacterial artificial chromosome (BAC) is a DNA construct whose length can exceed 350 thousand base pairs (Shizuya et al., 1992), and this feature makes it applicable for large-insert cloning of genes or their promoter regions (Zhuang et al., 2005). In this case, transgenesis is carried out by introducing BAC into the zygote pronucleus (Richardson-Jones et al., 2010). Genetic knockout and knock-in are usually obtained in two steps: first, the gene of interest is inserted into the embryonic stem cells using homology-directed repair (To get knockout, the sequence replaces the gene of interest.) and then the modified cells are introduced into the blastocyst. Adult chimeric animals are mated with wild-type animals, and their heterozygous offspring are selected (Zhuang et al., 2005). These offspring are also crossed among themselves and animals carrying the insertion in the

homozygous state are selected from the third generation (Richardson-Jones et al., 2011).

To examine the serotonergic system with above approaches, different lines of transgenic animals have been generated and most of them are based on the Cre-LoxP or Flp-FRT recombinations. Cre and Flp recombinases recognize the LoxP and FRT sequences, respectively, and catalyze the recombination of the flanked DNA fragment to remove or invert it. The flanked region of DNA can be a gene or a stop cassette in front of the gene, so its removal turns the expression off or on (Muñoz-Jiménez et al., 2017). Based on this recombination, several lines of transgenic animals have been raised for targeting the serotonin system, where Cre recombinase expresses under the control of a specific promoter (*sert*, *pet-1*, or *tph2*) (Hainer et al., 2016). These animals are used for obtaining genetically modified animals with conditional knockout (Liu et al., 2010) and conditional induction of the serotonin system genes (Piszczek et al., 2013) to study the contribution of particular genes (Features and design of these and other genetic models are described in the Table.) For example, the same approach proves the necessity of Pet-1 for not only launching the serotonin program in neurons but also maintaining the serotonergic phenotype in the future (Liu et al., 2010).

Moreover, use of both Cre and Flp recombinases allows studying individual neuron subtypes in the serotonin system, as well as its development, by analyzing the descendants of certain rhombomeres (Kim et al., 2009). There are also many other methods to produce transgenic animals without recombinases. An example is the CRISPR/Cas9 technology, by which the first transgenic pigs with *tph2* knockout were obtained (Li et al., 2017).

Despite the undeniable contribution of genetically modified animals with constitutive changes in gene expression described previously, their main disadvantage is that they are unfit for studying the effects of a single alteration in the neurotransmitter system, because during the development of the nervous system they may experience a compensatory effect of other neurotransmitter systems, as well as aberrations in neurons in general (Hainer et al., 2016).

Inducible expression models

Inducible genetic models permit one not only to avoid adaptive effects but also to study the development of the serotonergic system in ontogeny. One of the widely used systems for gaining spatial and temporal control over transgene expression is the inducible system Cre-ERT2. Cre-ERT2 recombinase is a chimeric protein constructed by fusion of Cre-recombinase and a mutant human estrogen receptor form, which binds the synthetic ligand tamoxifen (TMX) instead of estradiol. After being activated by tamoxifen treatment, chimeric recombinase passes through the nuclear membrane into the nucleus, and induces flanked region recombination, resulting in temporal control of the gene of interest (Kristianto et al., 2017).

Another way to control transgene expression without using Cre recombinases involves ligand-inducible systems, such as Tet-ON/Tet-OFF. Administration of tetracycline-like compounds causes changes in the tetracycline-dependent transactivator (rtTA or tTA) conformation. Depending on the type of system, this protein becomes able to bind (Tet-ON) or not to bind (Tet-OFF) the tetO sequence, thereby initiating or blocking transgene expression, respectively (Das et al., 2016). Besides, there is a modification of this system in which a gene under control of a tissue-specific promoter encodes a chimeric tetracycline-dependent transactivator protein fused with the KRAB domain, a strong repressor. The resulting chimeric protein strongly suppresses the expression of the gene of interest by binding to the tetO upstream of it (Richardson-Jones et al., 2011). Several genetic models based on the Tet-OFF and Tet-ON systems have been produced for examining the serotonergic system (Weber et al., 2012; Donaldson et al., 2014; Hilber et al., 2015). For example, the effect of reduced expression of autoreceptors 5-HT1A on anxiety behavior in adulthood has been discovered (Donaldson et al., 2014). However, unlike Cre activated recombinases, the effect of the tetracycline-inducible system is reversible, because it does not change the DNA sequence, and this feature expands the range of possible applications of this system. The disadvantage of this method is that doxycycline (the most stable and common tetracycline-like compound) has its own effects on the animal behavior and the expression of some genes (Shishkina et al., 2017), which impede the interpretation of behavioral test results.

Viral vectors

Viral vectors are another promising approach for studying the serotonergic system. They are stereotaxically delivered to the raphe nuclei at a certain developmental stage and do not affect foregoing ones. Many studies have been conducted to apply the viral vector approach to transgenic animals. Most of them are based on Cre/LoxP recombination (Tye, Deisseroth, 2012; Verheij et al., 2018), so that vectors should ensure the expression of their transgene in cells where Cre recombinase is synthesized. Nowadays, there are over 250 Cre transgenic mouse lines available as part of "Gene Expression Nervous System Atlas" project in collaboration with the Intramural Research Program of the National Institute of Mental Health (http://www.gensat.org) (Gong et al., 2007) and from commercial repositories such as the Jackson Lab (http://jaxmice.jax.org).

Along with that, efforts are being made to create viral vectors specifically expressing a transgene in the serotonergic system not only in transgenic animals but also in wild-type animals or selection lines. Benzekhroufa et al. (2009) designed a vector for serotonin neurons where a two-step transcription amplification system (TSTA) was used to increase the expression of the gene of interest. In this system, the enhancement of the *tph2* promoter fragment was achieved by adding the UAS enhancer and the GAL4-p65 chimeric *cis*-regulator, as well as many variations of the UAS enhancer cassettes to the promoter. This approach allows achieving high and specific transgene expression in serotonin neurons. Based on these constructs, lentiviral and adeno-associated viral vectors were created for serotonin neuron targeting (Benzekhroufa et al., 2009). Moreover, these vectors were improved by optimizing the IRES site and adding the WPRE sequence to increase the expression level of the genes delivered by the retroviral vectors (Nishitani et al., 2019).

Another interesting approach takes advantage of viral delivery and RNA interference. The interaction of small interfering RNAs with the mRNA of the target gene leads to the mRNA destruction, thus preventing its translation and inhibiting the

Genetic models used to study the serotonergic system

Genetic model	Model design	Model features	Example
Transgenic (BAC)	Injection of a genetic construct with a gene of interest under the control of a cell-specific promoter into an animal pronucleus	Genetic construct persists in all cells lifelong, not reversible, can be transmitted to offspring	Zhao et al., 2011
Targeted viral transduction	Stereotaxic injection of a genetic construct with a gene of interest under the control of a cell-specific promoter in the region of interest	Genetic construct is stereotaxically delivered in the area of interest and can provide inducible and reversible specific expression of the gene of interest	Benzekhroufa et al., 2009
Knockout	An existing gene is replaced or disrupted with an artificial piece of DNA (STOP cassette or a new gene)	The gene is absent from all cells of the body throughout life	Mosienko et al., 2012
Conditional knockout	An animal bearing the Cre gene downstream (and under the specific control) of the cell-specific promoter and an animal bearing the LoxP gene are crossed	Gene expression is absent only from CRE-expressing cells	Liu et al., 2010
Conditional induction	An animal bearing the Cre gene downstream of the cell-specific promoter and an animal bearing a STOP sequence flanked by LoxP are crossed	Gene expression is observed only in CRE-expressing cells	Piszczek et al., 2013
Inducible knockout	An animal bearing the gene for inducible CRE-ERT2 recombinase downstream of the cell-specific promoter and an animal bearing a gene of interest flanked by LoxP are crossed	Gene expression is absent only from CRE-expressing cells after tamoxifen treatment. The system provides time- and space-specific irreversible lack of gene expression	Liu et al., 2010
Inducible transgenic	An animal bearing the tetO sequence upstream of a gene of interest and an animal bearing a gene for tetracycline transactivator (tTA or rtTA) protein downstream of the cell-specific promoter are crossed	Modulation of gene expression occurs only in cells expressing tTA or rtTA after treatment with tetracycline-like compounds. The system provides modulation of temporal- and space-specific reversible gene expression	Hilber et al., 2005
Inducible suppression	An animal bearing the tetO sequence upstream of a gene of interest and an animal bearing a gene for tetracycline transactivator (tTA or rtTA) protein fused with KRAB downstream of the cell-specific promoter are crossed	Modulation of gene expression occurs only in cells expressing tTA-KRAB or rtTA-KRAB after tetracycline-like compounds treatment. The system provides modulation of time- and space-specific reversible gene expression	Richardson-Jones et al., 2011
Knock-in	The insertion of a gene or promoter sequence before the gene of interest	The expression of the endogenous gene of interest is preserved	Sachs et al., 2013
Intersectional transgenic	An animal bearing sequences flanked by loxP and FRT (LoxP - STOP - LoxP - FRT - gene 1 - STOP2 - FRT - gene 2) and an animal bearing Cre and Flp recombinases under the specific control of two different cell-specific promotors are crossed	Gene expression depends on the expression patterns of Flp and Cre recombinases; suitable to study different subtypes of neurons in neurotransmitter systems	Kim et al., 2009
shRNA suppression	Injection of short hairpin RNA (shRNA), which depletes target gene expression, into the region of interest in the brain	The expression of the endogenous gene of interest is suppressed for a short period (several days after a single injection)	Verheij et al., 2018

synthesis of the encoded protein (Rao et al., 2009). Thereby, miRNAs control gene expression not at the transcriptional level as in the above systems but immediately after it. In addition, the effect of a single injection of siRNA lasts only for a few days (Albert et al., 2014). For viral targeting, a functional miRNA analogue, small hairpin RNA (shRNA), is used (Rao et al., 2009). The main feature of this system is its targeting. A miRNA can work only in those cells where the mRNA of its specific gene is synthesized. However, this is the main obstacle for its use: the gene of interest must be endogenous and tissue-specific. This method is also applicable to studies of the serotonergic system (Gautier et al., 2017; Verheij et al., 2018). For example, when examining the effects of *tph2* gene expression suppressing in bulbospinal serotonin neurons, their role in modulating the perception of neuropathic and inflammatory pain was shown (Gautier et al., 2017).

In general, the delivery of DNA and/or RNA using recombinant viral particles holds promise not only in the study of serotonergic neurons but also in medicine, in particular, in genetic therapy. However, it has its drawbacks. For example, the organism may develop an immune response, resulting in the loss in delivery effectiveness with repeated injections (Lukashev, Zamyatnin, 2016). Another problem is the stereotaxic method of delivery of the virus. Invasive intervention injures the brain, which may adversely affect the research results.

Optogenetic and chemogenetic approaches

The methods described above for achieving cell-specific gene expression are used in optogenetics and chemogenetics. Both approaches permit one to control a certain type of neurons in a selective manner to study their functions, firing activity, and signal transduction. In optogenetics, cell-specific promoters are used to govern the expression of genes for light-activated ion channels or pumps genes in the neuron type of interest. Optogenetic proteins change the permeability of the cell membrane for certain ions, which makes it possible to control the discharge activity of the cell with high temporal resolution (Lammel et al., 2016). For optogenetic studies of serotonergic neurons, a BAC line of transgenic Tph2-ChR2 (H134R)-EYFP mice has been developed, in which the transcription of membrane-depolarizing Channelrhodopsin-2 (ChR2) is controlled by the *tph2* promoter (Zhao et al., 2011). The same promoter has been used to create mice in which the ChR2 (C128S) gene is located after the tetracycline-dependent operator (tetO), while tTA is under the control of the *tph2* promoter. By using this line of mice with inducible expression of ChR2 in serotonin neurons, the contribution of these cells to the modulation of anxiety-like behavior and patience to wait for a delayed reward was studied (Miyazaki et al., 2014; Ohmura et al., 2014).

There is one more general approach that does not require the creation of a separate line of animals for the expression of each opsin type in target cells. It is the use of lines expressing Cre recombinase under the control of the *sert* or *pet-1* promoter. In this approach opsins are delivered to neurons by transduction with adeno-associated viruses. The vector contains the opsin gene in the inverted orientation. The gene is flanked by DIO (Double-floxed Inverted Orientation) on both sides and located downstream from the strong neuronal promoter *hSyn*. As an example, Sert-Cre mice were injected with viral vectors containing depolarizing Channelrhodopsin (AAV-hSyn-DIO-ChR2) and hyperpolarizing halorhodopsin (AAV-hSyn-DIO-NpHR) to elucidate the role of the serotonergic system in social deficit in the mouse model of autism (Walsh et al., 2018). Other studies using the same approach to deliver ChR2 to serotonergic neurons of the dorsal raphe nucleus revealed the roles of these cells in suppressing spontaneous discharge activity in olfactory neurons (Lottem et al., 2016), in the neurological response to the expected reward (Li et al., 2016), and in suppressing spontaneous locomotor activity in the open field and reducing the speed of movement (Correia et al., 2017). In addition to the Sert-Cre line, where the *sert* promoter is used to achieve specific expression of Channelrhodopsin in serotonin neurons, the similar Pet1-Cre line is used in optogenetic studies (Challis et al., 2014; Liu et al., 2014; Luo et al., 2017).

To deliver optogenetic proteins to serotonergic neurons of animals that had not been subjected to genetic modifications, lentiviral vectors based on the rat and mouse *tph2* promoters were designed. They made it possible to study the differences in the depressive-like and anxious behavior of these species, stimulating the neurons of the dorsal raphe nucleus (Nishitani et al., 2019).

In chemogenetics, receptors designed to interact with chemical ligands that are inert in the body are used. Nowadays, there are a number of receptors associated with G-proteins called DREADD (Designer Receptors Exclusively Activated by Designer Drugs), which have been obtained by modification of human muscarinic receptors. These receptors no longer respond to acetylcholine nor to any other endogenous molecule, but they bind to clozapine N-oxide (CNO) instead (Alexander et al., 2009). For example, one of the most commonly used DREADDs, hM3Dq, binding to CNO, triggers an intracellular cascade via Gq protein and activation of phospholipase C, which leads to depolarization of neurons and increases their discharge activity (Urban, Roth, 2015). Another chemogenetic receptor, hM4Di, inhibits the discharge activity of neurons through Gi protein (Zhu, Roth, 2014).

Currently, several options are available to achieve the expression of DREADD in genetically determined cells. There are lines of genetically engineered mice in which hM3Dq expression is controlled by the Tet-OFF system (Alexander et al., 2009; Garner et al., 2012) or Cre-Lox recombination (Teissier et al., 2015). In addition, a growing number of promoters are being used in many viral vectors for cell-specific expression of DREADDs, including modified herpes simplex viruses (Ferguson et al., 2011), adeno-associated viruses (Zhu et al., 2014; Scofield et al., 2015) and lentiviruses (Mahler et al., 2014; Vazey, Aston-Jones, 2014).

Numerous studies using chemogenetic approaches were conducted to perceive the functions of serotonergic neurons and their projections. In such experiments mice of the Sert-Cre line are injected with adeno-associated viruses containing the sequence hM4Di or hM3Dq (AAV-hSyn-DIO-hM4Di/hM3Dq) to obtain its expression in the serotonergic neurons (Urban et al., 2016; Fernandez et al., 2017; Singh et al., 2017). For example, it was found that serotonergic projections from the median raphe are essential for regulating the memory of objects and the synaptic plasticity of the hippocampus (Fernandez et al., 2017). In another study, pet1-Cre mice were similarly used to activate and inhibit serotonergic neurons in order to show that serotonergic neurons in the medial raphe nucleus play a key role in regulating anxiety- and depression-like behavior (Li et al., 2018).

Conclusion

Serotonergic neurons form an intricate and extensive network of axon projections throughout the brain. The main task of analyzing these neuronal circuits is to understand how serotonergic networks are related to the numerous functions of this neurotransmitter system. Several new techniques for manipulating subpopulations of serotonergic neurons have been designed in recent years: various lines of animals have been created using the double recombination strategy to achieve transgene expression exclusively in serotonergic neurons. Inducible systems for temporal control of gene expression,

DREADDs and optogenetics, were also successfully used to investigate serotonergic neurotransmission. Depending on the goals of the experiments, the researchers choose which of the available variants of gene expression regulation to use, because each of them has its own advantages and limitations. It is important to note that these models are not completely free of nonspecific effects. Adequate controls should be included in the experimental design for the most accurate and informative interpretation of the results. Nevertheless, the tools and methods depicted in this review, both individually and in combinations, open up new possibilities for the study of the serotonergic neurotransmitter system.

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Acknowledgement. This work was financially supported by the Russian Foundation for Basic Research, project 18-315-00114 mol_a, and the Russian Academy of Sciences, project 0324-2019-0041.

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

Received December 28, 2018. Revised February 25, 2019. Accepted March 1, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):456-464
DOI 10.18699/VJ19.514

Effect of neonatal dexamethasone treatment on cognitive abilities of adult male mice and gene expression in the hypothalamus

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The early postnatal period is critical for the development of the nervous system. Stress during this period causes negative long-term effects, which are manifested at both behavioral and molecular levels. To simulate the elevated glucocorticoid levels characteristic of early-life stress, in our study we used the administration of dexamethasone, an agonist of glucocorticoid receptors, at decreasing doses at the first three days of life (0.5, 0.3, 0.1 mg/kg, s.c.). In adult male mice with neonatal dexamethasone treatment, an increase in the relative weight of the adrenal glands and a decrease in body weight were observed, while the basal level of corticosterone remained unchanged. Dexamethasone treatment in early life had a negative impact on the learning and spatial memory of adult mice in the Morris water maze. We analyzed the effect of elevated glucocorticoid levels in early life on the expression of the *Crh*, *Avp*, *Gr*, and *Mr* genes involved in the regulation of the HPA axis in the hypothalami of adult mice. The expression level of the mineralocorticoid receptor gene (*Mr*) was significantly downregulated, and the glucocorticoid receptor gene (*Gr*) showed a tendency towards decreased expression ($p = 0.058$) in male mice neonatally treated with dexamethasone, as compared with saline administration. The expression level of the *Crh* gene encoding corticotropin-releasing hormone was unchanged, while the expression of the vasopressin gene (*Avp*) was increased in response to neonatal administration of dexamethasone. The obtained results demonstrate a disruption of negative feedback regulation of the HPA axis, which involves glucocorticoid and mineralocorticoid receptors, at the level of the hypothalamus. Malfunction of the HPA axis as a result of activation of the glucocorticoid system in early life may cause the development of cognitive impairment in the adult mice.
Key words: neonatal dexamethasone treatment; HPA; glucocorticoid receptor; mineralocorticoid receptor; spatial memory; gene expression.

For citation: Bondar N.P., Reshetnikov V.V., Burdeeva K.V., Merkulova T.I. Effect of neonatal dexamethasone treatment on cognitive abilities of adult male mice and gene expression in the hypothalamus. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):456-464. DOI 10.18699/VJ19.514

Влияние неонатального введения дексаметазона на когнитивные способности взрослых самцов мышей и экспрессию генов в гипоталамусе

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Ранний постнатальный период является критическим для развития нервной системы. Стресс в этот период вызывает негативные отдаленные последствия, которые отражаются как на поведенческом, так и на молекулярном уровне. В нашем исследовании для моделирования повышенного уровня глюкокортикоидов, характерного для стрессирующего воздействия в раннем возрасте, мы использовали введение дексаметазона, агониста глюкокортикоидных рецепторов, в понижающихся дозах в первые три дня жизни (0.5, 0.3, 0.1 мг/кг, п/к). У взрослых самцов мышей с неонатальным введением дексаметазона было найдено увеличение относительного веса надпочечников и снижение веса тела, при этом базальный уровень кортикостерона в крови не изменялся. Введение дексаметазона в раннем возрасте оказало негативное воздействие на скорость обучения и формирование пространственной памяти в водном лабиринте Морриса у взрослых животных. Мы проанализировали влияние повышенного уровня глюкокортикоидов в раннем возрасте на экспрессию генов *Crh*, *Avp*, *Gr*, *Mr*, участвующих в регуляции гипоталамо-гипофизарно-надпочечниковой системы (ГГНС), в гипоталамусе взрослых животных. Уровень экспрессии гена минералокортикоидного рецептора (*Mr*) был снижен достоверно, а гена глюкокортикоидного рецептора (*Gr*) – на

уровне тенденции (p = 0.058) у самцов мышей с неонатальным введением дексаметазона по сравнению с введением физиологического раствора. Уровень экспрессии гена, кодирующего кортикотропин-рилизинг гормон (*Crh*), не изменялся, тогда как экспрессия гена вазопрессина (*Avp*) повышалась под влиянием неонатального введения дексаметазона. Полученные данные демонстрируют возможное нарушение механизмов негативной регуляции ГГНС на уровне гипоталамуса, в которую вовлечены глюкокортикоидный и минералокортикоидный рецепторы. Нарушение функции ГГНС при активации глюкокортикоидной системы в раннем возрасте может быть причиной развития когнитивных нарушений у взрослых животных.
Ключевые слова: неонатальное введение дексаметазона; ГГНС; глюкокортикоидный рецептор; минералокортикоидный рецептор; пространственная память; экспрессия генов.

Introduction

As is well known, the early postnatal period is the most important for the development of the central nervous system and of the behavioral phenotype (Teicher et al., 2016). In particular, clinical trials have demonstrated that stressful events in early childhood may have adverse effects on the individual's cognitive and emotional functions at different stages of life, including in adulthood (Pervanidou, Chrousos, 2018; Weems et al., 2018). Similarly, studies in rodents have associated early postnatal stress with some behavioral and cognitive deficits in adult animals (Ladd et al., 2000; Lehmann, Feldon, 2000; Schmidt, 2010). Because the hypothalamic-pituitary-adrenal (HPA) axis plays a key role in stress response, it is surmised that the long-term effects of stress may be associated with persistent changes in the functionality of different molecular constituents of this axis (De Kloet, 2013; Pervanidou, Chrousos, 2018).

According to the classic interpretation, HPA activation in response to stress starts with an increase in the expression levels of the *Crh* and *Avp* genes in the paraventricular nucleus of the hypothalamus (PVN). The products of these genes – corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) – stimulate the expression of the *Pomc* gene and the secretion of adrenocorticotropic hormone (ACTH) by the adenohypophysis (Harno et al., 2018). ACTH, in turn, stimulates the synthesis of glucocorticoid hormones in the adrenals (van Bodegom et al., 2017; Gjerstad et al., 2018). In the brain, the action of these hormones is mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) (De Kloet, 2013). GRs are expressed in all brain structures, peaking in CRH neurons of the hypothalamic PVN and in adenohypophyseal corticotrophs (Sapolsky et al., 1983; van Eekelen et al., 1991). It is known that, by inhibiting *Crh* and *Pomc* expression (Drouin et al., 1993; Malkoski, Dorin, 1999), activation of GRs in these cells triggers a negative feedback mechanism that reduces HPA activity and terminates the response to the received hormonal signal (McEwen et al., 1992). MR expression is largely confined to the limbic system, peaking in the hippocampus (Sapolsky et al., 1983; van Eekelen et al., 1991). It is surmised that MRs like GRs participate in the control of HPA activity, mediating "proactive" feedback involved in the maintenance of its basal level and in the control of the inhibitory hippocampal effect on HPA function (Berardelli et al., 2013). Additionally, it is well known that GRs and MRs are involved in the formation of cognitive functions, emotional reactions and behavioral reactions (De Kloet, 2013; Paul et al., 2015).

Glucocorticoid levels can be changed by exposure to stressful factors at early ages or to drugs with effects on HPA activity. One of the most popular models of early-life stress is prolonged separation of pups from their mothers (maternal separation for 3 h once a day) within the first two weeks of life. As was established in rodent experiments, this kind of stress leads to substantial behavioral and cognitive impairment in adults animals (Pryce, Feldon, 2003; Aisa et al., 2007; Suri et al., 2013; Bondar et al., 2018) and has delayed effects on the expression of stress-response genes (Reshetnikov et al., 2018b). Studies exploring the effects of this kind of stress on HPA gene expression revealed that adult animals have reduced *Gr* expression in the hippocampus (Ladd et al., 2004; Aisa et al., 2007), frontal cortex (Navailles et al., 2010) and striatum (Wong et al., 2015) and enhanced *Avp* expression in the PVN of the hypothalamus (Sanchez et al., 2001; Ladd et al., 2004; Murgatroyd et al., 2009). Our previous work showed enhanced *Avp* expression in the hypothalamuses of adult mice who had experienced maternal separation during the early postnatal period. Additionally, these animals were found to have reduced *Crhr1* mRNA levels in the hippocampus and an increased Mr/Gr mRNA ratio in the hippocampus and hypothalamus (Reshetnikov et al., 2018b).

Administration of the synthetic glucocorticoid hormone dexamethasone during the first postnatal days is another tool used for modeling early postnatal stress in rodents (Wong et al., 2015; Yates et al., 2016). Behavioral deficits in animals like these have been well studied by rat experiments. Those animals were shown to have changes in anxious and depression-like behaviour, learning and memory deficits (Kamphuis et al., 2004; Neal et al., 2004; Claessens et al., 2012; Vazquez et al., 2012; Ko et al., 2014). A few works on mice that use the dexamethasone model have revealed changes in anxious behavior and impairment in novel object recognition (Li et al., 2014a). Although there is a large number of works that explore the effects of neonatal administration of glucocorticoids, delayed effects of these hormones on stress-response genes are not sufficiently well studied – and to a still lesser extent so in mice. Some works demonstrated delayed effects of this "pharmacological" stress on separate HPA genes: a reduction in amount of striatal GR in mice (Wong et al., 2015) and reduced hippocampal *Gr* expression in rats (Vazquez et al., 2012). However, the hypothalamus, this brain's region with importance for HPA function, remains to be poorly studied in the context of the delayed effects of the neonatal rise in glucocorticoids. In this work, the delayed effects of HPA activation during animals' early life were explored using a pharmacological approach, which included the direct activation of the glucocorticoid system by administration of the synthetic glucocorticoid dexamethasone.

The aim of this work was to assess the impact of neonatal dexamethasone administration on cognitive function in adult mice and to trace the delayed effects of this drug on the expression of key genes regulating HPA function at the hypothalamic level. A comparison of original data with experimental data on delayed effects of early-life stress (maternal separation) (Reshetnikov et al., 2018b) will allow the similarity and differences between these two types of exposure to be assessed.

Materials and methods

Animals. The experiment animals were C57Bl/6 mice. The animals were kept under standard conditions in the conventional animal facility of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (RFMEFI62117X0015) (Novosibirsk, Russia) with a 12-hour light and 12-hour dark cycle. Food and water were available *ad libitum*. All animal procedures were performed in compliance with the EU Council Directive 86/609-EEC and were approved by the Ethics Committee at the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (protocol No. 39 of September 27, 2017).

Experimental Design. Animals started to receive dexamethasone (KRKA, Slovenia) on postnatal day 1 (PND1), the day of birth being designated as PND0. Dexamethasone injections were administered according to the following scheme: 0.5 μg/g body weight on PND1, 0.3 μg/g body weight on PND2, and 0.1 μg/g body weight on PND3. The control group received injections of saline solution (10 μl per animal). All injections were administered subcutaneously once a day between 9 am and 10 am. The administration scheme and doses were chosen according to previous research (Kamphuis et al., 2003; Ko et al., 2014; Li et al., 2014a, b).

On PND30, pups were weaned and grouped by sex and litter before testing. A further experiment involved only males. All experimental procedures were run on adult mice (~PND90). Two cohorts of animals were used for measuring gene expression and assessing cognitive function. Animals in the first cohort (six neonatally injected with saline and six neonatally injected with dexamethasone) were sacrificed by rapid decapitation, blood was collected, the adrenals were removed and weighed, the hypothalamuses were removed and frozen at –70 °C. To obtain serum, blood was kept at room temperature for 1 h and then centrifuged for 10 min at 3000 g; serum was collected and stored at –70 °C until used. The relative adrenal weight was calculated as the ratio of the total weight of two adrenals (in milligrams) to the body weight (in grams). Animals in the second cohort (17 neonatally injected with saline and seven neonatally injected with dexamethasone) were tested in the Morris water maze.

The Morris water maze test. This test assesses animals' ability to develop and maintain spatial memory (Morris, 1984; Vorhees, Williams, 2006). A Morris water maze is a pool 100 cm in diameter, with four equally spaced visual cues on the pool wall to mark four quadrants with different starting locations (Target, Opposite, Sector 1 and Sector 2). A platform 10 cm in diameter was set in Target sector, slightly beneath the water. Water in the pool was made opaque with the addition of powdered milk, to keep the platform unseen. The water temperature was 24 ± 1 °C.

Testing lasted five days. First, mice were trained for four consecutive days and received four training trials per day, a total of 16 training trials, each starting at the same time of day. In each training trial, a mouse was placed in one of the sectors and allowed to search for the hidden platform for 1 min. If the mouse did not find the platform within 1 min, the experimenter led the animal to it. After the platform was located, the mouse was left on it for 15 sec to memorize the spatial cues. After that the mouse was taken out and placed in a cage for 15 sec for resting before the next trial. Throughout the experiment, the platform remained at its original position. Finally, on day 5, a probe trial was given: the platform was removed, the mouse was placed in the Opposite sector and the time spent in each sector within 1 min was measured. The test was recorded and processed using EthoStudio software (Kulikov et al., 2005).

RNA extraction and real-time PCR. RNA was extracted from frozen tissue specimens using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. RNA was purified using paramagnetic RNAClean XP beads (Beckman Coulter, Germany) and dissolved in double-distilled water. RNA quality and quantity were evaluated using a NanoDrop 2000 spectrophotometer. Complementary DNA (cDNA) was synthesized using kits produced by Syntol (Russia). The reaction included 1 μg of RNA, all procedures were carried out according to the manufacturer's protocols.

Gene expression was assessed by real-time PCR using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). We assessed the expression of the glucocorticoid receptor genes *Gr* and *Nr3c1*, the mineralocorticoid receptor genes *Mr* and *Nr3c2*, the corticotropin-releasing factor gene *Crh*, and the arginine vasopressin gene *Avp*. PCR outputs were analyzed using the ΔΔCt method and normalized to the expression of the β-actin gene (*Actb*) as a reference gene. The *Avp*, *Gr* and *Mr* amplification products were detected using EVAGreen; the *Crh* amplification products, using TaqMan probes. Primers and probes (Table 1) were designed using Primer BLAST (NCBI) and the PrimerQuest design tool (IDT Technology). The reaction parameters were as follows: 95 °C for 5 min followed by 38 cycles at 95 °C for 10 sec and at 60 °C for 20 sec. After the completion of the PCR reaction for the systems with the intercalating dye EVAGreen, product specificity was assessed by analysis of melting curves. Each reaction was performed in triplicate. The amplification efficiency was from 90 to 110 % for each primer pair.

Measuring corticosterone concentrations. Serum corticosterone concentrations were measured by ELISA using a Corticosterone ELISA Kit (Enzo, New York, NY, USA) according to the manufacturer's protocols. Each reaction was performed in duplicate.

Statistical data processing. Statistical processing of the Morris water maze test data was performed by ANOVA and Fisher's LSD as a post hoc test. A comparative analysis of the body weight, relative adrenal weight, corticosterone concentrations and gene expression levels was performed using Student's *t*-test. Differences between the groups were considered statistically significant at $p < 0.05$ and showing a tendency to significance at $p < 0.1$. Statistical analyses were performed using Statistica 6.0.

Table 1. Forward and reverse primer sequences

Gene name		Sequence (5′→3′)		Product size, bp
Gr (*Nr3c1*)	*Nuclear receptor subfamily 3, group C, member 1*	For	ATGTATGACCAATGTAAACACA	132
		Rev	GCTCTTCAGACCTTCCTTAG	
Mr (*Nr3c2*)	*Nuclear receptor subfamily 3, group C, member 2*	For	GTGTGTGGAGATGAGGC	155
		Rev	GGACAGTTCTTTCTCCGAAT	
Crh	*Corticotropin releasing hormone*	For	GGAGAAGAGAGCGCCCCTAA	152
		Rev	AAGAAATTCAAGGGCTGCGG	
		Probe	5,6-ROX-ATGCTGCTGGTGGCTCTGTCGTCC-3BHQ-2	
Avp	*Arginine vasopressin*	For	TCTCCGCTTGTTTCCTGAGC	230
		Rev	GGGCAGGTAGTTCTCCTCCT	
Actb	*Beta-actin*	For	TATTGGCAACGAGCGGTTCC	140
		Rev	TGGCATAGAGGTCTTTACGG	
		Probe	5,6-ROX-CCAGCCTTCCTTCTTGGGTATGGAATCC-3BHQ-2	

Results

The body weight, relative adrenal weight and corticosterone concentrations. In the neonatal dexamethasone-treated group, the body weight was lower [t (1, 35) = 4.54, $p < 0.001$], the relative adrenal weight was higher [t (1, 35) = 2.26, $p = 0.030$], and corticosterone concentrations did not differ [t (1, 10) = 0.84, $p = 0.419$] compared to the respective variables in the saline-treated group (Fig. 1).

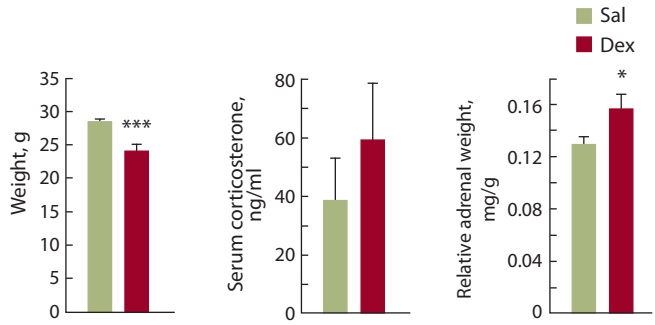

Fig. 1. Effects of neonatal dexamethasone treatment on adult mice's body weight, corticosterone concentrations and relative adrenal weight.

Sal, saline-treated group; Dex, dexamethasone-treated group. * $p < 0.05$; *** $p < 0.001$ compared with the Sal group.

The Morris water maze test. Our studies in adult animals neonatally injected with dexamethasone and given this test showed that administration of this drug at early ages hindered learning in the training trials and adversely affected visual cue-based spatial orientation performance in the probe trial. The repeated measures ANOVA revealed an effect of the factor 'drug' on latency to find the platform in the Morris water maze test. Mice neonatally injected with dexamethasone showed a longer latency to find the platform compared to the controls: trial 7 [F(1, 24) = 7.65, $p = 0.011$], trial 9 [F(1, 24) = 6.48, $p = 0.018$], trial 10 [F(1, 24) = 10.08, $p = 0.004$], trial 11 [F(1, 24) = 10.18, $p = 0.004$], trial 12 [F(1, 24) = 10.74, $p = 0.003$], trial 13 [F(1, 24) = 5.02, $p = 0.034$], and trial 14 [F(1, 24) = 4.63, $p = 0.042$] (Fig. 2, *a*).

The probe trial, with the platform removed, was given 24 h after the last training trial. Memory acquisition was considered to be displayed if there was a preference for the Target sector compared to any other sector. ANOVA revealed differences in time spent in different sectors by each mouse in the saline-treated group [F(3, 68) = 5.25, $p = 0.003$] and the lack of differences in preferences for sectors shown by any mouse in the neonatal dexamethasone-treated group [F(3, 28) = 0.71, $p = 0.549$]. Mice in the saline-treated group displayed a preference for the Target sector compared to any other sector (Target sector vs Sector 1, $p = 0.013$; Target sector vs Opposite sector, $p = 0.006$; Target sector vs Sector 2, $p = 0.001$). Mice administered with dexamethasone at early ages displayed no preference for the Target sector and spent equal amounts of time in all water maze sectors, suggesting impaired memory acquisition (Fig. 2, *b*).

Changes in the hypothalamic expression of genes involved in the glucocorticoid system. We assessed the expression of the main genes involved in the glucocorticoid system: the glucocorticoid receptor gene, mineralocorticoid receptor gene, corticotropin-releasing factor gene, and arginine vasopressin gene *Avp*. The expression levels of both glucocorticoid (*Gr*) [t (1, 8) = 2.21, $p = 0.058$, a tendency] and mineralocorticoid (*Mr*) [t (1, 8) = 3.28, $p = 0.011$] receptors were lower in dexamethasone-treated mice than in the controls; however, the ratios of the mRNA of these receptors (Mr/Gr) did not differ between the groups. The expression level of *Avp* was higher in dexamethasone-treated mice than in the controls [t (1, 8) = 2.47, $p = 0.039$], while the expression level of *Crh* was unaffected (Fig. 3).

Discussion

Stress- or glucocorticoid-induced increased HPA activation during early life leads to substantial changes in adult animals' brain structures and behavior. Our study demonstrated that administration of the GR agonist dexamethasone to mice during their first days of life leads to impairment in spatial memory in their adulthood. Along with this impairment, a change in HPA activity was observed in the form of a decrease in the hypothalamic expression of the *Gr* and *Mr* genes in adult animals. GRs and MRs are involved in the regulation

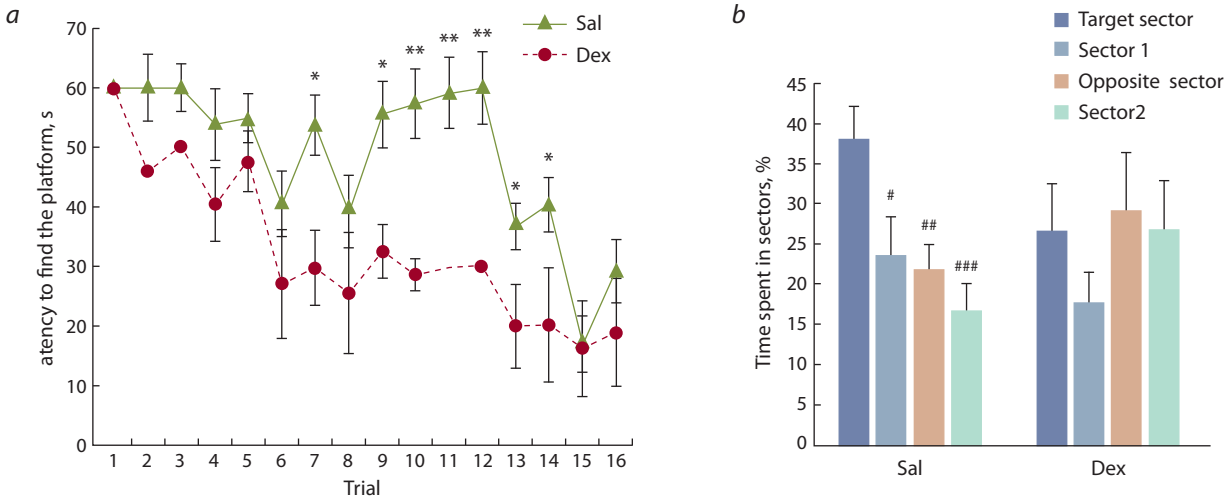

Fig. 2. Effects of neonatal dexamethasone treatment on the behavior of adult mice in the Morris water maze. *a*, Latency to find the platform in 16 daily training trials; *b*, Time spent in the each sector in the probe trial (data are presented as the percentage of the probe trial time).

Before the probe trial, the hidden platform was removed, and the mice were placed in the pool for 1 min. Sal, saline-treated group; Dex, dexamethasone-treated group. * $p < 0.05$; ** $p < 0.01$ compared with the Sal group; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ compared with the time spent in the Target sector (Fisher's LSD as post hoc).

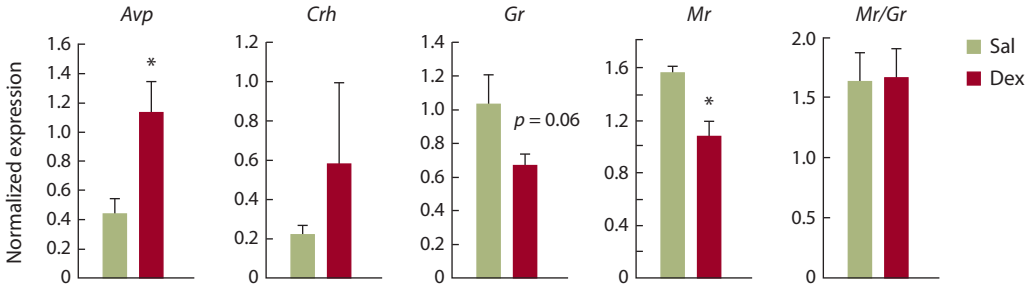

Fig. 3. Effects of neonatal dexamethasone treatment on hypothalamic gene expression in adult mice.

Expression levels of *Avp*, *Crh*, *Gr*, and *Mr* mRNA were normalized to *Actb*. Sal, saline-treated group; Dex, dexamethasone-treated group.
* $p < 0.05$ compared with the Sal group.

of stress response and mediate negative feedback during HPA activation, so decreases in their expression suggest a possible disruption in negative regulation of the hormonal response in the hypothalamus.

As is known, the effects of exogenous glucocorticoids strongly depend on the animal species, HPA axis maturity and drug dosage. Adult rats administered with dexamethasone at high doses on postnatal day 5–10 demonstrate reduced neophobia and anxiety in the Light/Dark Box and the Open Field tests, without change in locomotor activity (Yates et al., 2016). At the same time, administration of dexamethasone at low doses to rats during the first three days of their life can cause increases in anxious behavior (Neal et al., 2004; Vazquez et al., 2012) as well as in depressive-like behavior (Ko et al., 2014; Li et al., 2014b). Or, conversely, it may have no effect on the locomotor activity, anxious and social behavior of rats in adulthood (Kamphuis et al., 2004). A few studies involving administration of dexamethasone to mice during the first three days of their life demonstrate that adult ICR strain mice administered with dexamethasone at low doses during the first three days of their life display increased anxiety, while their locomotor and exploratory activities remain unaffected (Li et al., 2014a). By contrast, neonatal low-dose dexamethasone administration to C57Bl6 strain mice demonstrated a decrease in anxious behavior in adult animals (Batluk et al., 2018), suggesting the strain-specificity of this drug. The most consistently observed and typical effects of both neonatal and prenatal dexamethasone administration that are reported by virtually all researchers working on no matter which animal species are developmental retardation and weight loss (Neal et al., 2004; Lin et al., 2006; Wang et al., 2010; Vazquez et al., 2012; Chiu et al., 2018). We have similar observations: adult mice neonatally treated with dexamethasone had lower body weight compared to the control conspecifics. It is possible that weight loss is associated with a direct effect of dexamethasone on protein catabolism (Weiler et al., 1997; Leret et al., 2004), leading to impairment in skeletal growth (Swolin-Eide et al., 2002).

In this work, we found increases in the relative adrenal weight of adult animals who had been neonatally administered with dexamethasone. Because the corticosterone levels in the adult animals has remained unchanged, the increases in adrenal weight is most likely due to body weight loss, although a possible change in HPA activity induced by

dexamethasone administration during early life may be a factor, too.

In rodent brains, glucocorticoid receptors are mainly located in the hippocampus, especially CA1, and that is why the neurons in this region are most sensitive to glucocorticoids (van Eekelen et al., 1991). As shown on mice and rats, dexamethasone increases caspase-3 activity and apoptosis in the hippocampus and cortex immediately after treatment (Feng et al., 2009; Bhatt et al., 2013; Lanshakov et al., 2016), reduces the number of neurons in the cortex and hippocampus (Kreider et al., 2006; Tijsseling et al., 2013) by PND21, and can also change the hippocampal ratio of the NMDA receptor subunits (Kamphuis et al., 2003), thus changing neuronal plasticity. Neonatal effects of dexamethasone on the hippocampus cause cognitive impairment. Experiments on rats with neonatal dexamethasone administration clearly show a slower learning curve and impaired spatial memory acquisition in the Morris water maze (Ferguson et al., 2001; Kamphuis et al., 2003; Machhor et al., 2004; Qaheri et al., 2013), impaired short-term memory (Claessens et al., 2012), slower learning in passive avoidance tests (Lin et al., 2006; Wang et al., 2010; Chiu et al., 2018) and the poor recognition of familiar and unfamiliar partners (Kamphuis et al., 2004; Wang et al., 2010). As far as mice are concerned, cognitive dysfunctions have been exemplified only with novel object recognition (Li et al., 2014a). Ours is one of the first studies to show impairments inflicted to the learning ability and spatial memory of adult mice by administration of dexamethasone during early life. Mice become slower to learn, as early as on test day 2 they fall behind the control animals in locating the platform. Furthermore, in the probe trial, which was given them 24 h after the last training trial, they did not show preference for the Target sector, where the platform was located, displaying impaired long-term spatial memory.

During the early postnatal period, mice and rats normally have a low stress response (aka hyporesponsive period) because of low blood ACTH and corticosterone concentrations and a reduced number of glucocorticoid receptors in tissues (Levine, 1994). Consequently, most weak stimuli like isolation, a new situation, or saline injection presented during early life did not cause HPA activation. However, powerful stresses like prolonged maternal separation or dexamethasone administration lead to increased HPA activation, which, in turn, causes developmental abnormalities. It is an often occurrence that glucocorticoid administration at early ages does not change basal corticosterone levels in adult animals (Neal et al., 2004; Claessens et al., 2012; Vazquez et al., 2012), which is consistent with our observations, however, what changes is the stress response. Postnatal dexamethasone administration leads to a blunted release of corticosterone in response to a stress being experienced in adulthood (Felszeghy et al., 2000; Flagel et al., 2002; Mesquita et al., 2009; Vazquez et al., 2012), and recovery to basal levels is delayed (Neal et al., 2004).

We assessed the expression of the genes associated with the regulation of HPA activity in the hypothalamus: *Gr*, *Mr*, *Avp* and *Crh*. We demonstrated decreases in the expression levels of the *Gr* and *Mr* genes in mice who had been neonatally administered with dexamethasone. There were no significant differences in the expression levels of the corticotropin-releasing factor gene *Crh* between the groups, however, the expression level of the *Avp* gene, of which the product is involved in the regulation of ACTH synthesis (Aguilera, Rabadan-Diehl, 2000), was significantly higher in the dexamethasone treatment group. Nevertheless, because of high variances within the groups, there were no differences in basal blood corticosterone levels between the groups, nor do these levels correlate with *Crh* expression levels – although, admittedly, they show a tendency towards an increase. GR levels are directly associated with the dynamics of the hormonal response to stress. As blood corticosterone levels increase, hypothalamic GR activation leads to a reduction in CRH release and, consequently, to a reduction in ACTH release in the hypophysis, normalizing the levels of stress-response hormones (De Kloet et al., 1998). The decrease in hypothalamic *Gr* expression revealed in our work may lead to, among other effects, a decrease in the level of the GR protein itself, which, in its turn, leads to errors in the operation of the mechanisms acting to restore normal levels of hormones after responding to stress. Decreased *Gr* expression levels or decreased amounts of this gene's protein product in the brain's regions are reported in some works on the delayed effects of glucocorticoid administration. Rats neonatally administered with dexamethasone were found to have decreased expression levels of *Gr* in the hippocampus (Vazquez et al., 2012) and a decreased capability of GR to bind the hormone in the hippocampus and hypothalamus (Felszeghy et al., 1996). A reduction in the amount of GR was also found in the mouse striatum after dexamethasone administration during early life (Wong et al., 2015). However, to the best of our knowledge, decreased *Gr* and *Mr* in the hypothalamuses of mice neonatally administered dexamethasone were not shown previously. Therefore, our data add to knowledge about delayed changes in the HPA axis after neonatal dexamethasone administration.

Because administration of dexamethasone during the first days of life can imitate a stressful event experienced at an early age, we compared our new data with those obtained previously on the effects of early-life stress on cognitive functions and hypothalamic gene expression in mice (Table 2) (Reshetnikov et al., 2018b). Unlike neonatal dexamethasone administration, prolonged separation of pups and their mothers during the first weeks of life does not affect the learning curve in the Morris water maze, for the experimental mice learned to locate the hidden platform as rapidly as did their control conspecifics. However, as soon as 24 h later the experimental animals stopped showing preference for the sector where the platform had been set up and then removed, suggesting impaired long-term spatial memory. Thus, more marked cognitive deficits result from neonatal dexamethasone administration than from the stress caused by maternal separation, because the former affects both learning and the reproduction of newly learned information. A comparison of available data on other aspects of cognitive functions shows that impairments in novel object recognition that are due to both early-life stress (Reshetnikov et al., 2018a) and neonatal dexamethasone treatment (Li et al., 2014a) are similar.

Stronger delayed effects of neonatal glucocorticoid administration than of early postnatal stress in the form of maternal separation can be revealed by comparing respective changes in gene expression. Thus, the expression levels of the *Gr* and *Mr* genes were not affected by early-life stress (maternal separation), but were decreased following neonatal dexamethasone administation. Early-life stress led to increased *Mr*/*Gr* ratios

Table 2. Comparison of the effects of prolonged separation of pups from mothers in early life (MS) and the effects of neonatal treatment with dexamethasone

Parameters	MS*	Dexamethasone
Weight	=	Reduced
Adrenal glands weight	=	Elevated
Level of corticosterone	=	=
Level of genes expression in hypothalamus		
Gr	=	Tendency to be reduced
Mr	=	Reduced
Ratio mRNA *Mr/Gr*	Elevated	=
Avp	Elevated	Elevated
Crh	=	=
Learning and memory in Morris water maze		
Learning	=	Reduced
Long-term memory	Reduced	Reduced

* Comparison with (Reshetnikov et al., 2018b).

both in the hypothalamus and hippocampus due to a slight increase in *Mr* expression. This fact explains a weaker effect of this stress on learning, because the balance of these receptors is involved in the stress response and the acquisition of long-term memory (De Kloet, 2013). Neonatal dexamethasone treatment did not affect the *Mr*/*Gr* ratio: a decrease in the expression of either type of receptor is accompanied by a decrease in the expression of the other, without a compensatory change in their balance that would, if it were present, act to restore HPA function. The increase in *Avp* expression levels is twice as high following neonatal dexamethasone administration as it is following early postnatal stress, while *Crh* expression remains unchanged, no matter which type of stress is applied. Thus, neonatal dexamethasone administration leads to stronger delayed effects on cognitive abilities and hypothalamic gene expression compared to the stress caused by maternal separation during the first weeks of life.

Conclusion

Our results demonstrate that adult mice who have experienced neonatal dexamethasone administration show a slower learning curve and impaired spatial memory acquisition in the Morris water maze. At the same time, adult mice so treated were found to have decreased expression levels of the *Gr* and *Mr* genes and increased *Avp* in the hypothalamus, suggesting delayed HPA dysregulation. Early postnatal stress in the form of maternal separation and postnatal dexamethasone administration lead to unidirectional changes in the cognitive abilities and HPA functions of adult animals, however, these changes are stronger following dexamethasone administration – probably because these different kinds of treatment lead to different HPA activation levels.

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Acknowledgement. This work was supported by the Russian Science Foundation as Project 16-15-10131.

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

Received November 7, 2018. Revised February 17, 2019. Accepted February 20, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):465-472
DOI 10.18699/VJ19.515

Genetic basis of depressive disorders

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Depression is a common mental disorder being one of the main causes of disability and mortality worldwide. Despite an intensive research during the past decades, the etiology of depressive disorders (DDs) remains incompletely understood; however, genetic factors are significantly involved in the liability to depression. The present review is focused on the studies based on a candidate gene approach, genome-wide association studies (GWAS) and whole exome sequencing (WES), which previously demonstrated associations between gene polymorphisms and DDs. According to the first approach, DD development is affected by serotonergic (*TPH1*, *TPH2*, *HTR1A*, *HTR2A*, and *SLC6A4*), dopaminergic (*DRD4*, *SLC6A3*) and noradrenergic (*SLC6A2*) system genes, and genes of enzymatic degradation (*MAOA*, *COMT*). In addition, there is evidence of the involvement of HPA-axis genes (*OXTR*, *AVPR1A*, and *AVPR1B*), sex hormone receptors genes (*ESR1*, *ESR2*, and *AR*), neurotrophin (*BDNF*) and methylenetetrahydrofolate reductase (*MTHFR*) genes, neuronal apoptosis (*CASP3*, *BCL-XL*, *BAX*, *NPY*, *APP*, and *GRIN1*) and inflammatory system (*TNF*, *CRP*, *IL6*, *IL1B*, *PSMB4*, *PSMD9*, and *STAT3*) genes in DD development. The results of the second approach (GWAS and WES) revealed that the *PCLO*, *SIRT1*, *GNL3*, *GLT8D1*, *ITIH3*, *MTNR1A*, *BMP5*, *FHIT*, *KSR2*, *PCDH9*, and *AUTS2* genes predominantly responsible for neurogenesis and cell adhesion are involved in liability to depression. Therefore, the findings discussed suggest that genetic liability to DD is a complex process, which assumes simultaneous functioning of multiple genes including those reported previously, and requires future research in this field.
Key words: depressive disorder; serotonin; hypothalamic-pituitary adrenal axis; neurotrophin; apoptosis; cytokines; GWAS; whole-exome sequencing.

For citation: Davydova Yu.D., Enikeeva R.F., Kazantseva A.V., Mustafin R.N., Romanova A.R., Malykh S.B., Khusnutdinova E.K. Genetic basis of depressive disorders. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):465-472. DOI 10.18699/VJ19.515

Генетические основы предрасположенности к депрессивным расстройствам

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Депрессия – это распространенное психическое расстройство, которое является одной из ведущих причин нетрудоспособности и смертности в мире. Несмотря на интенсивные исследования, проводимые в течение последних десятилетий, этиология депрессивных расстройств все еще остается не до конца изученной, однако генетические факторы, безусловно, играют важную роль в предрасположенности к депрессии. Настоящий обзор сфокусирован на результатах работ, основанных на генно-кандидатном подходе, полногеномных (Genome-Wide Association Studies, GWAS) и полноэкзомных (Whole Exome Sequencing, WES) исследованиях, продемонстрировавших связь полиморфных локусов генов с депрессивными расстройствами. Согласно первому подходу, формирование депрессивной симптоматики находится под влиянием генов серотонинергической (*TPH1*, *TPH2*, *HTR1A*, *HTR2A*, *SLC6A4*), дофаминергической (*DRD4*, *SLC6A3*) и норадренергической (*SLC6A2*) систем, а также генов ферментов их метаболизма (*MAOA*, *COMT*). Кроме того, имеются данные об участии генов гипоталамо-гипофизарной системы (*OXTR*, *AVPR1A*, *AVPR1B*) и рецепторов половых гормонов (*ESR1*, *ESR2*, *AR*), генов нейротрофического фактора мозга (*BDNF*) и фермента метилентетрагидрофолатредуктазы (*MTHFR*), нейронального апоптоза (*CASP3*, *BCL-XL*, *BAX*, *NPY*, *APP*, *GRIN1*) и воспалительной системы (*TNF*, *CRP*, *IL6*, *IL1B*, *PSMB4*, *PSMD9*, *STAT3*) в развитии депрессивных расстройств. Результаты второго подхода

(GWAS и WES) демонстрируют, что гены белков пикколо (*PCLO*) и сиртуина (*SIRT1*), фактора пролиферации стволовых клеток (*GNL3*), гликозилтрансферазы (*GLT8D1*), α-трипсинового ингибитора (*ITIH3*), мелатонинового рецептора (*MTNR1A*), костного морфогенного белка (*BMP5*), ломкой гистидиновой триады (*FHIT*) и киназного супрессора (*KSR2*), протокадгерина (*PCDH9*) и активатора транскрипции *AUTS2*, преимущественно участвующие в процессах нейрогенеза и клеточной адгезии, вовлечены в развитие депрессии. Таким образом, эти и другие литературные данные подтверждают, что формирование генетической предрасположенности к депрессивным расстройствам – сложный процесс, затрагивающий функционирование большого числа генов, в том числе тех, которые ранее не обсуждались в связи с депрессией, что требует обратить особое внимание на них в дальнейших исследованиях.
Ключевые слова: депрессивное расстройство; серотонин; гипоталамо-гипофизарная система; нейротрофин; апоптоз; цитокины; GWAS; полноэкзомное секвенирование.

Inroduction

According to the World Health Organization (WHO), unstable social, economic and ecological factors in the modern society result in the constant increase in the distribution frequency of socially significant diseases with a specific attention given to depressive disorders (DDs). Primarily, such attention is caused by a high distribution of depression among the population, the annual increase in morbidity, the difficulties of diagnosis, prevention and treatment of this disease (Smulevich, 2015). Moreover, depression is one of the leading causes of disability worldwide. To date more than 322 million of individuals with DDs differing in age were registered and the total number of individuals with depressive disorders increased by more than 18.4 % over the last decade. Suicidal behavior (SB) represents an increasing depression-associated problem being the second leading cause of mortality among individuals aged 15–29 years (WHO, 2017).

Depression is a mental disorder characterized by a pathologically reduced mood, inhibited intellectual and motor activity, reduced vital impulses with individual pessimistic assessment and future (Smulevich, 2015). Different medical classifications are based on a variety of diagnostic criteria of depression. The international classification of diseases (ICD-10) diagnoses depression (F32 − depressive episode) depending on the number and severity of symptoms including reduced mood, anhedony, strength decline and fatigue, psychomotor retardation or arousal, guilt and humiliation ideas, suicidal thoughts, decreased attention concentration and sexual motivation, impaired sleep and appetite (Smulevich, 2015; ICD-10, 2018).

Nowadays, the most common validated scales for DDs diagnostics include the Hospital Anxiety and Depression Scale (HADS), Hamilton Depression Rating Scale (HAM-D), Beck Depression Inventory (BDI), Montgomery-Asberg Depression Rating Scale (MADRS), which are used in clinical practice by psychiatrists for the assignment of treatment strategy (Cusin et al., 2010).

The multifactorial nature of depressive disorders

Depression is a multifactorial mental disorder caused by a wide range of psychological, social, neurochemical, and hereditary factors and their interaction (Smulevich, 2015). According to the results of twin studies, the coefficient of inheritance of depression is 29–46 % differing for various DDs (Kendler et al., 2006). The psychosocial predictors of DDs distinguish a special style of individual negative thinking, which is characterized by a focus on the negative aspects of life, conflict child-parent relationships in childhood, maternal depression or a large number of stressful life events (Daches et al., 2018). The results of numerous studies suggest that the development of depression may be related to the individual reaction on diagnosed psychiatric disorder (Kim et al., 2018) causing social disadaptation. DDs are widely assumed to cause or be caused by suppressed anger and aggression (Sahu et al., 2014).

Interestingly, DDs appear to be sex-specific. It was reported that the frequency of depression was twice higher in women than in men (WHO, 2017). This sexual dimorphism is related to the differences in nervous and endocrine systems functioning and to the sex-specific transcription profiles of the genes (Gerhard, Duman, 2018). Moreover, such frequency is assumed by fact that men with depression symptoms less likely seek for the medical help than women (Girgus, Yang, 2015).

Neurophysiological studies of depressive disorders

The neurophysiological approach used for DD study suggests an important role of the detection of the primary impairments in the brain structures involved in the regulation of emotional processes and motivation. However, the data on such neurophysiological markers are scarce to date due to the significant clinical and etiological heterogeneity of DDs.

Functional, structural and post-mortem studies evidence that abnormalities in the subgenual part of the cingulate gyrus are the most stable neurophysiological markers of DDs. A decreased volume or increased metabolic activity of the subgenual part was observed in patients at the early stages of the disease and in individuals with a family history of depression (Hajek et al., 2008). Magnetic resonance imaging demonstrated reduced volumes of the frontal areas, especially the anterior cingulate gyrus, orbitofrontal and prefrontal cortex, as well as a decrease in the volume of the hippocampus, putamen and caudate nucleus in DD patients (Koolschijn et al., 2009). Moreover, temporal and insular lobes together with cerebellum, which demonstrate a decreased activity, are involved in DD pathophysiology (Fitzgerald et al., 2008).

Significant impairments in the activity of the hypothalamic-pituitary-adrenal (HPA) axis have been detected more than in a half of DD patients. Namely, the hypothalamus of patients was characterized by an increase in the number of neurons producing corticotropin-releasing hormone, which chronic

increase results in the enhancement of the toxic damage of monoaminergic neurons thus reducing their number in depressive states (Naughton et al., 2014).

Molecular genetic studies of depressive disorders

Monoaminergic systems genes. The hypothesis of monoaminergic neurotransmission deficiency in DDs proposed in the 60s of the last century, triggered the study of gene polymorphisms involved in neurotransmitter turnover and affecting serotonin, dopamine and norepinephrine synthesis, degradation and neurotransmission (Gatt et al., 2015; Shadrina et al., 2018). Inconsistent findings revealed in multiple studies of DDs were subsequently systematized in meta-analyses (Kishi et al., 2013; Zhao et al., 2014; Liu et al., 2016; Wang et al., 2016; Bleys et al., 2018; Culverhouse et al., 2018; Rui et al., 2018; Taylor, 2018), which allowed to confirm or deny initial hypotheses on the involvement of certain monoaminergic system genes in the development of DDs (Table). According to the results of the meta-analyses, increased risk for developing depression was associated with polymorphic loci located in the serotonin transporter (*SLC6A4*) and receptors (*HTR1A*, *HTR2A*), tryptophan hydroxylase (*TPH2*), dopamine transporter (*SLC6A3*) and receptor (*DRD4*), norepinephrine transporter (*SLC6A2*), monoamine oxidase A (*MAOA*), and catechol-O-methyltransferase genes (*COMT*).

The role of the methylenetetrahydrofolate reductase gene. Methylenetetrahydrofolate reductase (MTHFR) is one of the key enzymes involved in the metabolism of folate and methionine, which, in turn, play an important role in the regulation of gene expression. The methylenetetrahydrofolate reductase gene (*MTHFR*; 1p36.22) was considered as a candidate one in the study of affective and bipolar disorders, schizophrenia, and depression (Gatt et al., 2015). The activity of this enzyme is known to decrease as a result of nucleotide substitutions in the *MTHFR* gene. One of these SNPs include *677C > T* (*rs1801133*) resulting in *Ala222Val* substitution in the catalytic domain of the enzyme and causing decreased enzymatic activity to 35 % (Weisberg et al., 1998). Subsequently, a number of meta-analyses confirmed the association of *rs1801133*T* allele and DD risk (Rai, 2017).

The role of brain-derived neurotrophic factor gene. Together with the study of neurotransmitter systems, analysis of the genes involved in the regulation of neurotoxic and neuroprotective responses to stress appears to be important. Neurotrophic factors (neurotrophins) represent a large group of polypeptides playing a key role in the development and maintenance of central nervous system (CNS) (Popova et al., 2017). The brain-derived neurotrophic factor encoded by the *BDNF* gene (11p14.1) is one of the most examined neurotrophic factors. According to the functional studies, Met-allele of the *BDNF Val66Met* (*196G > A*; *rs6265*) causes a reduced level of neurotrophic factor in the prefrontal cortex and brain stem and is associated with an increased risk of depression compared to *Val*-allele carriers (Youssef et al., 2018). Previously, a modulating effect of several environmental factors including chronic stress, childhood maltreatment, brain injury, and season of birth on the association of the *Val66Met* and depression (Bîlc et al., 2018) or increased anxiety (Kazantseva et al., 2015) was reported.

The role of neuronal apoptosis genes. The study of neuronal apoptosis as the mechanism underlying the maintenance of cellular homeostasis in nervous system is becoming highly important due to the increased distribution of psychiatric diseases nowadays. To date scarce studies were conducted on the model objects indicating the relationship between neuronal apoptosis and depression or stress, which can represent the primary basis for further molecular-genetic research in humans. For example, enhanced level of caspase-3 (encoded by the *CASP3* gene) – a proteolytic enzyme inducing cellular apoptosis – was observed in the cerebral cortex of rats, which experienced chronic mild stress (Bachis et al., 2008). Moreover, a prolonged exposure to stress results in a reduced expression of the antiapoptotic *BCL-XL* gene and enhanced expression of the proapoptotic *BAX* gene; whereas, reduced stress exposure promoted expression of the brain-derived neurotrophic factor (*BDNF*), nerve growth factor (*VGF*) and neuropeptide (*NPY*) genes in the hippocampus (Jiang et al., 2014).

Together with above-mentioned invasive approaches to the study of molecular mechanisms of complex pathophysiological states, other methods include reconstruction and analysis of associative genetic networks describing the relationship between molecular-genetic objects associated with neuronal apoptosis. Thus, the analysis of neuronal apoptosis genes conducted via gene prioritization approach revealed that neuronal apoptosis was regulated by the proteins encoded by the *BDNF* (with the highest priority), glutamate receptor (*GRIN1*), amyloid beta precursor protein (*APP*), coagulation factor II thrombin receptor (*F2R*), tumor necrosis factor superfamily ligand (*FASLG*), and transcriptional co-activator of steroid and nuclear receptors (*PPARGC1A*) genes. Most of these genes have not been previously discussed with respect to DDs thus providing the field of their examination in the future studies of depressive states (Yankina et al., 2017).

The role of hypothalamic-pituitary-adrenal axis genes. The functioning of the hypothalamic-pituitary-adrenal (HPA) axis represents another key mechanism regulating mental functions. Notably, the concentration balance between oxytocin and vasopressin, as the components of HPA axis, regulates various types of emotional reactions, while an impaired balance accompanied anxiety, autism and depression (Neumann, Landgraf, 2012). The oxytocin action is mediated by its interaction with oxytocin receptor (OXTR). One of the most studied the *OXTR* gene polymorphisms is the G > A (rs53576) substitution in the intron 3, which was reported to be associated with social behavior and depression (Kushner et al., 2018). Namely, *OXTR G/G*-genotype carriers demonstrated a significant increase in depressive symptoms (Kushner et al., 2018).

The data on the vasopressin role in the development of depression are scarce. A wide range of psychological functions of vasopressin (encoded by the *AVP* gene) is realized by its binding to two types of receptors: V1A (*AVPR1A*) and V1B (*AVPR1B*), expressed in the paraventricular and supraoptic hypothalamic nuclei (Neumann, Landgraf, 2012). Post-mortem studies established that DD patients were characterized by elevated levels of *AVP* and *AVPR1A* gene expression (Wang et al., 2008), whereas association studies of the *AVPR1B* gene polymorphisms and affective-related traits showed conflicting results (Kazantseva et al., 2014).

Meta-analyses based on associations of monoaminergic systems genes and depressive disorders

Gene	SNP/VNTR	Comparison groups	Number of studies (N)[1]	OR[2] (*p*)[3]	Risk allele/genotype	Reference
TPH2 (12q21.1)	*rs4570625*	*G* vs. *T*	6 (2754)	0.83 (0.001)	*G*	Gatt et al., 2015
	rs17110747	*A* vs. *G*	5 (2536)	0.84 (0.02)	*A*	
HTR1A (5q12.3)	*rs6295*	*C* vs. *G*	15 (9732)	0.87 (0.007)	*C*	Kishi et al., 2013
	rs878567	*C* vs. *T*	5 (4775)	0.83 (0.0002)	*C*	
HTR2A (13q14.2)	*rs6311*	*A* vs. *G/G*	15 (5539)	1.20 (0.03)	*A*	Zhao et al., 2014
SLC6A4 (17q11.2)	*5-HTTLPR*	*S* vs. *L*	51 (51449)	1.18 (< 0.0001)	*S*	Bleys et al., 2018
			31 (38802)	1.25 (0.02)		Culverhouse et al., 2018
DRD4 (11p15.5)	*VNTR 48* bp	*2R* vs. (*3R*, *4R*, *5R*, *6R*, *7R*)	5 (1132)	1.73 (0.0003)	*2R*	Gatt et al., 2015
SLC6A3 (5p15.33)	*VNTR 40* bp	9/10 vs. 10/10	3 (423)	2.06 (< 0.01)	*9/10*	»
SLC6A2 (16q12.2)	*rs5569*	*A/A* vs. *G*	18 (8798)	1.19 (0.02)	*A/A*	Rui et al., 2018
MAOA (Xp11.3)	*VNTR 30* bp	*L* vs. *S*	9 (4223)	1.23 (0.03)	*L*	Gatt et al., 2015
	rs1137070	*T* vs. *C*	39 (18824)	1.26 (0.0006)	*T*	Liu et al., 2016
COMT (22q11)	*rs4680*	*Val* vs. *Met/Met*	17 (5308)	1.18 (0.02)	*Val*	Wang et al., 2016
		Val vs. *Met*	49 (10925)	0.98 (0.68)	–	Taylor, 2018

Notes: [1] N – number of individuals included in meta-analysis; [2] OR – odds ratio; [3] *p* – significance level (*p*-value).

The role of sex hormone genes and their receptors. As noted above there is a strong evidence of depression sex-specificity (Girgus et al., 2015; Gerhard, Duman, 2018). Therefore, the study of the effects of sex hormones on the human behavior is becoming increasingly popular. Estrogens represent a group of female sex hormones, which affect CNS activity via genomic and non-genomic mechanisms due to their interaction with estrogen receptors ERα and Erβ encoded by the *ESR1* and *ESR2* genes, respectively. The majority of association studies of these genes is focused on four SNPs, namely, *rs2234693* (*–397T > C*) and *rs9340799* (*–351 A > G*) in the *ESR1* gene and *rs1256049* (*1082G > A*) and *rs4986938* (*1730 G > A*) in the *ESR2* gene, which are assumed to affect gene expression and are associated with DD development (Keyes et al., 2015).

Androgens are male sex hormones also involved in the regulation of CNS activity. Testosterone as a main androgen demonstrates a number of physiological functions including the regulation of the psycho-emotional sphere in men. A decreased testosterone level due to age-related andropause was reported to significantly increase DD risk (Dreval, 2017). Moreover a reduced expression of the androgen receptor gene (*AR*, Xq12) associated with the presence of extended polyglutamine $[CAG]_n$ repeats in the exon 1 correlated with a higher risk of depression (Sankar, Hampson, 2012); however, others failed to detect such association (Gardiner et al., 2017).

The role of inflammatory genes. Recently, the hypothesis of inflammation as a DD predictor has being widely developed, which is evidenced by the data on increased expression of proinflammatory cytokines, in particular, C-reactive protein in the acute phase of inflammation (encoded by the *CRP* gene), tumor necrosis factor alpha (*TNF* gene), interleukin-1β (*IL1B* gene) and interleukin-6 (*IL6* gene) (Liu et al., 2012; Köhler et al., 2017) in patients with depressive episode. This observation could be explained by the fact that any stress is accompanied by an increased blood cytokines level and permeability of the blood-brain barrier. Such changes result in the ability of circulating cytokines to penetrate into the brain, trigger neuroinflammatory reactions, which can contribute to the development of DDs and other psychopathologies. Meanwhile, a strong evidence of a reduced synaptic availability of monoamines caused by inflammatory mediators, which is known to be one of the main mechanisms in DD pathogenesis, exists (Miller, Raison, 2016).

T-cells dysfunction and impaired immune response are also considered in the context of the inflammatory theory of depression. Thus, M.L. Wong et al. (2008) revealed that 47.8 % of the population risk of depression was caused by genetic variations in the *PSMB4* gene (*rs2296840*), which encodes β4-proteasome subunit, and in the *TBX21* gene (*rs17244587*) encoding transcription factor and involved in T-lymphocytes differentiation. Moreover, T-cells functioning and response to antidepressant therapy was significantly related to the activity of genes encoding ε-subunit of T-lymphocytes co-receptor (*CD3E*), β-subunit of glycosidase II (*PRKCSH*), signal protein (*STAT3*) and proteasome protein (*PSMD9*) (Wong et al., 2008). Therefore, they are considered as candidates in DD study.

Genome-wide association study (GWAS)

Nowadays genome-wide association study (GWAS) represents one of the most prospective approaches for the study of complex behavioral traits, which includes simultaneous examination of thousands of single nucleotide polymorphisms (SNPs) in genes involved even in unknown pathogenetic mechanisms causing DD development. One of the first GWAS demonstrated an association of the *rs2522833* in the

PCLO gene ($p = 6.4 \times 10^{-8}$) with an increased depression risk; however, it remained statistically insignificant under the appropriate level of statistical significance ($p < 5 \times 10^{-8}$) (Sullivan et al., 2009). Subsequent study confirmed the role of this protein in DD regulation, since *PCLO rs2715157* was associated with DD ($p = 2.91 \times 10^{-8}$) (Mbarek et al., 2017). The *PCLO* gene encodes the protein located in presynaptic terminals and plays a key role in monoaminergic neurotransmission of the brain (Sullivan et al., 2009), which fits into the contemporary ideas of neurotransmitter theory of depression.

As a result of Major Depressive Disorder Working Group of the Psychiatric Genomics Consortium (PGC-MDD) in 2013, 15 SNPs residing 3p21.1 were associated with a combined phenotype including the *PBRM1* gene involved in chromatin remodeling, G-protein nucleolar 3 gene (*GNL3*), glycosyltransferase 8 domain containing 1 gene (*GLT8D1*), *ITIH1-ITIH3-ITIH4* genes cluster, etc. (Major Depressive Disorder Working Group…, 2013). The association of *rs2535629* neighboring the *ITIH3* gene (inter-alpha-trypsin inhibitor heavy chain 3) and DD was the most significant ($p = 5.9 \times 10^{-9}$); however, it requires additional study.

According to the subsequent genome-wide association studies, SNPs residing the genes involved in circadian rhythms regulation such as sirtuin 1 (*SIRT1*), melatonin receptor 1A gene (*MTNR1A*), fragile histidine triad (*FHIT*) (CONVERGE consortium, 2015; Demirkan et al., 2016; Direk et al., 2017) are of interest. These findings suggest a significant role of circadian rhythms in the regulation of individual psycho-emotional sphere and point to the necessity to consider them in the DD research for the understanding of biological mechanisms causing this affective disorder.

Other GWAS based on the combined sample of CONVERGE, PGC and 23andMe demonstrated that *rs7973260* ($p = 1.8 \times 10^{-9}$), *rs62100776* ($p = 8.5 \times 10^{-9}$) and *rs9540720* ($p = 1.69 \times 10^{-8}$) located in the *KSR2* (12q24.22–q24.23), *DCC* (18q21.2) and *PCDH9* (13q21.32) genes, respectively, were associated with DD (Okbay et al., 2016). Notably, the proteins encoded by the protocadherin 9 (*PCDH9*) and kinase suppressor of ras 2 genes (*KSR2*) interacting with DCC netrin 1 receptor (*DCC*) are involved in synaptic plasticity, cellular adhesion, and axonal guidance (Guo et al., 2016; Xiao et al., 2018). Moreover, an increased expression of the *DCC* gene in prefrontal cortex resulted in a depressive-like behavior in mice (Torres-Berrío et al., 2017), while an increased expression of the *PCDH9* gene was observed in hippocampus and frontal lobe of depressed individuals, which represents a marker of enhanced risk of depression in humans (Xiao et al., 2018).

A whole-genome association study conducted by D.M. Howard et al. (2018) based on the UK Biobank cohort demonstrated the association of 17 SNPs and depression risk, including *rs10127497* (*SGIP1*), *rs6424532* (*LOC105378800*), *rs7548151* (*ASTN1*), *rs6699744* (*LOC105378797*), *rs112348907* (*KCNQ5*), *rs3132685* (*HCG9*), *rs5011432* (*TMEM106B*), *rs2402273*, *rs1554505* (*MAD1L1*), *rs3807865* (*TMEM106B*), *rs263575* (*LOC105375983*), *rs10929355* (*NBAS*), *rs40465* (*LOC105379109*), *rs1021363* (*SORCS3*), *rs10501696* (*GRM5*), *rs9530139* (*B3GLCT*), and *rs28541419*. These genes were suggested to be responsible for synaptic plasticity and neurogenesis (Howard et al., 2018). Another neurogenesis-related gene (*AUTS2*) was also involved in developing DD, which was reported in GWAS examining response efficacy to antidepressant therapy (*rs7785360* and *rs12698828*, $p = 1.60 \times 10^{-8}$) (Myung et al., 2015).

Therefore, the results obtained under GWAS evidence in the involvement of multiple genes in depression development (including those previously unexamined in psychopathologies) involved in different stages of neurogenesis, synaptic plasticity and circadian rhythms regulation. This is congruent with the existing theory of polygenic architecture of DD and demonstrates the direction for the future molecular-genetic research in this field.

Whole-exome sequencing (WES)

The development of next generation sequencing (NGS) technologies resulted in a trend for the sequencing of genetic regions containing only coding parts (i.e. exons). The total length of exons is known to be about 1% of the genome: however, it is assumed that the majority of pathogenic mutations are exon-specific. Hence, whole-exome sequencing (WES) represents one of the important approaches to solve a number of diagnostic and research tasks.

To date several whole-exome studies of DDs were conducted. The first WES study on the pharmacogenetics of antidepressants (Tammiste et al., 2013) was carried out by a group of scientists from the University of Tartu ($n = 510$). Tammiste et al. demonstrated the association of *rs41271330* in bone morphogenetic protein 5 (*BMP5*) gene with response to antidepressant therapy. Another WES study based on the RS (the Rotterdam Study) and ERF (Erasmus Rucphen Family) studies revealed a missense mutation *rs77960347* (*Asn396Ser*) observed with a population frequency of 1% in the *LIPG* gene encoding endothelial lipase, which was associated with depressive symptoms ($p = 5.2 \times 10^{-8}$) (Amin et al., 2017b). This enzyme is assumed to be involved in the metabolism of steroids, cholesterol and thyroid hormones, while *Asn396Ser* substitution caused reduced enzymatic activity (Amin et al., 2017b). In addition, subsequent WES analysis performed by the same group of scientists in DD patients detected mutation in the *NKPD1* gene ($p = 3.7 \times 10^{-8}$), which is involved in sphingolipids synthesis (Amin et al., 2017a), and in the *RCL1* gene ($p = 1.0 \times 10^{-4}$) (Amin et al., 2018). The sphingolipids are known to be widely present in the nervous tissue and are involved in myelination, which impairment could result in neuronal degeneration. At the same time, the *RCL1* gene is widely expressed in astrocytes and neurons in the cerebral cortex; however, its effect on their functioning and the potential role in DD pathogenesis remain incompletely studied and require additional studies.

Conclusion

Numerous results of genome-wide and whole-exome analyses summarized in the present review conclude that the key processes involved in developing DDs include neurogenesis, cell adhesion, axonal guidance and synaptic plasticity, which modifications have been considered as the main pathogenetic factors of cognitive impairments and neurodegenerative disorders. Moreover, the data on the association of genes encoding the proteins involved in the regulation of circadian rhythms, inflammation and hormonal regulation with an increased risk

of depression were discussed. These observations suggest that DD development is a highly complex process caused by impairments in a whole cascade of reactions and genes functioning with a small contribution of each of them in depression pathogenesis.

The studies of epigenetic factors including methylation and modifications of histones, microRNAs, and long non-coding RNAs examined in details in the previous review (Mustafin et al., 2018) are expected to make a significant contribution to unravel the nature of DDs. The role of gene-environmental interactions, ethnicity-geographic and socio-cultural factors in manifestation of depressive symptoms via epigenetic mechanisms of gene expression regulation representing a particular interest in further research in this field could not be excluded.

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Acknowledgements. The present study was supported by the Russian Foundation for Basic Research (project No. 17-29-02195) "Genomics of aggressive and depressive human behavior".

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

Received December 1, 2018. Revised February 18, 2019. Accepted March 9, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):473-481
DOI 10.18699/VJ19.516

Population genetics of spinocerebellar ataxias caused by polyglutamine expansions

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Hereditary disorders of the neuronal system are some of the most important problems of medicine in the XXI century. The most interesting representatives of this group are highly prevalent polyglutamine spinocerebellar ataxias (SCAs). It has a basement for quick progression of expansion among different groups all over the World. These diseases are SCA1, 2, 3, 6, 7 and 17, which phenotypically belong to one group due to similarities in clinics and genetics. The substrate of these genetic conditions is CAG trinucleotide repeat of Ataxin genes which may expand in the course of reproduction. For this reason a characteristic feature of these diseases is not only an increase in patient numbers, but also a qualitative change in the progression of their neurological symptoms. All these aspects are reflected in the structure of the incidence of polyglutamine SCAs, both at the global level and at the level of individual population groups. However, most scientific reports that describe the population genetics of polyglutamine SCAs are limited to quantitative indicators of a specific condition in a certain area, while the history of the occurrence and principles of the distribution of polyglutamine SCAs are poorly understood. This prevents long-term predictions of the dynamics of the disease and development of strategies for controlling the spread of mutations in the populations. In this paper we make a detailed analysis of the polyglutamine SCAs population genetics, both in the whole world and specifically in the Russian Federation. We note that for a better analysis it would be necessary to cover a wider range of populations in Africa, Asia and South America, which will be possible with the development of new methods for molecular genetics. Development of new methods of detection of polyglutamine SCAs will allow the scientists to better understand how they lead to the brain disease, the means of their spread in the population and to develop better methods for therapy and prevention of these diseases.
Key words: spinocerebellar ataxia; polyglutamine diseases; population genetics; epidemiology.

For citation: Shuvaev A.N., Belozor O.S., Smolnikova M.V., Yakovleva D.A., Shuvaev Andr.N., Kazantseva O.M., Pozhilenkova E.A., Mozhei O.I., Kasparov S. Population genetics of spinocerebellar ataxias caused by polyglutamine expansions. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):473-481. DOI 10.18699/VJ19.516

Популяционная генетика полиглутаминовых спиноцеребеллярных атаксий

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Наследственные заболевания нервной системы – одна из наиболее актуальных проблем медицины XXI века. Особо выделяются те, которые доминируют в этой группе. К таким заболеваниям, безусловно, можно отнести полиглутаминовые спиноцеребеллярные атаксии (СЦА), имеющие в своей основе молекулярные механизмы быстро прогрессирующей экспансии среди различных групп населения нашей планеты. Это СЦА1, 2, 3, 6, 7 и 17, фенотипически объединенные в одну группу по принципу развития мозжечковой атаксии вследствие специфических генетических причин. Субстратом данных генетических заболеваний является ЦАГ тринуклеотидная

последовательность (цитозин-аденин-гуанин), которая имеет тенденцию к увеличению при передаче генома последующим поколениям. Поэтому характерная особенность этих заболеваний – не только количественное увеличение больных, но и качественное изменение течения их неврологической симптоматики. Все это отражается в структуре заболеваемости полиглутаминовыми СЦА как на глобальном общемировом уровне, так и на уровне отдельных групп населения конкретных регионов. Однако большинство работ, посвященных популяционной генетике полиглутаминовых СЦА, ограничиваются количественными показателями конкретной нозологии на определенной территории, тогда как история возникновения и принципы распространения полиглутаминовых СЦА на сегодняшний день исследованы недостаточно хорошо. Это не позволяет делать долгосрочные прогнозы относительно динамики в последующих поколениях и управлять мутагенным процессом. В настоящей работе представлен детальный анализ популяционной генетики полиглутаминовых СЦА, выведены общие генетические и частные закономерности их развития и особенности популяционной динамики в мире и в Российской Федерации. Обозначены проблемы в исследовании и выявлении таких заболеваний. Для лучшего понимания необходимо охватить широкий пласт популяций Африки, Азии и Южной Америки, что будет возможно при развитии новых методов молекулярной генетики. Такой подход к исследованию полиглутаминовых СЦА позволяет обозначить их место в контексте генетических заболеваний головного мозга и определить особенности распространения и методов генетической профилактики.
Ключевые слова: спиноцеребеллярная атаксия; полиглутаминовые заболевания; популяционная генетика; эпидемиология.

Introduction

In 1991 a new type of mutations in the human genome was discovered, the so-called "dynamic mutations". These mutations cause an increase in the number of copies (expansion) of the simple repeating sequences (Kremer et al., 1991; Warren 1996). As was revealed later, there are numerous simple (CAG, GCG, GCC, GAA, CTG) (Verkerk et al., 1991; Brook et al., 1992; Matilla et al., 1993; Kawaguchi et al., 1994; Koide et al., 1994; Gedeon et al., 1995; Campuzano et al., 1996; David et al., 1997; Zhuchenko et al., 1997; Babovic-Vuksanovic et al., 1998; Brais et al., 1998; Xiang et al., 1998; Vincent et al., 2000; O'Hearn et al., 2001), and more complex repetitive sequences (CTGG, ATTCT, CCCCGCCCCGCG) (Lalioti et al., 1997; Liquori et al., 2001; Potaman et al., 2003). The most common and heterogeneous group of such diseases is the group caused by expansion of CAG triplet in relevant genes (Table 1). CAG encodes the amino acid glutamine, so these diseases are called "polyglutamine" (Zoghby, Orr, 1999).

Today, we know of at list nine such conditions. The first one, which was described in 1991, linked to CAG expansion and causing the progressing degeneration of motor neurons, was the spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease (La Spada et al., 1991). Subsequently also this pathological mechanism has been found in eight other diseases, including Huntington's disease (HD) in the huntingtin gene, dentato-rubral-pallido-luysian atrophy (DRPLA or Haw River syndrome) in the *ATN1* gene, and six types of spinocerebellar ataxias (SCA1, 2, 3, 6, 7, and 17) (Gardian et al., 2005).

Among all polyglutamine diseases there, SCAs have the most similar pathophysiology, progression and clinical signs. Their genetics, however, is not identical (see Table 1).

Features of mutagenesis in polyglutamine SCA progression

Mechanisms of mutagenic process are dramatically different from those of the static mutations. This explains the dominance of polyglutamine SCAs over SCAs caused by static mutations. In comparison to point mutations, which occur spontaneously and stochastically, dynamic mutations have a substrate, the CAG sequence, which initially is repeated only several times (Dunnen, 2017). CAG sequence expansion usually takes place during mitosis of somatic and germ cells. Trinucleotide repeat expansion occurs by replication-dependent (Kovtun, McMurray, 2001), and reparation-dependent (Kovtun et al., 2007) mechanisms. These disturbances are the cause of the phenomenon called "anticipation", when the disease occurs progressively earlier and is more severe in subsequent generations. It was discovered that, the longer the allele is, the more unstable it becomes. The average number of CAG repeats expanded during reproduction depends on the SCA type (from +0.5 CAG repeats in SCA3 to +12 CAG repeats in SCA7) (Stevanin et al., 2000). Paternal alleles are more unstable during transmission which is probably due to a larger number of mitotic divisions during sperm cell maturation compared to oocytes during gametogenesis. However, it could also be linked with decreases in repair DNA protein concentration and activity (Pearson et al., 2005).

The prevalence of polyglutamine SCAs

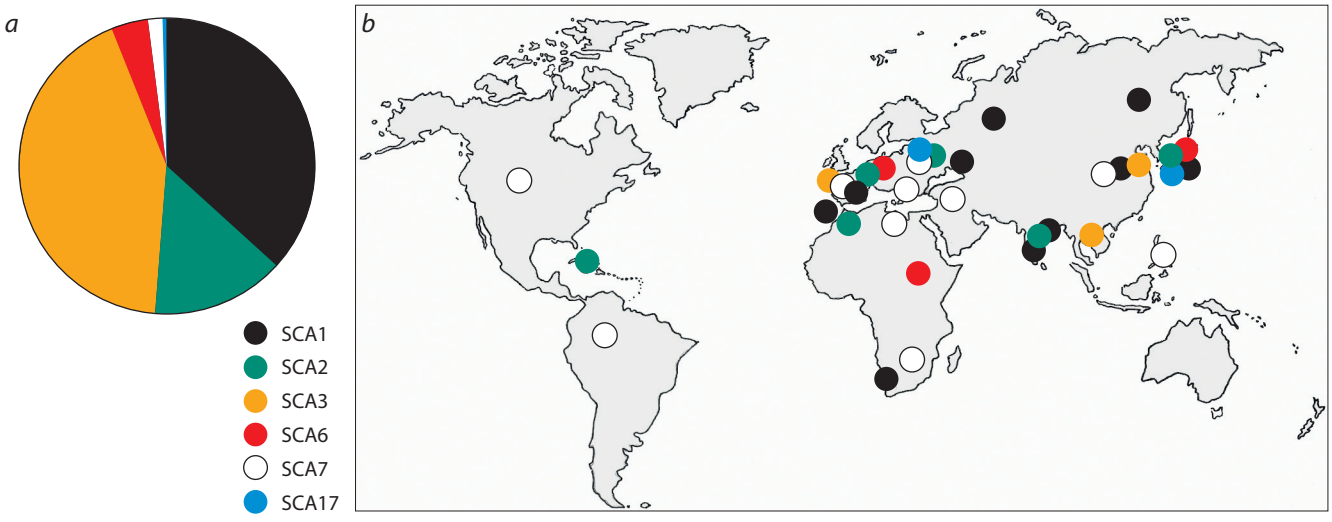
The total morbidity of polyglutamine SCAs is dramatically variable and varies around 1–9 per 100.000 with more accurate accounts being 4–5 per 100.000.

SCA1 is observed, approximately, at a frequency of 1–2 people per 100.000 of "general" population (Manto, 2005). SCA2 has been revealed in 14 % of cases of all SCAs, which makes up about 0.6 per 100.000 population (Cancel et al., 1997; Geschwind et al., 1997a; Riess et al., 1997). SCA3 at some areas is the most common autosomal dominant SCA (Schols et al., 2004; Bauer et al., 2005). The frequency of SCA3 is ~1.5–2 persons per 100.000 population (van de Warrenburg et al., 2002). The world-wide incidence of SCA6 comes near 0.02–0.31 per 100.000 population (Geschwind et al., 1997b; Ikeuchi et al., 1997; Matsumura et al., 1997; Matsuyama et al., 1997; Riess et al., 1997; Stevanin et al., 1997; Schöls et al., 1998; Pujana et al., 1999; Jiang et al., 2005). The incidence of SCA7 in several studies was 2 % of all SCAs, but according to the most accurate calculations it is 0.05–0.2 (an average 0.08) per 100.000 (Filla et al., 2000; Storey et al., 2000). Less than 100 families with SCA17 have been described to date (~0.0015 per 100.000) (Maruyama et al., 2002; Alendar et al., 2004; Craig et al., 2005) (Figure, *a*).

Overall, about 60 % of all clinical SCAs are polyglutamine SCAs, while the other identified and accurately diagnosed forms comprise less than 5 %. It is therefore important to note

Table 1. Molecular characteristics of polyglutamine SCAs

Disease	Locus	Gene	Protein	Polyglutamine chain	
				Norma	Pathology
SCA1	6p23	*Atxn1*	Ataxin-1	6–39	41–83
SCA2	12q24	*Atxn2*	Ataxin-2	14–32	34–77
SCA3	14q24-q31	*Atxn3*	Ataxin-3	12–40	55–86
SCA6	19p13	*CACNA1A*	α_{1A} P/Q Ca^{2+} channel	4–18	21–33
SCA7	3p21–p12	*Atxn7*	Ataxin-7	7–18	37–306
SCA17	6q27	*TBP*	TATA-box-binding protein	25–43	45–63



Worldwide distribution of polyglutamine SCAs.

a – the pie chart shows the percentage ratio of each polyglutamine SCA relative to the total number calculated per 100.000 population (SCA17 is only 0.037 % of the total, this value is not seen in the graph); *b* – on the World map is shown the place of occurrence of corresponding disease haplotype (black and white circles).

that 35–40 % of SCAs have no established genetic base and are only characterized by the phenotype (Bird, 1998; Jayadev, Bird, 2013). At the same time, among polyglutamine SCAs, SCA1 and SCA3 comprise 2/3 of all registered cases (37 and 42 % respectively) (see Figure, *a*).

Polyglutamine SCAs prevalence rate in Russia

Research into polyglutamine diseases in Russia has been carried out for over 20 years. The prevalence of polyglutamine SCAs in Russia is similar to those in the European populations, but there are also differences. From 105 Russian families (excepting the Yakut population) with polyglutamine diseases, SCA1, 2, 3, 6, 7 and 17 was diagnosed in 61 families. The prevalence of SCAs are: SCA1, 28 families (46 %); SCA2, 21 (34.4 %); SCA3, 7 (11.5 %); SCA6, 1 family; and SCA7, 1 family (per 1.6 %). SCA17 was found in 3 families (Klyushnikov et al., 2016, 2017). Thus, characteristic in the Russian population is a low incidence of SCA3, while in populations of the USA and Japan it is the most frequent pathology (Klyushnikov et al., 2008). Notably, the prevalence of SCA17 is very high in comparison to the distribution in global population.

It is interesting that the preponderance of SCA1 over SCA3 is common in yet another population of the Eastern Europe, Poland. The morbidity of polyglutamine SCAs in Polish population is as follows: SCA1, 83.6 %; SCA2, 13.9 %; SCA3, 0.6 %; and SCA17, 1.8 % (Krysa et al., 2016).

Genetic aspects of the origin and expansion of polyglutamine SCAs genesis

Advancements in population genetics and paleogenetics have allowed scientists to trace when polyglutamine SCAs appeared in the human population. The mutation of a gene is quite an unusual phenomenon, therefore such diseases are characterized by the presence of the founder, the person who for the first time gained a certain mutation and became the origin of the unique genetic profile called “haplotype”. Analyses of haplotypes help reveal the dynamics, common factors that affect the disease and predict the development of this pathology in the future. In polyglutamine SCAs, several haplotypes of founders were traced, with at least 2–3 separate haplotypes in each population (Lund et al., 2001; Bettencourt, Lima, 2011) (see Figure, *b* and Table 2). Usually, the mytation rate is higher and/or the mutation is older when it can be detected in wider populations or when it can be detected with a higher incidence (Lund et al., 2000; Bettencourt, Lima, 2011). However, due to the “anticipation” phenomenon, these two factors are mutually

Table 2. Worldwide distribution of poliglutamine SCAs

Mutation rate	SCA	Haplotype	Place of inhabitance	References
Slow (time of origin 80.000 years ago and later)	SCA3	Asiatic	Taiwan, India, Japan, Australia	Gaspar et al., 2001; Verbeek et al., 2004
		Portuguese (global distribution due to the great geographical discoveries)	North America, Germany, France, Portugal, Brazil, India, China, Australia	Bettencourt, Lima, 2011; Martins et al., 2012
		Cambodian (possibly a variation of the Portuguese haplotype)	Cambodia, United States	Jayadev et al., 2006
	SCA6	Paleolithic (before the division of humanity into races)	England, Japan, Brazil, Finland	Craig et al., 2008
		German	North Rhine-Westphalia	Dichgans et al., 1999
		Japanese	Tottori Prefecture	Mori et al., 2001
Fast (time of origin 900 years ago and later)	SCA1	Japanese	Miyagi and Yamagata Prefectures	Wakisaka et al., 1995; Zhou et al., 2001
		Yakut	Russia (Yakutia), China	Gouriev, 2004; Osakovsky et al., 2004; Zhou et al., 2001
		Hindustani	India (Bihar)	Mittal et al., 2005; Sinha et al., 2004
		Tamil	India (Tamil Nadu)	Mittal et al., 2005; Rengaraj et al., 2005
		Western Cape (3 haplotype may be present at once)	South Africa (Western Cape)	Ramesar et al., 1997
		Spanish	Girona, Catalonia	Pujana et al., 1999
		Italian	Southern Italy	Jodice et al., 1993
		Eastern European	Russia, Ukraine, Belarus	Popova et al., 2001
		Bashkir	Republic of Bashkiria	»
		Polish	Wielkopolska and Lodz Voivodeship	Krysa et al., 2016
	SCA2	Western European	Germany, France	Didierjean et al., 1999
		Pan-European	France, Germany, Serbia	»
		North African	Morocco, Libya	»
		Caribbean	Jamaica, Cuba	»
		Indian	India (Bihar)	Choudhry et al., 2001
		Japanese	Gunma Prefecture	Mizushima et al., 1998
	SCA7	Haplotype of Continental Europe	Belgium, Finland, France, Germany, Sweden and UK	Stevanin et al., 1999
		Italian	Italy	»
		Asiatic	Korea and Philippines	»
		Middle Eastern	Israel	»
		Anglo-Saxon	UK, USA	»
		North African	Algeria, Morocco, Tunisia	»
		South American	Brazil	»
		Scandinavian	Sweden	Jonasson et al., 2000
		South African	South African	Greenberg et al., 2006
	SCA17	Japanese	Niigata Prefecture	Koide et al., 1999
		German (high instability of CAG repeats)	Northern Germany	Zühlke et al., 2005
		German (low instability of CAG repeats)	Germany	De Michele et al., 2003; Zühlke et al., 2003

exclusive in polyglutamine SCAs. This conclusion could be suspected by comparing the haplotype numbers and SCA time of origin (see Table 2).

According to the mutation speed, age of clinical presentation and time of origin, polyglutamine SCAs can be divided conditionally into two groups. The first comprises diseases with early presentation and low speed of mutations, SCA3 and SCA6. 2–3 haplotypes are known for each polyglutamine disease.

In the case of SCA6, the size of the area occupied by the diseased population directly correlates with the time of its origin. The worldwide distribution of this pathology charac-

terized by a very low degree of mutation can be linked to the presence of a Paleolithic haplotype which arose before the division of humanity into races (about 80.000 years ago) (Craig et al., 2008). The Asiatic haplotype of SCA3 arose in the prehistoric period (about 7.000 years ago). People with this haplotype live in South and Southeast Asia, and also in northeastern part of Australia (Martins et al., 2012). However, the worldwide distribution of SCA3 is explained differently. The Portuguese haplotype arose ~1400 years ago and was local until the era of great geographical discoveries (Bettencourt, Lima, 2011). The active exploration of the World Ocean by the Portuguese resulted in this haplotype quickly spreading to the countries of Asia and the New World (Martins et al., 2012). While these haplotypes make a significant contribution to the incidence of SCA3 and SCA6, there are also "younger" local haplotypes all over the World (Dichgans et al., 1999; Mori et al., 2001; Jayadev et al., 2006).

The widespread distribution of SCA1, SCA2, SCA7 and SCA17 in the world population is due to a different reason than SCA3, SCA6. These diseases are characterized by a high level of mutations. The mutations that cause these diseases are relatively young: in most cases, they are present only in 1–20 generations and are common within various ethnic groups. Patients with these polyglutamine SCAs exhibit numerous haplotypes, in some cases these are just individual haplotypes in a single population.

SCA1 is distributed worldwide, however, the main proportion are local haplotypes. It could be seen clearly in populations of islands and remote areas with low migration rates (Wakisaka et al., 1995; Wadia et al., 1998; Sinha et al., 2004; Mittal et al., 2005; Rengaraj et al., 2005). In other regions, such as Eastern Europe, migration was always high and it lead to the spread of SCA1 to different parts of the Eurasian continent. The prevalence of SCA1 in the Russian population is due to the presence of three specific haplotypes (Eastern European, Yakut and Bashkir) (Popova et al., 2001; Gouriev, 2004).

The same tendency could be seen in other polyglutamine SCAs with high mutation rates (SCA2, SCA7 and SCA17). Here there is a large number of "young" haplotypes all over the World (Mizushima et al., 1998; Didierjean et al., 1999; Koide et al., 1999; Stevanin et al., 1999; Jonasson et al., 2000; Choudhry et al., 2001; De Michele et al., 2003; Zühlke et al., 2003, 2005; Greenberg et al., 2006). There are huge "white spots" on the World Map, which may contain a large number of yet unknown haplotypes of the polyglutamine SCAs with unique genetic signatures and clinical features. Within Africa, the northern and southern parts are relatively well studied, and several haplotypes were found among peoples living in these territories (Ramesar et al., 1997; Stevanin et al., 1999; Greenberg et al., 2006), whereas the central part of Africa remains completely unexplored.

The data obtained by Japanese researchers confirm that the incidence of polyglutamine SCA is not static, it is a dynamic process that continues and changes continuously. For example, a Japanese girl with SCA17 from Niigata Prefecture was described who had a *de novo* duplication from her father's allele (Koide et al., 1999). Therefore, she could be "founder" of a new haplotype.

The study of the haplotypes of polyglutamine SCA is important for practical medicine. In the German population, there are two haplotypes of SCA17. The first haplotype is characterized by a high instability of CAG repeats during transmission (Zühlke et al., 2005). The second haplotype is more stable (De Michele et al., 2003; Zühlke et al., 2003). For this reason, the identification of a haplotype is important not only for assessing the incidence in a single population, but also for predicting the course of the disease and for medical-genetic counseling of patients.

Isolation as a factor in the incidence of polyglutamine SCAs enhancement

Isolation is a strong factor contributing to polyglutamine SCAs. Isolation could be seen not only in areas with a low migration history due to the remoteness of the place or ethnic characteristics, but also it could be linked to traditions leading to closely related marriages (Dedov et al., 2004; Maximova et al., 2008). The most vivid example of natural isolation is the population of the small island named Flores of the Azores archipelago, where there is the highest incidence of SCA3 in the world, 1 of 140 people (Lima et al., 1998). Another striking case of natural isolation is the larger Yakut population, where the incidence of SCA1 is about 46 cases per 100 thousand population (Platonov et al., 2016). Until the XX century, the population of the Japanese islands remained in relative isolation from other peoples. Confirmation of this can be found in the official data of SCA1 incidence in Miyagi and Yamagata Prefectures where the migration rate is the lowest in Japan (Wakisaka et al., 1995). However, geographical isolation is always a relative phenomenon. Thus, in China, not only Chinese but also Yakut and Japanese haplotypes have been reported (Zhou et al., 2001).

However, a high level of genetic isolation of a certain group of people may be due not only to geographical factors. The ethological (cultural, ethnic etc.) isolation of small populations in India, who obey a cast system, also leads to impressive consequences. Thus, SCA1, which is uncharacteristic of India, is observed in Tamil families living exclusively in two villages of Rajapalayam and Kottamedu of Tamil Nadu state, with an incidence of 1 SCA1 patient per 15 healthy residents (Rengaraj et al., 2005). In general, isolation has no global effect on morbidity (Mori et al., 2001), but it is a decisive factor for a specific group of people who live for a long time in the same territory.

Conclusions

Polyglutamine SCAs have appeared a long time ago and are spread all over the World. Insufficient information on polyglutamine SCAs does not allow accurate determination of the incidence and prevalence among some population groups. Extensive regions such as Africa, India, Southeast Asia, remain virtually unexplored in terms of these diseases. However, analysis of available data from population genetics reveals a number of features of polyglutamine SCAs. The common features with other hereditary diseases are the presence of the founder, the dependence of the prevalence on the age of the mutation and the frequency of mutagenesis, as well as the prevalence of SCAs in isolated populations. The specifics of SCAs is determined by their mechanism since they occur not due to spontaneous mutagenesis but result from duplications of pre-existing CAG repeats. This mechanism explains the

prevalence of polyglutamine SCAs over others SCAs in the global population, and also the phenomenon of "anticipation". The anticipation needs to be taken into account in order to explain the dependence of the time of presentation of the disease on the rate of mutation.

One may speculate that 5–7 thousand years ago mainly diseases with a low mutation rate (*SCA3* and *SCA6*) were present, since types with high rates (*SCA1*, *SCA2*, *SCA7* and *SCA17*) lead to a rapid elongation of CAG repeats and the manifestation of the disease at younger ages in subsequent generations, which inevitably leads to the exclusion of the affected individuals from the process of reproduction.

The study of polyglutamine SCAs from the point of view of population genetics makes it possible to better determine their place in the context of genetic diseases of the brain and to understand the biological, ethnic and social features of these diseases. The development of molecular genetics will allow the scientists to cover, in the future, a wide range of populations in Africa, Asia and South America, which might not only change our understanding of the time and pattern of distribution of polyglutamine SCAs, but also reveal new mechanisms of the mutation process and disease progression.

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Acknowledgements. The scientific article was written with support of Russian Foundation of Basic Research (RFBR) grant KO_a 17-54-10005 and grant "Competitiveness Enhancement Program" for Immanuel Kant Baltic Federal University.

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

Received November 22, 2018. Revised March 19, 2019. Accepted March 22, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):482-488
DOI 10.18699/VJ19.517

The role of olfactory transport in the penetration of manganese oxide nanoparticles from blood into the brain

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There is no doubt that various nanoparticles (NPs) can enter the brain from the nasal cavity. It is assumed that NPs can penetrate from blood into the central nervous system (CNS) only by breaking the blood–brain barrier (BBB). The accumulation of NPs in CNS can provoke many neurological diseases; therefore, the understanding of its mechanisms is of both academic and practical interest. Although hitting from the surface of the lungs into the bloodstream, NPs can accumulate in various mucous membranes, including the nasal mucosa. Thus, we cannot rule out the ability of NPs to be transported from the bloodstream to the brain through the olfactory uptake. To test this hypothesis, we used paramagnetic NPs of manganese oxide (Mn_3O_4-NPs), whose accumulation patterns in the mouse brain were recorded using T1-weighted magnetic resonance imaging. The effect of intranasal application of endocytosis and axonal transport inhibitors on the brain accumulation patterns of intranasally or intravenously injected Mn_3O_4-NPs was evaluated. A comparative analysis of the results showed that the transport of Mn_3O_4-NPs from the nasal cavity to the brain is more efficient than their local permeation through BBB into CNS from the bloodstream, for example with the accumulation of Mn_3O_4-NPs in the dentate gyrus of the hippocampus, and through the capture and transport of NPs from the blood by olfactory epithelium cells. Also, experiments with the administration of chlorpromazine, a specific inhibitor of clathrin-dependent endocytosis, and methyl-β-cyclodextrin, inhibitor of the lipid rafts involved in the capture of substances by endothelium cells, showed differences in the mechanisms of NP uptake from the nasal cavity and from the bloodstream. In this study, we show a significant contribution of axonal transport to NP accumulation patterns in the brain, both from the nasal cavity and from the vascular bed. This explains the accumulation of different sorts of submicron particles (neurotropic viruses, insoluble xenobiotics, etc.), unable to pass BBB, in the brain. The results will add to the understanding of the pathogenesis of various neurodegenerative diseases and help studying the side effects of therapeutics administered intravenously.
Key words: nanoparticles; olfactory transport; magnetic resonance imaging; intravenous injection.

For citation: Romashchenko A.V., Sharapova M.B., Morozova K.N., Kiseleva E.V., Kuper K.E., Petrovskii D.V. The role of olfactory transport in the penetration of manganese oxide nanoparticles from blood into the brain. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):482-488. DOI 10.18699/VJ19.517

Роль ольфакторного транспорта в проникновении наночастиц оксида марганца из кровеносного русла в мозг

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Возможность поступления из носовой полости в головной мозг наночастиц (НЧ) различной природы не вызывает сомнения. Как уже было показано ранее, накопление НЧ в центральной нервной системе (ЦНС) может спровоцировать целый ряд неврологических заболеваний, поэтому понимание механизмов данного процесса представляет интерес как с научной, так и с практической точек зрения. Предполагается, что из крови НЧ могут проникнуть в ЦНС, исключительно преодолев гематоэнцефалический барьер (ГЭБ). Попав с поверхности легких в кровеносное русло, НЧ могут накапливаться в различных слизистых оболочках, в том числе и в слизистой носовой полости. Таким образом, нельзя исключать возможность транспорта НЧ из кровотока в мозг за счет их захвата окончаниями обонятельных нейронов. Для проверки этой гипотезы мы использовали парамагнитные НЧ оксида марганца (Mn_3O_4-НЧ), паттерны накопления которых в структурах мозга мыши регистрировали с помощью Т1-взвешенной магнитно-резонансной томографии. В настоящем исследовании была проведена оценка влияния интраназальной

аппликации ингибиторов эндоцитоза и аксонального транспорта на накопление Mn_3O_4-НЧ в структурах ЦНС при их введении в носовую полость или в кровоток. Сравнительный анализ полученных результатов показал, что перенос Mn_3O_4-НЧ из носовой полости в мозг эффективнее их проникновения в ЦНС из кровеносного русла, которое может осуществляться как за счет локального преодоления ГЭБ, например при накоплении Mn_3O_4-НЧ в зубчатой извилине гиппокампа, так и через захват и транспорт НЧ из крови клетками ольфакторного эпителия. При этом эксперименты с введением хлорпромазина, специфического ингибитора клатрин-зависимого эндоцитоза, и метил-β-циклодекстрина, вещества, разрушающего липидные рафты, участвующие в захвате веществ клетками эндотелия, продемонстрировали различия в механизмах захвата НЧ из носовой полости и кровеносного русла. В результате проведенного исследования нам удалось показать значимый вклад аксонального транспорта в поступление наночастиц в головной мозг как из носовой полости, так и из сосудистого русла. Это объясняет накопление в мозге субмикронных частиц различной природы (нейротропные вирусы, нерастворимые ксенобиотики и др.), которые не способны преодолевать ГЭБ. Полученные результаты будут полезны как для понимания патогенеза различных нейродегенеративных заболеваний, так и для исследования побочных эффектов терапевтических препаратов, вводимых внутривенно.

Ключевые слова: наночастицы; ольфакторный транспорт; магнитно-резонансная томография; внутривенное введение.

Introduction

People, like other mammalians, are constantly exposed to solid aerosols, which may include a great many of xenobiotics. Sedimentation of submicron and nanosized aerosols on the surface of the upper and lower respiratory tracts is followed by their penetration into blood and migration into internal organs, including the brain. Numerous experimental studies show that the accumulation of particulate matter in the brain of an animal leads to disruption of the dopaminergic and serotonergic systems of the brain and, as a consequence, to neurodegeneration (Tranfield, Walker, 2012). In addition, it is shown that people living than 50 m apart from motorways are at a dramatically higher risk of Alzheimer's disease. Analysis of the dependence of epidemiological situations on the concentration of solid aerosols and exhaust gases in the atmosphere shows that it is the concentration of solid particles in the air rather than the components of exhaust gases that influences the risk of neurodegenerative disorders (Chen et al., 2017).

Despite this, the respiratory and cardiovascular systems are considered the main targets for nanoaerosol toxicity (Donaldson et al., 2002; Chen et al., 2008; Brook et al., 2010; Kampfrath et al., 2011). Accordingly, most of the research is focused on the portal role of lungs in the penetration of nanoparticles into blood and their accumulation in internal organs, including the brain. At present, though, there is a significant body of experimental evidence for the key role of nasal epithelium in transporting nanoaerosols directly from the environment to the brain (Kreyling, 2016). For several viruses, the main route of penetration into the central nervous system of mammals is their transport from the nasal cavity to the brain. These include: herpes virus (HSV-1, HSV-2), (Kennedy, Chaudhuri, 2002), influenza A virus (Tanaka et al., 2003), bornaviruses (Sauder, Staeheli, 2003), rhabdoviruses, including rabies virus (Astic et al., 1993), parainfluenza (Mori et al., 2004), and prions (Zanusso et al., 2003). The basis of the olfactory transport of viruses and nanoparticles (Mistry et al., 2009) from the nasal cavity to the brain, is the uptake of the particles by the endings of the olfactory nerves, followed by movement inside the axons and passage through synaptic transmissions (Mori et al., 2005).

Since the nasal cavity is densely vascularized, NPs may enter the brain from the bloodstream. To assess the contribution of this process to the formation of observable spatiotemporal patterns of NP distribution in the mouse brain, we compared the accumulation of NPs in CNS after their intranasal and intravenous administrations. In this study, nanoparticles of manganese oxide (Mn_3O_4-NP) were used as an effective particulate paramagnetic agent detectable by magnetic resonance imaging (MRI). Assessment of Mn_3O_4-NP accumulation, based on the change in MRI signals in T1-weighted images of brain divisions of mice, was carried out 12 h after intranasal/intravenous administration. According to the literature and our preliminary experiments, this time corresponds to the maximum accumulation of intranasally introduced nanoparticles in olfactory bulbs (OB) (Khlebtsov, Dykman, 2011). To identify the role of olfactory neurons in the transport of Mn_3O_4-NPs from the nasal cavity or bloodstream to the brain, the effect of preliminary nasal application of endocytosis and axonal transport inhibitors on the level of the MRI signal in CNS was investigated.

Materials and methods

Animals. Experiments were conducted with SPF Balb/c male mice (25–32 g, age 10–12 weeks, $n_{total} = 64$). Manipulations were carried out at the Center for Genetic Resources of Laboratory Animals of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk. Experimental mice were kept in unisexual family groups of 2–5 animals in individually ventilated (IVC) cells of the OptiMice system (Animal Care Systems) under controlled conditions, at 22–26 °C, relative humidity 30–60 %, and the light:dark schedule 14:10 with dawn at 01:00. Food (Ssniff, Germany) and deionized water enriched with "Severyanka" (St. Petersburg) mineral mixture were provided to animals *ad libitum*.

Nanoparticles. We used commercially available manganese oxide Mn_3O_4 nanoparticles (Mn_3O_4-NPs, US3340, US-NANO, USA).

The crystal structure of the purchased manganese nanoparticles was determined by X-ray powder diffraction on the VEPP-3 accelerator complex with the following synchrotron

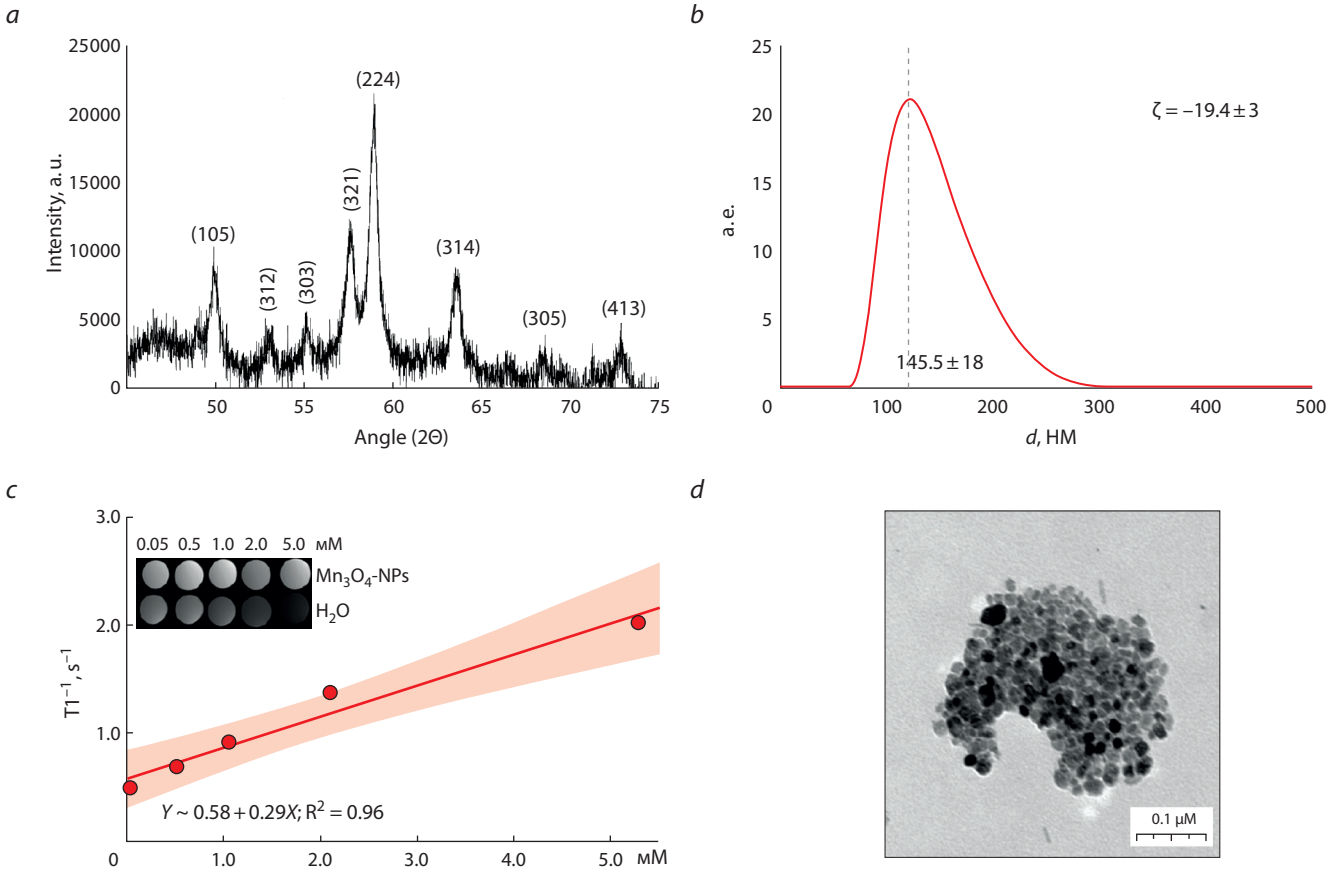

Fig. 1. Physicochemical characteristics of Mn_3O_4-NPs.

a – characterization of the crystalline phase in a sample of Mn_3O_4-NPs analyzed by powder X-ray diffraction; *b* – the values of the hydrodynamic radius (*d*) and zeta potential (ζ) of Mn_3O_4-NPs assessed by dynamic light scattering; *c* – dependence of the reciprocal of the relaxation time T1 on manganese concentration in the Mn_3O_4-NP samples; *d* – high-resolution TEM image of the Mn_3O_4-NPs.

radiation parameters: monochromatic beam wavelength λ = 0.1516 nm and angular range 2θ from 45° to 75° (Fig. 1, *a*). The hydrodynamic diameter of Mn_3O_4-NPs in a colloidal solution was determined by dynamic light scattering (angle 90°, temperature 22 ºC) and the zeta potential using electrophoresis in a U-shaped cell as recommended by the manufacturer Zetasizer NanoZS (Malvern, England) (see Fig. 1, *b*). The morphology and shape of the nanoparticles were studied by transmission electron microscopy (TEM). The JEM 1400 microscope (JEOL, Japan) was equipped with a digital camera Veleta (SIS, Germany) (see Fig. 1, *d*).

As shown in our previous work, relaxation values (R1, ms^{-1}, see Fig. 1, *c*) of Mn_3O_4-NPs correlate with manganese concentration in the samples (Romashchenko et al., 2017). *In vivo* experiments showed that the amplitude of the MRI signal in OB 12 hours after the intranasal application of Mn_3O_4-NPs was directly proportional to the manganese concentration in the tissue (Romashchenko et al., 2017). OB were taken for calibration, since the concentration of nanoparticles after the intranasal or inhalation administration is the highest in this region (Moshkin et al., 2014). Earlier, we demonstrated low solubility of Mn_3O_4-NPs at different pH (4–7), which entitled us to regard the observed changes in the MRI signal in mouse brain divisions after intranasal/intravenous administration of Mn_3O_4-NPs as a result of accumulation of insoluble manganese rather than Mn^{2+} in the nervous tissue (Romashchenko et al., 2017) and use the level of the T1-weighted MRI signal to assess NP accumulation in the tissue.

Experimental design. Animals were injected with 10 μL of a colloidal solution of particles (5.5 mg/mL) in one nostril or 100 μL of Mn_3O_4-NPs intravenously (retroorbital sinus). Five minutes before use, the NPs were dispersed for 1 min with an ultrasonic homogenizer at 20 kHz and 300 W. It had been shown in pilot experiments that the values of the MRI signal in olfactory epithelium (OE) and OB reached their maximum in both intravenous and intranasal injections 12 h after the NP injection. To determine the patterns of Mn_3O_4-NP accumulation, MRI of the mouse brain was performed twice: 24 h before and 12 h after administration. Comparison of the obtained values of the MRI signal before and after the presentation of the NPs made it possible to assess the significance of manganese accumulation in brain divisions.

In order to investigate the role of olfactory neurons in capturing and transporting manganese nanoparticles, mice were treated with 10 μL of the following compounds before intranasal/intravenous administration of nanoparticles into each nostril:

- chlorpromazine (C8138 SIGMA, Sigma-Aldrich) is a spe-

cific inhibitor of clathrin-dependent endocytosis (Wang et al., 1993; Boucrot et al., 2015). The drug was administered at a dose of 0.4 mg/kg;
- methyl-β-cyclodextrin (332615 SIGMA, Sigma-Aldrich) destroys lipid rafts (Brownell et al., 2011), participating in the capture of substances by endothelium cells. The drug was administered at a dose of 0.4 mg/kg;
- zinc chloride (229997 SIGMA, Sigma-Aldrich), inducer of olfactory epithelial cell death (Burd, 1993). The drug was administered at a dose of 20 mg/kg;
- colchicine (C9754 SIGMA, Sigma-Aldrich) inhibits tubulin polymerization, endocytosis and cellular transport (Castel, 1990). The drug was administered at a dose of 0.2 mg/kg.

For each substance, eight animals were tested. Colchicine and chlorpromazine were injected 20 min and zinc chloride solution 24 h before the intranasal application of Mn_3O_4-NPs with respect to the onset of the effect.

MRI studies. The accumulation of paramagnetic nanoparticles in brain divisions of the mouse was investigated using MRI on the Ultra-High-Definition BioSpec 117/16 USR Tomograph (Bruker, Germany) – 11.7 T. MRI scans and the subsequent processing of the obtained images were carried out in accordance with previously developed protocols (Romashchenko et al., 2017).

Statistics. To compare the two means, we used the Mann–Whitney U test. Multiple mean comparisons were performed using the LSD test (Least Significant Difference). Data are expressed as mean ± SE.

Results

To analyze the patterns of Mn_3O_4-NPs distribution with two routes of their administration, we selected mouse brain regions demonstrating a significant increase in MRI signal amplitude 12 h after the administration in comparison to control (Fig. 2, *a*). In both groups, injection of Mn_3O_4-NPs resulted in a statistically significant increase in manganese accumulation in OB and OE (see Fig. 2, *b*). In experiments with both intranasal and intravenous Mn_3O_4-NPs administration, the maximum signal level was recorded in OB (see Fig. 2). The level of MRI signal in OE and OB was higher when Mn_3O_4-NPs was injected into the nasal cavity than in the retroorbital sinus, and manganese accumulation in the dentate gyrus of the hippocampus (DG) was higher after intravenous administration of Mn_3O_4-NPs (see Fig. 2, *b*).

Thus, the propagation of Mn_3O_4-NPs after intranasal or intravenous injection was limited mainly by the structure of the olfactory system (see Fig. 2). This raises the question of the role of the olfactory epithelium in the penetration of nanoparticles into the brain from both the nasal cavity and the vascular bed. Data from the literature suggest that the mechanism of nanoparticle nose-to-brain transport is their endocytosis by olfactory neurons with subsequent axonal transport into the glomerular layer of OB, where they cross the synaptic contact and migrate to the mitral cells. We hypothesized that Mn_3O_4-NPs accumulate in OB and areas of the olfactory tract through the uptake of NPs by olfactory neurons from blood. To test this hypothesis, we used an inhibitor of clathrin-dependent endocytosis (chlorpromazine), an inhibitor of axonal transport (colchicine), a substance that destroys lipid rafts involved in the uptake of substances by endothelial cells (methyl-β-cyclodextrin, (András et al., 2012)) and zinc chloride, an inducer of the death of olfactory epithelium cells. Both the inducer of OE cell death and the inhibitor of axonal transport almost completely prevented the accumulation of Mn_3O_4-NPs in OB during their preliminary intranasal application (Fig. 3). The inhibitor of clathrin-dependent endocytosis, being introduced into the nasal cavity, reduced the accumulation of Mn_3O_4-NPs in OE and OB only after their

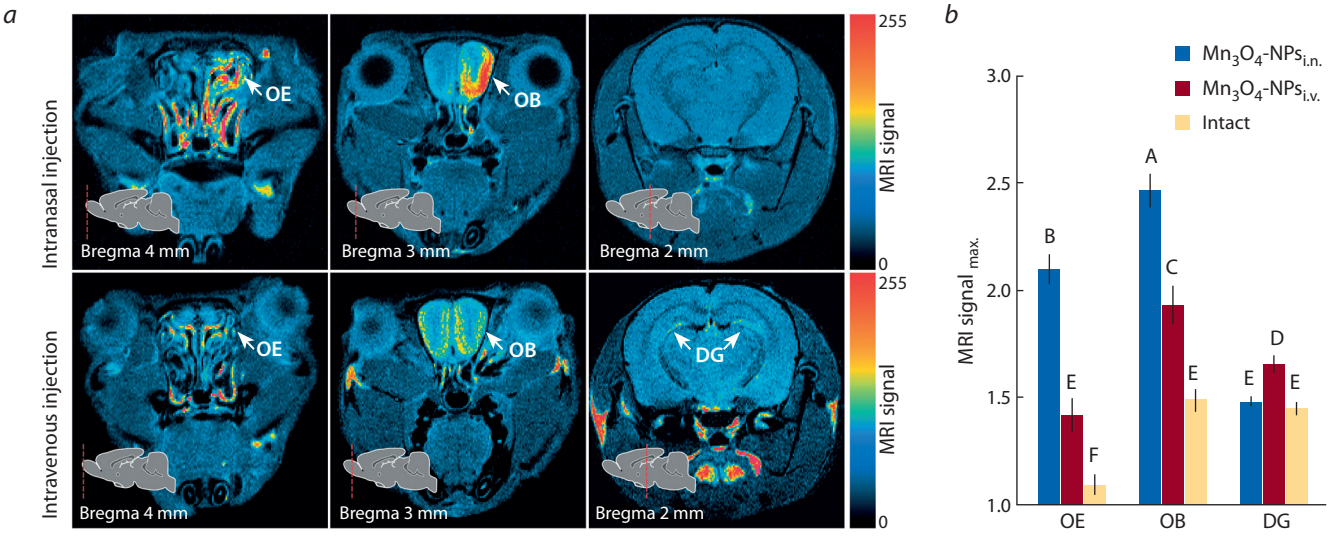

Fig. 2. Patterns of Mn_3O_4-NP accumulation in olfactory bulbs (OB), olfactory epithelium (OE) and dentate gyrus (DG) with their intranasal (*a*, top panel) and intravenous (*a*, bottom panel) administration.

White arrows indicate hyperintense sites corresponding to the accumulation of Mn_3O_4-NPs. The patterns of manganese particle accumulation on the MRI scan were visualized by pseudo staining; *b* – quantitative assessment of changes in the level of MRI signal in OB, OE, and DG 12 h after intranasal (i.n.)/intravenous (i.v.) administration of Mn_3O_4-NPs. To assess the accumulation of nanoparticles in the region, the MRI signal normalized relative to the reference was used. Intact. – averaged values of the MRI signal in OB, OE, and DG in animals before intranasal/intravenous injection of NPs. A–F – the significance of differences in mean values (LSD test, $p < 0.05$).

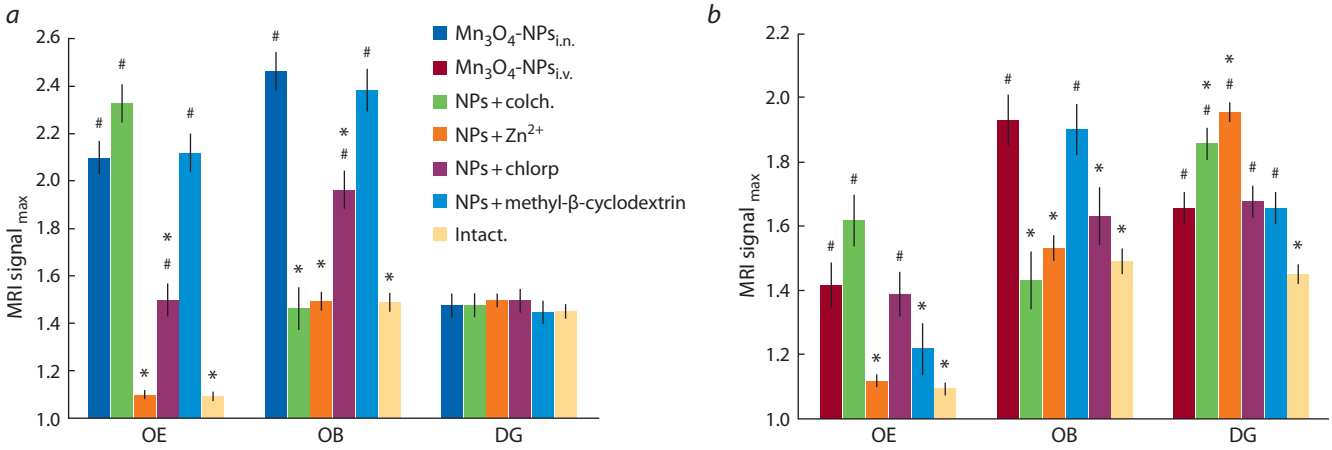

Fig. 3. Effect of intranasal application of inhibitors of endocytosis and axonal transport on the accumulation of Mn_3O_4-NPs in olfactory bulbs (OB), olfactory epithelium (OE), and the dentate gyrus (DG) of a mouse 12 h after their introduction into the nasal cavity (*a*) or the bloodstream (*b*).
To assess the accumulation of nanoparticles in the region, the MRI signal normalized relative to the reference was used. Intact. – averaged amplitudes of the MRI signal in OB, OE, and DG in animals before intranasal/intravenous injection of NPs; * – significant differences compared with the control group, which was administered only NPs (Mann–Whitney U test, $p < 0.05$); # – significant differences compared with intact animals, before the introduction of the NPs (Mann–Whitney U test, $p < 0.05$); colch – colchicine; chlorp – chlorpromazine; mβcd – methyl-β-cyclodextrin.

presentation in the nasal cavity. Intranasal administration of methyl-β-cyclodextrin significantly reduced the accumulation of Mn_3O_4-NPs in OE and OB only after their intravenous injection. None of the inhibitors applied to the nasal cavity had a significant effect on the level of MRI signal in the dentate gyrus (see Fig. 3). The results indicate a significant role of olfactory transport in the penetration and propagation of nanoparticles within the olfactory tract from both the surface of the nasal cavity and the bloodstream.

Discussion

In this work, we investigated the patterns of accumulation of Mn_3O_4-NPs in brain divisions of mice after their intravenous and intranasal presentation. The presence of manganese in brain tissue is apparent from the enhancement of the signal in T1 weighted MRI images, whose amplitude directly depends on manganese concentration (Lin, Koretsky, 1997). In particular, the microelement analysis of OB isolated from mice immediately after the MRI study showed a very close correlation between the intensity of the tomographic signal and the Mn content in the tissue (Romashchenko et al., 2017). All these observations provide grounds for considering the MRI signal amplitudes a semiquantitative indicator of the saturation of the brain tissue with manganese. The particles used in the work are practically insoluble in blood and slightly soluble at a pH corresponding to the acidic medium of lysosomes (Romashchenko et al., 2017). Therefore, the patterns of MRI contrast recorded in the first 12 h after Mn_3O_4-NPs administration are likely to reflect the accumulation of NPs but not manganese (II) ions, known to penetrate into the intracellular space (Lin, Koretsky, 1997). The Mn_3O_4-NPs used by us had a sufficiently large hydrodynamic radius (~130 nm), limiting their ability to force BBB. Therefore, after intravenous administration of Mn_3O_4-NPs, we observed the localized rather than the distributed nature of T1-weigthed MRI signal, associated with the accumulation of particles, only in OE, OB, and DG. Similar accumulation patterns were observed after the intranasal application of Mn_3O_4-NPs. Based on the level of the MRI signal, the highest concentrations of particles after injection in vein or the nasal cavity were recorded in OB and OE. In both cases, the MRI signal amplitude in OB was significantly higher than in OE, and this difference can be attributed to features of OB anatomy. Each globule in OB is innervated by several olfactory neurons (Dhuria et al., 2010), which can cause concentration of the intranasally administered contrast in OB. The congruence of the accumulation patterns obtained with two routes of NP administration convinced us that in both cases the olfactory transport plays an important role in the nose-to-brain transport of NPs, which is possibly due to the endocytosis of particles by nasal epithelial cells and their subsequent axonal transport (Mori et al., 1995; Dhuria et al., 2010; Munster et al., 2012; Hopkins et al., 2014; John et al., 2014).

It had been shown that the application of dissolved zinc salts (5%) to the surface of OE led to the almost complete death of olfactory neurons and supporting cells during the day (Burd, 1993). In our experiments, a preliminary (24 h before) introduction of zinc chloride solution caused the almost complete abolition of the MRI signal increase in OB. Subsequent experiments with the provision of colchicine, inhibiting axonal transport (Ribak et al., 1978), confirmed the hypothesis of the key role of olfactory neurons in the accumulation of Mn_3O_4-NPs in OB in both routes of administration. Also, experiments with the introduction of chlorpromazine, a specific inhibitor of clathrin-dependent endocytosis (Wang et al., 1993), and methyl-β-cyclodextrin, an inhibitor of lipid rafts (András et al., 2012), demonstrated differences in the mechanisms of NP uptake from the nasal cavities and bloodstream.

The application of inhibitors did not affect the accumulation of Mn_3O_4-NPs in DG. A statistically significant increase in the MRI signal in this region after intravenous administration of Mn_3O_4-NPs can be associated with intense neurogenesis in DG requiring additional structural (membrane) and energy resources. As a result, the trapping of substances from the

bloodstream, partly by endocytosis, may increase. In turn, this process may increase the intensity of nanoparticle uptake.

Conclusion

We demonstrate a significant contribution of axonal transport to the entry of nanoparticles into the brain, from both the nasal cavity and the vascular bed. This explains the accumulation of various submicron particles (neurotropic viruses, insoluble xenobiotics, etc.) unable to permeate through BBB in the brain. The results will add to the understanding of the pathogenesis of various neurodegenerative diseases, as well as to studies of side effects of drugs administered intravenously.

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Acknowledgements. The study was supported by the Russian Science Foundation, project 17-74-10081. Use of the equipment of the shared access center was supported by the Russian Ministry of Education and Science, unique identifier of the project RFMEFI62117X0015.

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

Received March 19, 2019. Revised April 16, 2019. Accepted April 16, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):489-495
DOI 10.18699/VJ19.518

The use of whole genome amplification for genomic evaluation of bovine embryos

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The integration of high technologies into livestock production has been actively occurring in the last decade in the countries with a developed animal breeding. First of all, we are talking about reproductive technologies (IVF) and genomic technologies (general genomic evaluation of animal and genomic evaluation of breeding value). Combining reproductive and genomic technologies is a promising approach that allows receiving high-quality breeding cattle in the shortest possible time. The basis of the proposed technology for accelerated reproduction of high-value breeding cattle is to obtain information about the genome of the embryo for genomic evaluation. The amount of genetic material that can be obtained for research is extremely limited, as it is necessary to preserve the viability of the embryo. The stage of the whole genome amplification was introduced to obtain a high quality of genetic material in a sufficient quantity. The main purpose of this work is to assess the possibility of using embryo biopsy specimens (bsp) for embryo genotyping using microarray chips and predicting the carrier status of lethal haplotypes at the embryo stage. We obtained 100 cattle embryos, of which 78 biopsy specimens were taken to analysis. For the biopsies obtained we performed the whole genome amplification. The quality and quantity of DNA for all the 78 samples after the whole genome amplification were satisfactory for further genotyping. The quality of the performed genotyping was satisfactory and allowed the assessment of lethal haplotype carriers (determining the sex of the animal and identification of the carrier status for seven Holstein lethal haplotypes). We tested 78 embryos. From the genotyping analysis, there was detected one carrier status for three lethal haplotypes, HH0 (Brachyspina), HH5, and HCD. The carrier status of HH0 and HH5 was confirmed by testing the casual mutation using PCR analysis. The carrier status for HCD has not been confirmed by casual mutation analysis. The situation in which an animal is an HCD carrier, but not the carrier of a casual mutation, can be explained. The putative ancestor of the haplotype is the bull HOCAN000000334489 WILLOWHOLME MARK ANTHONY (year of birth is 1975), but a casual mutation associated with this disease has arisen only in his descendant HOCAN000005457798 MAUGHLIN STORM (year of birth is 1991). The results obtained confirm the importance of testing the casual mutation in the animals that are carriers of lethal haplotypes according to the genotyping data.
Key words: cattle; dairy direction; breeding; genomic evaluation; breeding animals.

For citation: Pantiukh K.S., Rukin I.V., Portnov S.M., Khatib A., Panteleev S.L., Mazur A.M. The use of whole genome amplification for genomic evaluation of bovine embryos. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):489-495. DOI 10.18699/VJ19.518

Применение полногеномной амплификации для генетической оценки эмбрионов коров

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В странах с развитым животноводством в последнее десятилетие активно происходит интеграция наукоемких технологий в племенное животноводство. В первую очередь речь идет о репродуктивных технологиях (ЭКО) и геномных технологиях (оценка носительства летальных гаплотипов и геномная оценка племенной ценности). Комбинирование репродуктивных и геномных технологий – перспективный подход, который позволит получать племенной скот высокого качества в кратчайшие сроки. В основе предлагаемой технологии ускоренного воспроизводства высокоценного племенного скота лежит получение информации о геноме эмбриона для проведения геномной оценки. Так как необходимо сохранить эмбрион живым, то количество генетического материала, который можно получить для исследований, крайне ограничено. Чтобы получить ДНК высокого качества и в достаточном количестве, при прове-

дении генотипирования на чипах вводится этап полногеномной амплификации ДНК. Основной целью работы была оценка возможности использования биоптата (бп) эмбрионов для генотипирования и предсказания носительства летальных гаплотипов на основе результатов генотипирования. Нами было получено 100 эмбрионов крупного рогатого скота, из которых удалось взять 78 биоптатов. Полученные биоптаты были использованы для проведения полногеномной амплификации и генотипирования с применением микроматрицы. Качество и количество ДНК после проведения полногеномной амплификации всех 78 образцов были удовлетворительными для дальнейшего генотипирования. Результаты генотипирования позволили провести расчет пола животного и определение статуса носительства семи основных летальных гаплотипов голштинской породы. Из 78 протестированных животных по результатам анализа генотипа были найдены 3 носителя летальных гаплотипов – HH0 (брахиспина), HH5 и HCD. Носительство летальных гаплотипов HH0 и HH5 было подтверждено тестированием мутации, влияющей на потерю фертильности (казуальной) с помощью ПЦР-анализа. Статус носительства гаплотипа HCD после тестирования казуальной мутации не был подтвержден. Отсутствие казуальной мутации HCD у животного-носителя гаплотипа HCD можно объяснить тем, что предположительным родоначальником гаплотипа HCD является бык HOCAN000000334489 WILLOWHOLME MARK ANTHONY (год рождения – 1975), в то время как казуальная мутация, связанная с появлением заболевания, возникла в этом гаплотипе уже у его потомка, быка HOCAN000005457798 MAUGHLIN STORM (год рождения – 1991). Полученные данные подтверждают важность тестирования казуальной мутации у животных-носителей летальных гаплотипов.
Ключевые слова: крупный рогатый скот (КРС); молочное направление; племенное разведение; геномная оценка; племенные животные.

Introduction

In dairy industry, cows are bred to produce a large quantity of milk and dairy products. In the Russian Federation, dairy products belong to the group of food products that are socially very important. In 2008, milk production in the Russian Federation amounted to about 32.3 million tons, and in 2015 milk yield decreased by 4.7 % to 30.8 million tons. The milk production is decreasing while a lot of citizens cannot afford a sufficient amount of milk and dairy products. According to the World Health Organization, the annual minimum level of milk and dairy product consumption is 359 kg per capita; however, this figure is only 249 kg in the Russian Federation. The absence in the Russian Federation of modern animal breeding programs for dairy cattle is the main cause of the current situation.

Milk production can be significantly increased by applying selection programs that are oriented towards increasing milk yield. The response to selection is measured as the annual genetic progress in a population (ΔG). The value of genetic progress in a population depends on the variability of the trait that animals are selected for, the selection intensity, the accuracy of estimated breeding value, and the generation interval. The implementation of genomic evaluation of breeding values can improve three of the four factors that affect the genetic progress in the population (Boicharda et al., 2016). In particular, genomic evaluations can increase the accuracy of estimation of breeding value by 40 % in comparison with the accuracy obtained from the traditionally estimates of the animal parent averages. In addition, generation interval can be reduced two to three times, and selection intensity can be greatly increased due to the ability of choosing candidate young bulls from a relatively larger number of animals (Food and Agriculture Organization of the United Nations, 2007). Consequently, the implementation of genomic evaluation of breeding values increases the annual genetic gain in population three times more than that achieved by traditional progeny testing, and reduces the cost for every unite of the genetic gain by 100-fold (Kuznetsov, 2015).

Another concern in the modern dairy industry is the decrease of cattle fertility. Ignoring fertility traits in selection programs and the intensive selection for increased milk yield for many years has been accompanied by declining the reproductive performance of dairy cattle (Ma et al., 2018). In addition, the decline in female fertility can be explained, in part, by genetic factors. In fact, there are unfavorable genetic correlations between milk yield and fertility. Furthermore, in the past few years, there have been identified many genetic defects that associate with the loss of fertility. These defects are mainly inherited in the autosomal recessive manner and cause the embryonic loss in homozygous state. An approach that was developed by P.M. VanRaden and his colleagues was used to detect most of these genetic defects. The concept of this approach is that lethal recessives can be discovered from haplotypes that are common in the population but are never homozygous in live animals (VanRaden, et al., 2011). These genetic defects have been called "lethal haplotypes". Using this method has resulted in identification of seven lethal haplotypes (HH1, HH2, HH3, HH4, HH5, HCD, and HH0) in Holsteins. Another new lethal haplotype, HH6, is now being tested in the Holstein breed (Fritz et al., 2018). The carrier status of lethal haplotypes is not included in the genomic evaluation of breeding value. Genetic monitoring to identify animal carriers of monogenic diseases and haplotypes is extremely important. The comprehensive information obtained from genetic monitoring of an animal for the carrier status for monogenic diseases, carrier status for lethal haplotypes, allelic composition for milk protein genes and other economically important traits should be included in – as can be called – the animal genetic passport.

The ability of carrying out the genomic evaluation of breeding value for the viable animal embryos and monitoring these embryos for genetic defects would considerably

accelerate the production of high-genetic-merit animals because only the embryos that are non-carriers for monogenetic diseases and have the highest breeding value will be selected and transplanted.

Materials and methods

Embryos production. To produce embryos, 17 Black-and-White bulls were primarily selected to serve as service sires.

One straw of semen was collected from each sire in a volume of 250 µl. The final selection of bulls was performed based on the quality of collected semen, pedigree analysis, and the estimated breeding value of ancestors of the bulls. As a result, 12 sires were selected from three farms: the head center for the reproduction of farm animals (6 sires), "Moskovskoe" for breeding work (3 sires), and "Alta Genetics Russia"(3 sires). Semen of bulls was transported in liquid nitrogen in a Dewar tank into the station where bovine oocytes are collected from donor cows.

Holstein cows from the farm "Permskaya Kraya" were selected to serve as donor cows. The selection was based on the age, high reproductive performance, and production indicators of the cow, taking into account the breeding schemes of the farm. Cows that were excluded were sick cows, cows that showed low levels of activities, cows that were in the period of progressive weight loss after calving, exhausted and obese cows. In order to obtain large numbers of oocytes from each donor cow, an echographic characteristics analysis of the ovarian was performed.

After the aspiration, liquid aspiration was washed using Dulbecco's buffer solution. The search of oocytes was conducted under a binocular loupe. The suitability of oocytes for maturation was visually evaluated. Oocytes that have been remained for the following *in vitro* maturation are those that met the following conditions: viable, evenly surrounded by cumulus cells, a fine-grained ooplasm that evenly fulfills the transparent shell of the oocyte, a homogenous thickness of the transparent shell with a round shape. Selected oocytes of the required quality were set to mature for 22 h in an IVM media. After maturation, oocytes were washed from the IVM media and transferred to the Fertilization Medium. Spermatozoa were washed by centrifugation on a discontinuous 45 : 90 Percoll gradient and prepared for oocytes fertilization *in vitro* (IVF). Oocytes cultivation was performed on a palate incubator under a constant temperature, regulated humidity and gaseous environment. On the 6th day of cultivation, in the incubator, the obtained embryos were evaluated and only high-quality embryos were selected for biopsy. Biopsy was performed at the blastocyst stage using a biopsy needle. 30–50 cells were taken from the trophoblasts of the blastocyst. Cells were counted while they were aspirated into a biopsy pipette. Biopsy specimens in biopsy pipette were released into a 2 µl PBS × 2 buffer. The drop containing the embryonic trophoblast cells was placed at the bottom of the LoBind tube whose bottom was previously prepared with a 2.5 µl drop of PBS × 2 buffer. After the biopsy, embryos were marked using necessary markers to identify them during the next stages of the experiment.

Embryo viability was monitored for 8–24 h prior to cryopreservation. All the pedigree information and the identification number of biopsy were saved for each embryo.

Whole genome amplification and genotyping. The method of isothermal multiple displacement amplification (IMDA) was used for the whole genome amplification (WGA). The whole genome amplification was performed using GenomiPhi V2 DNA amplification kit (Illumina, USA) considering the standard recommendations for it. The whole genome amplification was performed for 78 biopsy specimens. After the whole genome amplification, the quantity of DNA for each sample was measured using NanoDrop ND1000-Technologies-Inc, Wilmington, DE, USA, while the quality of DNA was checked using agarose gel electrophoresis. The DNA concentration was adjusted to 50 ng/µl. For genotyping, 4 µl from each sample was taken, and BovineSNP50 v3 DNA Analysis BeadChip was used considering the instructions provided in the manual protocol for this microarray: Infinium® HD Assay Ultra, Manual Experienced User Card (Part # 11328095 Rev. B, Illumina, USA).

Identification of the carriers of lethal haplotypes. For the subsequent analysis, only high-quality genotypes (call rate > 95 %) were chosen. For each of the tested animals, the carrier status for the seven lethal Holstein haplotypes was determined by analyzing the existence of alleles that are included in the haplotype. The animal has been recorded as carrier for a haplotype if it has been identified the alleles combination for that haplotype.

Amplification of individual DNA fragments. All the samples were tested for HCD using the method described by (Menzi et al., 2016). The method can be summarized by using three pairs of primers: the wild type forward primer (WF) 5'-GGTGACCATCCTCTCTCTGC-3', the wild type reverse primer (WR) 5'-AGTGGAACCCAGCTCCATTA-3', and the mutant forward primer (MF) 5'-CACCTTCCGCTATTCGAGAG-3'. The primers WF and WR ensure amplification of the DNA fragment that does not contain the mutation (249 bp in size), while the WF and MF amplify the fragment that contains the insertion (436 bp in size).

PCR was performed under the following conditions: 3 min at 94 °C, followed by 35 cycles each consisting of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, ending with 5 min at 72 °C. The PCR was performed for two reaction mixes, each of them being 10 µl in the final volume; the first mix contained 2 µl of 5 × Mix (PCR-mix 5 × MasCFETaqMIX-2025), 0.4 µl of HCD WF primer (2.5 pmole/µl), 0.4 µl of HCD WR primer (2.5 pmole/µl), and 6.2 µl of H_2O. The second mix contained: 2 µl of 5 × Mix (PCR-mix 5 × MasCFETaqMIX-2025), 0.4 µl of HCD MF primer (2.5 pmole/µl), 0.4 µl of HCD WR primer (2.5 pmole/µl), and 6.2 µl of H_2O.

Samples were tested for brachyspina mutation (HH0) using the allele-specific PCR method described by (Charlier et al., 2012). The first pair of primers, forward Across_UP1 5'-TCACAAAAGGGTAGGAGACTACCTG-3' and reverse Across_DN1 5'-GCTTATTGTTTACCCTTGACAGTGG-3', were used to amplify the DNA fragment

that does not contain the deletion. The size of the fragment is 551 bp. The second pair of primers ensures amplifying the fragment containing the mutation; forward BY Within_F1 5'-GCT-CAA-GTA-GTT-AGT-TGC-TCC-ACT-G-3' and reverse BY Within_R1 5'-ATA-AAT-AAA-TAA-AGC-AGG-ATG-CTG-AAA-3'. The fragment size is 421 bp (Charlier et al., 2012).

PCR for brachyspina was performed using the following conditions: 3 min at 94 °C, followed by 35 cycles each consisting of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, ending with 5 min at 72 °C. The first PCR-mix (10 µl) contained 2 µl of 5 × Mix (PCR-mix 5 × MasCFETaqMIX-2025), 0.4 µl of Across_UP1 primer (2.5 pmole/µl), 0.4 µl of BY Across_DN1 primer (2.5 pmole/µl), and 6.2 µl of H_2O. The second mix (10 µl) contained 2 µl of 5 × Mix (PCR-mix 5 × MasCFETaqMIX-2025), 0.4 µl of BY Within_F1 primer (2.5 pmole/µl), 0.4 µl of BY Within_B1 primer (2.5 pmole/µl), and 6.2 µl of H_2O.

Three primer pairs were used to identify carriers for HH5 mutation. The (HH5_F) forward primer 5'-AGATATGCTAAAGTTTACCTAGAAGAA-3', and two reverse primers (HH5_WT_R) 5'-CTGAAGCTCCATTCTGAGTCAT-3', and (HH5_Del_R) 5'-TGCTCTATGAATTTTGTGAATGGT-3'. The primers HH5_F and HH5_WT_R were used to amplify the DNA fragment that does not contain the mutation producing a fragment, which is 442 bp in size, while HH5_F and HH5_Del_R amplify the fragment containing the mutation, and the size of the obtained fragment is 256 bp.

Two PCR reaction mixes were used, each of them being 10 µl. The first mix was 2 µl of 5× Mix (PCR-mix 5 × MasCFETaqMIX-2025), 0.4 µl of HH5_F primer (2.5 pmole/µl), 0.4 µl of HH5_WT_R primer (2.5 pmole/µl), and 6.2 µl of H_2O. The second mix contained 2 µl 5 × Mix (PCR-mix 5 × MasCFETaqMIX-2025), 0.4 µl of HH5_F primer (2.5 pmole/µl), 0.4 µl of HH5_Del_R primer (2.5 pmole/µl), and 6.2 µl of H_2O.

The PCR amplification products were analyzed on 4 % TAE-based agarose gel with a voltage of 120 V for 40 min using 1 × TAE (0.04 M Tris base, 0.02 M acetic acid, 0.5 M EDTA) buffer and ethidium bromide staining for visualization. The DNA Ladder M-50 (DIALAT Ltd., cat. no. MWM-50RL) was used for determining the size of fragments.

Results

Choosing the breed of animals for embryo production. The Black-and-White holsteinized breed was chosen for embryo production. Nowadays, there are more than 300 breeds of *Bos taurus* around the word (Durov et al., 2013); only 120 of them are breeds for milk production and only 30 breeds are the most widely spread across the world. The most common dairy breed in the world is the Holstein (Dunin et al., 2013). In the Russian Federation the most common dairy breeds are Black-and-White, Simmental, Kholmogory, Red-and-White, Holstein, Red Steppe, Ayrshire, and Yaroslavl. The animals of Black-and-White breed make up about 58 % of the total Russian dairy cattle population. The largest number of these animals is concentrated in the European part of Russia. The total number of dairy cattle in Russia is about 1.587 million; from them 939.5 thousands are Black-and-White animals.

According to the previous information and statistics, we can say that the Black-and-White breed is the best and most popular dairy breed in Russia, and it is necessary to start the genomic evaluation of breeding values for its animals. However, in order to improve the milk production in their herds, farmers usually use Holstein bulls for insemination. This has led to a high degree of holsteinization for this breed. In fact, the proportion of Holstein "blood" in the Black-and-White breed could be in excess of 50 % (Tikhonova et al., 2015).

Choosing bulls for embryo production. Assessment of semen quality of the bulls was based on the sperm concentration (the number of sperm in millions/mL), the mobility of sperm (in percent) taking into account the percent of progressive motility, non-progressive motility, and immobility sperm.

Embryo production. Five follicular aspirations were carried out for 36 donor cows. As a result, we obtained 379 cumulus-oocyte complexes (COCs); 322 of them were used for *in vitro* maturation (IVM). From the 322 COCs 100 embryos were obtained that reached the blastocyst stage (7th day of embryo development). Considering the quality of the embryos obtained, 80 embryos were selected and transferred to individual petri dishes for biopsy. From the 80 embryos, 78 biopsy specimens were obtained for which the whole genome amplification (WGA) was performed (Fig. 1).

Results of genotyping after WGA. The average concentration of DNA after amplification was 288.16 ng/µl (minimum 39.3 ng/µl, and maximum 567.4 ng/µl). Of the 78 samples, one had a concentration less than 50 ng/µl. The obtained DNA concentrations are comparable to those obtained by similar studies (Polisseni et al., 2010; Shojaei et al., 2014), and they are sufficient for carrying out genotyping by DNA microarray.

Genotyping was performed using BovineSNP50 V3 DNA Analysis BeadChip (Illumina, USA). All genotypes for the 78 samples were of satisfactory quality (call rate of sample > 95 %). 46 biopsy specimens of the 78 were identified as males and 33, as females. The distribution of DNA concentration values and the quality of genotyping of samples are shown in Fig. 2.

Identification of the carriers of lethal haplotypes. The genotypes for all the 78 embryo biopsy specimens were analyzed to identify the samples that are potential carriers of lethal haplotypes. Finding carriers for HH0 (brachypine), HH5 and HCD was expected since the frequencies of these haplotypes in the cattle population are higher than the frequencies of other haplotypes. The frequency of HH0 in the French Holstein cattle population is 7.4 % (Fritz et al., 2013), and it is 3.9 % and 6.7 % for HH5 and HCD, respectively, in the German Holstein cattle population (Schütz et al., 2016).

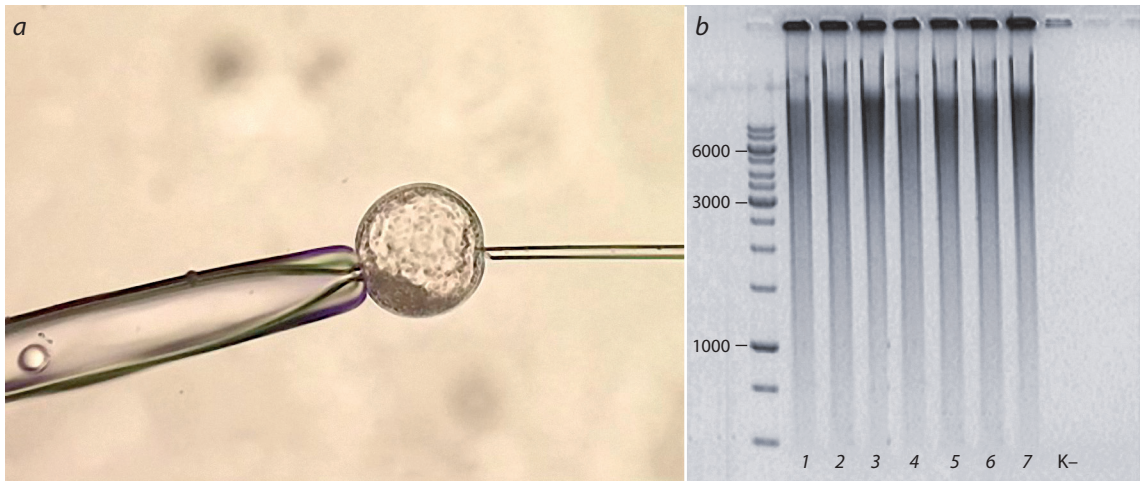

Fig. 1. Embryo biopsy and agarose gel electrophoresis for the WGA.

a – embryo biopsy using a needle; *b* – agarose gel electrophoresis for the products of the WGA (4 % gel, 120 V, 60 min, Thermo Scientific™ GeneRuler™ 1kb DNA Ladder 1kb DNA ladder, USA).

Among the 78 embryos, one carrier status for HH0, HH5, and HCD was detected in samples 14, 5, and 72, respectively.

The microarray probes do not contain DNA sequences for the direct detection of mutations associated with these lethal haplotypes, so the samples that were detected as being carriers of lethal haplotypes by analyzing the genotyping data were analyzed for the presence of casual mutations, using PCR analysis followed by gel electrophoresis. As a result, the casual mutation was confirmed by PCR analysis for the samples carriers for HH0 (brachispine) and HH5, while it has not been confirmed for the sample that is carrier for HCD (Fig. 3).

To confirm the status of carrier for HCD, but not carrier for the casual mutation, we tested the parents of this embryo for the presence of casual mutation for HCD. From the dam, we obtained hair for DNA extraction and sperms from the sire. As was expected, neither the mother nor the father were carriers for the HCD casual mutation (Fig. 4).

After genotyping and analyzing the genomic data for the presence of lethal haplotypes, the samples were tested for the presence of casual mutations using PCR (Fig. 5).

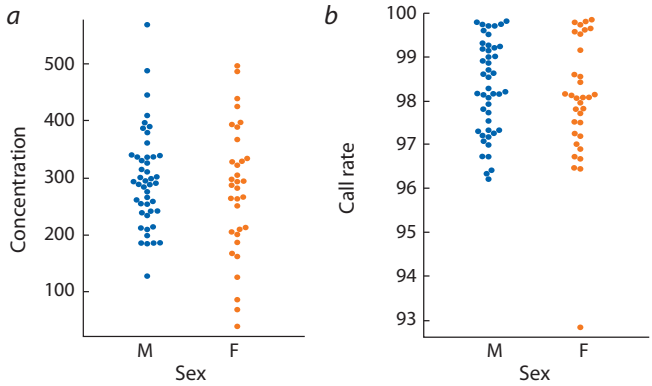

Fig. 2. The distribution of DNA concentration values and the quality of genotyping of samples (call rate).

a – DNA concentrations of biopsy specimens after the WGA; *b* – the quality of genotyping of biopsy specimens after the WGA.

Discussion

The situation in which an animal is carrier for HCD haplotype but not carrier of the casual mutation has been studied before and it is not associated with low quality of genotyping (the call rate of the genotype was 96.7 %). Large-scale studies on cattle populations have shown that animals that are homozygous for HCD can be either completely healthy or suffer from cholesterol synthesis deficiency. The pedigree analysis of these animals has shown that the primary source of the normal version of HCD haplotype is the bull HOCAN000000334489 WILLOWHOLME MARK ANTHONY born in 1975, and the casual mutation within this haplotype has occurred in its descendant, the bull HOCAN000005457798 MAUGHLIN STORM born in 1991. Thus, the bull HOCAN000005457798 MAUGHLIN STORM is considered the ancestor of the defective haplotype and its descendants carry the casual mutation, while the descendants of the bull HOCAN000000334489 WILLOWHOLME MARK ANTHONY whose family tree do not include the bull HOCAN000005457798 MAUGHLIN STORM are carriers for normal version of the haplotype, but not carriers for the casual mutation causing the disease (Kipp et al., 2015; Duff et al., 2016).

Conclusions

The results of this study show the feasibility to obtain a high quantity and quality of DNA after the whole genome amplification for embryo biopsy specimens. That indicates the possibility to perform high-quality genotyping on em-

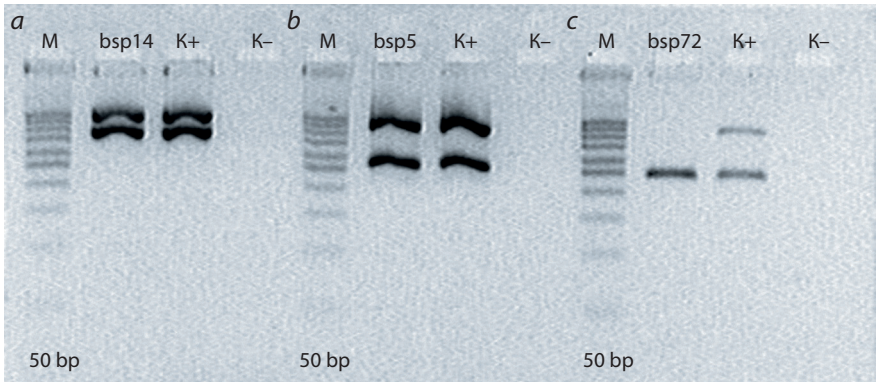

Fig. 3. Gel electrophoresis for PCR products for the samples carriers of lethal haplotypes according to microarray genotyping data.

a – HH0 (brachispine); *b* – HH5; *c* – HCD; M – 50 bp DNA marker; "K+" – positive control; "K–" – negative control; bsp14, bsp5 and bsp72 – samples analyzed.

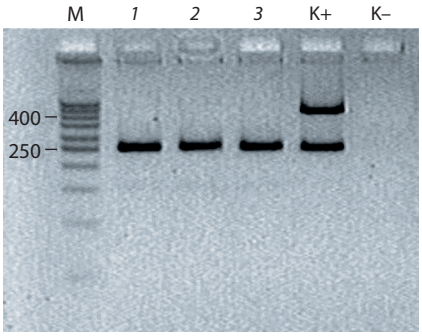

Fig. 4. Gel electrophoresis of PCR products for HCD.

1 – dam; *2* – sire; *3* – sample 72; M – 50 bp DNA marker; "K+" – positive control; "K–" – negative control.

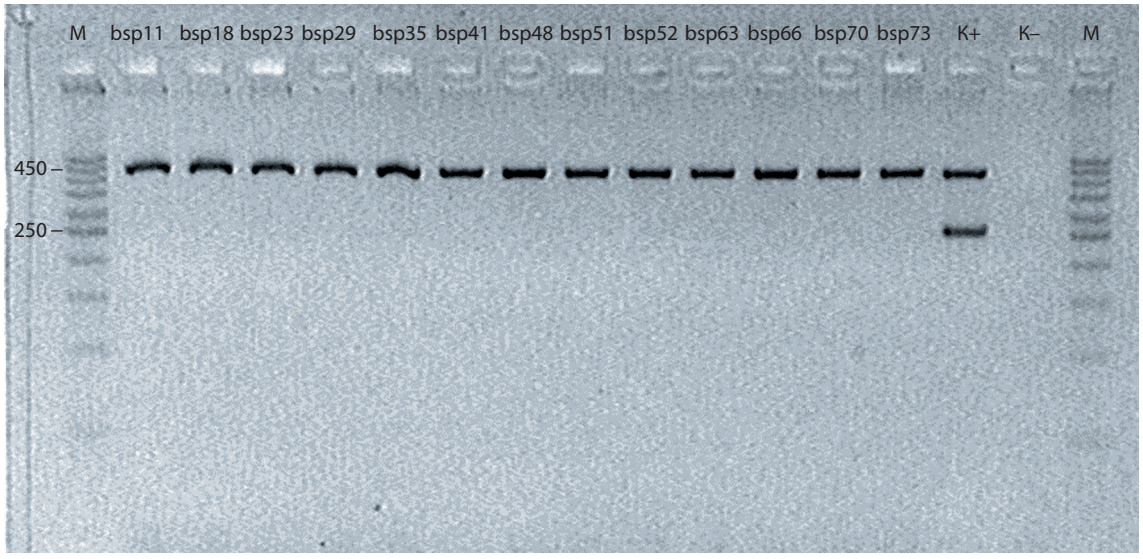

Fig. 5. Agarose gel electrophoresis of PCR products for HH5 casual mutation.

bsp11, bsp18, bsp23, bsp29, bsp35, bsp41, bsp48, bsp51, bsp52, bsp63, bsp66, bsp70, and bsp73 – samples analyzed. M – 50 bp DNA marker; "K+" – positive control; "K–" – negative control. According to the results, no carriers of HH5 casual mutation were identified.

bryos and to perform the genomic evaluation of animals at the embryo stage. The reliability of results obtained from the genotyping analysis can be confirmed by molecular genetic aspects using PCR methods – the classical methods for determining the carrier status of animals for monogenic diseases and lethal haplotypes. The embryos, from which genetic material was obtained and analyzed, were transferred to surrogate mothers. After the birth of animals, it is planned to verify the results obtained during the embryo analysis (gender, carrier status of monogenic diseases, and genotyping data) by re-genotyping the animals after birth. It is also planned to calculate the breeding value for these animals.

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Acknowledgements. This work was supported by a grant from the Ministry of Science and Higher Education of the Russian Federation within the framework of the project implementation under the grant agreement No. 14.579.21.0147. The unique identifier of the work (project) is RFMEFI57917X0147.

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

Received December 28, 2018. Revised March 28, 2019. Accepted April 19, 2019.

Вавиловский журнал генетики и селекции. 2019;23(4):496-502
DOI 10.18699/VJ19.519

Conjugacy of two types of phenotypic variability of small-leaved linden

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The properties of five bilaterally symmetrical features of the leaf blades of the small-leaved linden (*Tilia cordata* Mill.) in four populations of the Moscow Region in 2014–2017 were studied. The angle trait was excluded, because it possessed the property of directional asymmetry. Instead, a new linear trait was used: the distance between the base of the second vein of the first order and the base of the first vein of the second order on the first vein of the first order. The population difference in fluctuating asymmetry (FA) was found only in the first two traits (leaf width and distance between the bases of the first vein of the first order and the second vein of the second order). The largest value of FA was in the urban environment, the smallest was in the rural areas. A weak negative correlation was obtained between the magnitude of linear characteristics and the value of FA, as well as a weak positive correlation relationship between the values of FA in five traits. The first trait had the highest fluctuation variability, and the second one had the highest plastic variability. The regression dependence of the fluctuation variability on the plastic variability (b_1 = 0.25, p <0.05) and the dependence of these two types of variability on the interaction of the factors "year" and "site of sampling" were revealed. Thus, the conclusion was made about the conjugacy of two types of variability: fluctuation and plastic. According to the authors, asynchronous growth, competition for light in conditions of high solar activity in 2014–2016 compared to the abnormal wet summer of 2017 led to an increase in FA due to destabilization of mechanisms of growth and regulation of gene expression, which contributed to a decrease in the stability of development. The increase in FA and the decrease in the developmental stability in urban ambient in 2016 could be due to: a) an intensive flow of vehicles in spring and summer, b) a high level of groundwater in this part of the city and c) increased hydrolytic acidity of the soil.
Key words: small-leaved linden; fluctuating asymmetry; phenotypic plasticity; stability of development; fluctuation variability.

For citation: Baranov S.G., Zykov I.E., Kuznetsova D.D. Conjugacy of two types of phenotypic variability of small-leaved linden. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2019;23(4):496-502. DOI 10.18699/VJ19.519

Сопряженность двух видов фенотипической изменчивости липы мелколистной

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Изучены свойства пяти билатерально симметричных признаков листовой пластины липы мелколистной (*Tilia cordata* Mill.) в четырех популяциях Московской области в 2014–2017 гг. Угловой признак был исключен, так как он обладал свойством направленной асимметрии. Вместо него использован новый линейный признак: расстояние между основанием второй жилки 1-го порядка и основанием первой жилки 2-го порядка на первой жилке 1-го порядка. Популяционное различие во флуктуирующей асимметрии (ФА) было найдено только по первым двум признакам (ширина листа и расстояние между основаниями первой жилки 1-го порядка и второй жилки 2-го порядка). Наибольшая величина ФА листовой пластины была в городской среде, наименьшая – в сельской местности. Получены слабая отрицательная корреляционная связь между величиной пяти линейных признаков листовой пластины и значением ФА, а также слабая положительная корреляционная связь между величиной ФА этих параметров. Наибольшей флуктуационной изменчивостью обладал первый признак, а наибольшей пластической изменчивостью – второй признак. Установлены регрессионная зависимость флуктуационной изменчивости от пластической изменчивости (b_1 = 0.25; p < 0.05) и зависимость этих двух видов изменчивости от взаимодействия факторов времени и места сбора листовых пластин. Сделан вывод о сопряженности двух видов изменчивости – флуктуационной и пластической. Асинхронный рост, конкуренция за свет в ус-

ловиях высокой солнечной активности в 2014–2016 гг. (по сравнению с аномальным летом 2017 г.) приводили к повышению ФА из-за дестабилизации механизмов роста и регуляции генной экспрессии, что способствовало снижению стабильности развития. Увеличение ФА и снижение стабильности развития в городских условиях в 2016 г. могли быть обусловлены: а) интенсивным потоком автотранспорта в весенне-летний период, б) высоким уровнем залегания грунтовых вод в этой части города и в) повышенной гидролитической кислотностью почвы.
Ключевые слова: липа мелколистная; флуктуирующая асимметрия; фенотипическая пластичность; стабильность развития; флуктуационная изменчивость.

Introduction

To determine the fluctuating asymmetry (FA, a small statistically insignificant deviation from the strict symmetry of the values of the right and left parts of a homologous bilaterally symmetric trait) and the level of development stability, the dimensional or countable bilateral symmetric traits with a wide range of response to stress effect factors are used (Palmer, Strobeck, 2003).

The most common opinion is raising the FA means a reduced developmental stability, which means a decrease in the body's ability to compensate and reduce a deviation from normal ontogenetic development along a specific canalization path (Debat, David, 2001; Lens et al., 2002; Klingenberg, 2003, 2016).

The characteristics with a wide range of fluctuating asymmetry include the most genotypic variable traits with phenotypic (ecological) plasticity. They are features of many species and, for example, in small-leaved linden trees are more pronounced than in brown birch, which is associated with their species-specific properties and their affiliation to different ecological groups.

While testing developmental stability, it is important to test the magnitude of phenotypic plasticity separately from the variability associated with developmental instability caused by stress factors. According to some authors, FA is distinguished into a special type of phenotypic variability, fluctuation variability, which depends on stochastic features at the molecular-genetic level (Tikhodeyev, 2013).

For natural populations, the duration of observation of factors that influence the change in the stability of development is essential. These include climate features, biotopic characteristics, soil physicochemical status, and terrain relief. In this study, phenotypic plasticity means the variability of the size of bilateral traits.

In previous works (Baranov et al., 2015; Zykov et al., 2015), only for some traits, a high dispersion of the difference between the right and the left values (R – L) and a statistically significantly difference in FA depending on the location of the population were determined. For example, those traits were the distance between the bases of the second and the third veins of the 2nd order and the distance between the ends of the veins. The aim of this paper was to answer the question as to how stable the properties of bilateral traits during prolonged monitoring are and how the two types of variability are associated. The objectives were: to find the magnitude of the variability of traits depending on climatic conditions and the location of the population and to compare the effect of environmental factors on the level of plastic variability and fluctuation variability.

Materials and methods

Leaf blades. The collecting of leaf blades was carried out in 2014–2017 in four populations of small-leaved linden in the generative stage of development. The first population (the first site) was located in the center of the city of Orekhovo-Zuyevo, Moscow Region, 30 m away from the petrol station "British Petroleum" (BP) (55°48'13.8'' N; 38°58'23.8'' E). The second place was chosen in the western part of Orekhovo-Zuyevo, 70 m south-west of the chemical plant "Karbolit", which produces plastics based on phenol-formaldehyde resins, and 30 m away from the road parallel to the territory of this plant (55°48' 13.1" N; 38°58'23.9" E). The third site was located on the territory of the State Humanitarian-Technological University (SHTU) in the eastern part of Orekhovo-Zuyevo (55°47'31" N; 38°56'14" E). Finally, the fourth site was located within the rural settlement of Davidovo, Orekhovo-Zuyevo District, 250 m away from the "Michelin" tire plant (55°36'9" N; 38°51'33" E).

In each population, leaf blades with half width from 3 to 4 cm were evenly collected from the lower parts of the crowns of ten even-aged trees. 100 sheets of 10 wood samples were used. Material processing was carried out according to the method of V.M. Zakharov and A.T. Chubinishvili in 2000 (Zakharov, Chubinishvili, 2001). A significant addition was the new trait, as an alternative to the angular trait (No. 5). In fact, connecting two branching points of the veins represented a segment of the secant to the angle and indirectly reflected the angle between the midrib and the first bilateral vein.

In early studies, the previously used angular trait was uneasy for measurement, due to a high degree of curvature of the first lateral vein (Baranov et al., 2015; Zykov et al., 2015). The remaining traits were used to determine FA by the formula of normalizing difference $FA = |R - L|/(R + L)$. A threefold measurement of the first trait in a randomly selected sample of leaf blade was conducted. The standard error of the FA was equal to 0.28 % of the trait size mean value $(R + L)/2$. Such a standard error value (less than 1 % of the trait size) is considered acceptable for statistically significantly fluctuating asymmetry (Palmer, Strobeck, 2003).

Statistical assays. After the measurements, the data was saved in Excel spreadsheets. To test antisymmetry, one of the types of bilateral asymmetry affecting the value of FA, the values of kurtosis in samples (R – L) were tested. Directional asymmetry (DA), as the dominance of one of the bilateral sides, was tested in by the paired t-test H_0: R = L. Preliminary F-test on equality of variances was provided.

The plastic variability (PL) was calculated as: $PL = 1 - (x/X)$, where x and X corresponded to the minimum and maximum values of the leaf blade trait (Bruschi et al., 2003).

The subsequent assays were conducted in the STATISTICA 10 (StatSoft Ink). They were:

– generalized regression analysis taking into account the components of variation (when the evaluating factors influence the variability);

– Kolmogorov–Smirnov test and the same test with the Lilliefors correction for normality;

– Kruskal–Wallis test and Spearman's non-parametric correlation analysis (for multiple comparisons of samples with a deviation from the normal distribution). In the evaluating criteria, the level of statistical significance α = 0.05 % was used in all methods.

Results

Primary data processing. The Kolmogorov–Smirnov test showed that the histogram of samples |R – L|/(R + L) grouped by location and by year of sampling deviated from the normal distribution. The Lilliefors test showed a similar result ($p < 0.01$). The magnitude of the trait and the value of FA showed a weak negative correlation (Spearman $r = –0.06–0.13$); (Table 1). The highlighted values of r (see Table 1 and Table 2) correspond to $p < 0.05$.

The reason for this dependence is supposedly the competition for sunlight, which results in a decrease in developmental stability and an increase in FA in a population with a small surface of leaf blades and, accordingly, with a small amount of homologous bilateral symmetrical traits (Venâncio et al., 2016). Based on this, an important part of the preliminary analysis was the homogenization of the primary data. According to the existing ideas, in the analyzed samples, the average value of the bilaterally symmetric trait in the samples should not be statistically different. Otherwise, the correlation between the FA and the size of the trait may distort the result of the comparative analysis (Palmer, Strobeck, 2003).

It was decided to screen high and low values in samples grouped into the population category. After screening, one-factor analysis of variance showed no difference in the size of each trait among the populations ($p < 0.05$).

The character of the histogram frequency in difference values (R – L) was investigated. 69 % of samples grouped by place and year of collection were characterized by kurtosis γ in the range of 0 ÷ 2. 30 % of samples showed a peak distribution with a value of γ = 2 ÷ 4. In 10 % of cases, in samples the kurtosis was less than zero, but not lower than –0.2. According to the tabular data obtained using permutation multiplication, the critical value γ, indicating antisymmetry, is equal to the value γ = –0.68 (α = 0.05; $n = 100$) (Palmer, Strobeck, 2003). Thus, in samples (R – L), grouped by site and year of sampling, antisymmetry was not detected.

Checking up for the presence of directional asymmetry in samples (R – L) confirmed the presence of DA in six cases in the sixth and in one case in the third trait (site Karbolit, 2017). These samples were not used in the work, since directional asymmetry, like antisymmetry, interferes with the evaluation of fluctuating asymmetry, which is the only indicator of fluctuation variability.

Debatable is the question of the usage of traits highly correlated in FA value. Spearman's correlation analysis showed a weak positive correlation between four pairs of traits (Table 2).

Table 1. Correlative association size – FA (Spearman's r)

Trait, No.	1	2	3	4	5
1	–0.05	–0.04	–0.04	–0.05	–0.02
2	–0.05	**–0.13**	0.05	0.01	–0.02
3	0.02	0.05	–0.04	–0.03	0.03
4	–0.03	**–0.07**	**–0.06**	**–0.07**	0.01
5	–0.02	**–0.06**	0.00	**–0.08**	**–0.10**

Notes: The highlighted values correspond to $p < 0.05$.

Table 2. Coefficients of pair correlation between FA value (Spearman's r; five traits)

Trait, No.	1	2	3	4	5
1	1.00	**0.06**	0.05	–0.01	**0.09**
2	**0.06**	1.00	0.04	0.03	**0.15**
3	0.05	0.04	1.00	0.02	0.04
4	–0.01	0.03	0.02	1.00	**0.13**
5	**0.09**	**0.15**	0.04	**0.13**	1.00

Notes: The highlighted values correspond to $p < 0.05$.

The weak Spearman's r indicated a weak positive correlation. In the case of high correlation, the traits could not be independent. The weak correlation dependence was quite natural, that is, an increase in the FA of one trait led to an increase in the FA in another trait. For example, close in location traits Nos. 2–5 showed a conjugate fluctuation with a correlation coefficient $r = 0.09–0.15$.

Population variability. Testing population variability by the non-parametric Kruskal–Wallis test did not show a difference in mean value of FA ($p > 0.05$). Analysis of the variability of each trait showed a statistically significant difference in the first and second traits (Fig. 1).

The first trait differed in the median test ($p = 0.01$), the second one differed significantly in both the median test ($p = 0.039$) and the Kruskal–Wallis test ($p = 0.001$). The pairwise comparison showed that the population in Davidovo differed from the populations in the State Humanitarian Technology University area ($p = 0.002$) and in the "British Petroleum" gas station area ($p = 0.003$). The site "State Humanitarian Technology University" had the highest value of FA and, accordingly, a reduced development stability of population.

Temporal dynamics of variability. The Kruskal–Wallis test showed that a statistically significant difference in fluctuating asymmetry depending on the year of collection was characteristic of traits Nos. 1–2 (Fig. 2).

In other traits the difference in FA was not revealed during four years of observation. In analysis of variance, the statistically significant difference revealed ca. in the same way as in the nonparametric one, i. e. the value of FA differed in 2014 and 2016 ($p = 0.001$). It should be noted that the first and the

second traits are the largest in size, and the high variance and heterogeneity of the values of R and L contributed to the exhibit of differences in FA. Increased FA in 2014 can be explained by a high temperature in May during the formation of linden leaf blades (the air temperature was 15 to 60 % above the norm according to the report of the Hydrometeorological Centre of Russia).

The relationship between the two types of variability. It is known that the ecological plasticity of plants is determined by the buffer capacity of morphological structures, which allows them to actively adapt to environmental conditions. There are different views on the question of the relationship of developmental stability and environmental plasticity. For example, there is an opinion about the adaptation role of FA and the correlation between plasticity and developmental stability, or their partial correlation (Debat, David, 2001; Klingenberg, 2003).

A regression analysis was performed to find the relationship between fluctuation variability and plastic variability. The year of sampling was used as a fixed component of variation, the factor "site", and the interaction of "year × site" registered as random factors. The results showed that plastic variability was affected by the amount of FA (1st trait) and by the year and the mixed interaction of the factors "site" and "year of sampling" (Table 3).

Thus, the greatest impact on the plastic variability influenced the FA of the first trait (width of blade) ($p = 0.004$). The climatic conditions of the year and the interaction of the factors "year × site" were also significant ($F = 11.0$ and $F = 6.97$). A similar study was conducted on the effect of plastic variability, the year and site of sampling on the fluctuation variability (Table 4).

Plastic variability of only one, the second, trait had a statistically significant effect on FA ($p = 0.001$). The combined effect of "year × site" was significant, as was the effect of FA on plastic variability ($F = 4.19$; $p = 0.0001$). The profile graph in 3D space made it possible to estimate the impact of the site and the year of sampling on the FA value and on the value of PL (Fig. 3).

The dependence profile showed the highest value of FA in the area of the State Humanitarian Technology University (SHTU). Increased plastic variability was observed in 2014–2015 and depended on the year of sampling of leaves. Parametric estimation using univariate analysis of variance also showed a statistically significantly dependence of PL on the year of sampling (df = 3; F = 17.28; $p = 0.000$).

The described relationship of developmental stability and plastic variability characterised the 1st and 2nd traits. The main difference in the two types of variability was in their response to the ambient factors of the site and the year of sampling. The value of the plastic variability of morphological structures depended significantly on the climatic conditions of the year, as well as on the combination of these conditions with the specifics of the population station.

Developmental stability did not depend on the location of the population or on the year of sampling, but depended on the effect of the interaction of both factors. Trait No. 2 (the distance between the bases of the veins) possessed highest plastic variability; it influenced the FA mean value. The greatest fluctuation variability possessed by trait No. 1 (leaf width), which, accordingly, influenced plastic variability.

Multiple regression analysis showed the dependence of fluctuation variability on plastic variability with a regression coefficient $b = 0.25$ ($p < 0.05$).

Discussion

The use of non-parametric estimation methods was reasonable, since the logarithm techniques did not lead to normalization, or only part of the samples were normalized. The angular trait was replaced by a linear one, which made it easier to determine the FA index. Both types of variability, fluctuation and plastic, showed a conjugate effect: FA of the first trait influenced PL, and plastic variability of the 2nd trait influenced fluctuation variability. Such a conclusion seems to be consensus, since in the literature on this issue there is an opinion on both the one-sided effect of FA on PL (Houle, 2003; Tonsor et al., 2013; Tucić et al., 2018), and on the effect of plastic variability on FA, for example, in *Iris pumila* plants (Sultan, 2003). In other words, the traits were characterized by conjugation of 2 types of variability. The predominance of one type of variability was compensated for by the weakness of

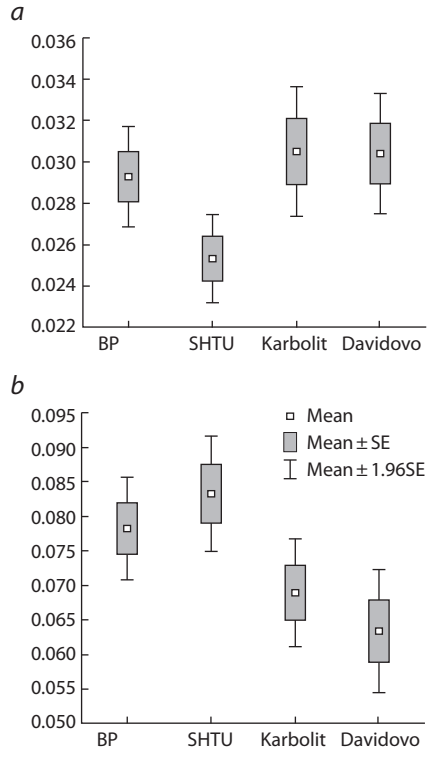

Fig. 1. Difference in FA value among populations (axis *OY*): A, *a* – first trait, *b* – second trait.

Here and in Fig. 2. Mean – mean value; SE – standart error.

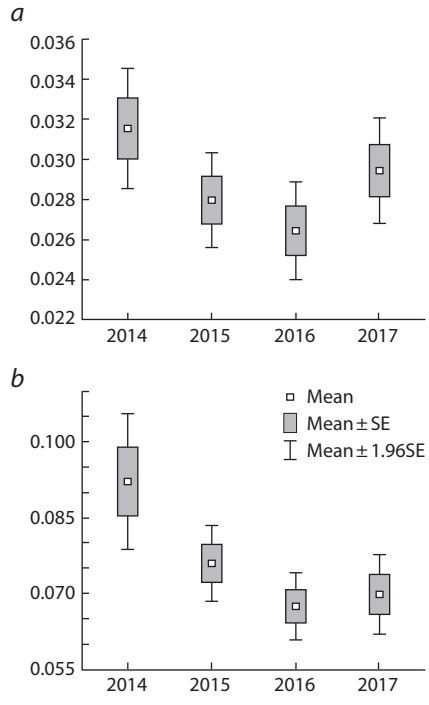

Fig. 2. Dependency FA value (axis *OY*) from year leaf blade sampling.
a – first trait, $p = 0.001$; *b* – second trait, $p = 0.012$.

Table 3. Effect of fluctuating asymmetry, year and site of sampling on plastic variability

Source	df effect	MS effect	df error	MS error	*F*	*p*
Fluctuation variability						
Trait 1	1	38.70	31.08	3.91	9.89	0.004
Trait 2	1	6.83	18.66	4.70	1.45	0.243
Trait 3	1	0.59	174.61	2.96	0.20	0.657
Trait 4	1	8.64	98.47	3.06	2.83	0.096
Trait 5	1	0.09	37.02	3.45	0.03	0.875
Site	3	10.61	8.51	37.57	0.28	0.837
Year	3	238.37	6.52	21.67	11.00	0.006
Site × year	7	17.03	1021.00	2.44	6.97	0.0001

Notes: Here and in Table 4: df – degree of freedom; MS effect – mean square; df error – df error degree of freedom of unexplained random error; MS error – mean square of the error; F – criterion of Fisher; *p* – statistical significance.

Table 4. Effect of factors plastic variability, year and site of sampling on fluctuation variability

Source	df effect	MS effect	df error	MS error	*F*	*p*
Plastic variability						
Trait 1	1	0.002	7.170	0.001	1.996	0.200
Trait 2	1	0.012	193.963	0.001	12.333	0.001
Trait 3	1	0.002	832.745	0.001	2.143	0.144
Trait 4	1	0.001	817.463	0.001	0.643	0.423
Trait 5	1	0.004	251.019	0.001	4.337	0.038
Site	3	0.003	7.382	0.004	0.627	0.619
Year	3	0.001	7.634	0.004	0.324	0.808
Site × year	8	0.003	1092.000	0.001	4.195	0.0001

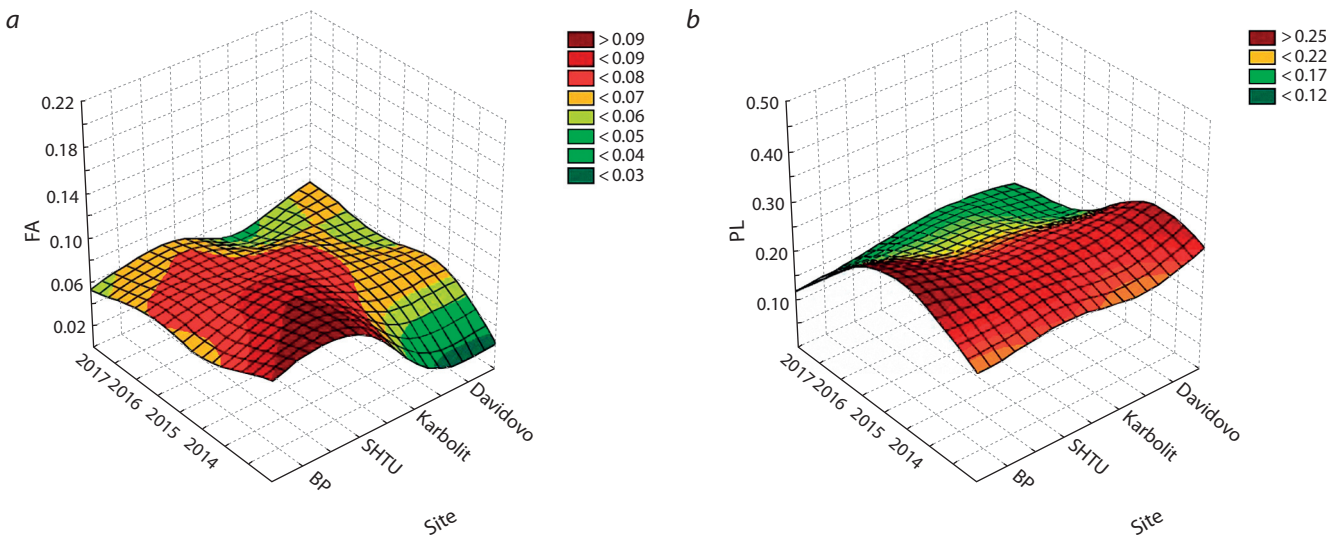

Fig. 3. Profile of the dependency of FA value (*a*) and plastic variability, PL (*b*) on the year and site of sampling.
In the tab: gradient profile FA and PL.

another type, for example, the weak fluctuation variability of trait No. 2 was compensated for by its high plastic variability.

In our opinion, asynchronous growth and competition for light in conditions of high solar activity in 2014–2016, compared with an anomalous summer of 2017, led to an increase in FA due to destabilization of the growth mechanisms and regulation of gene expression, which contributed to a decrease in developmental stability in the State Humanitarian Technology University area.

Evaluation of the components of the variance of plastic variability showed that the effect of the "year" was 26.2 % of the total dispersion, and the interaction of the factors "year" and "site" was 5.1 %.

The dispersion of fluctuating asymmetry was explained by a small fraction of the dispersion (about 2 %), which included the variance of the PL factors and "year × site".

We associated the increase in FA and the decrease in developmental stability in the State Humanitarian Technology University area in 2016: a) with a high level of intensity of the flow of vehicles, especially in spring and summer, b) with a high level of groundwater in that part of the city and c) with increased hydrolytic acidity soil. According to unpublished data, in the soil samples from Davidovo and "British Petroleum" petrol stations this indicator was 3.1–3.8 mg × eq[H^+]. In the area of SHTU, the hydrolytic acidity index was significantly higher, 5.7 mg × eq[H^+].

Environmental plasticity appears to be a highly heterogeneous type of variability, which depended on the year of sampling leaves. An abnormally wet year (the precipitation rate was over three times that in 2015) rather favorably affected the stability of the development of small-leaved linden populations in the decrease of the level fluctuating asymmetry.

It is known that the term "ecological plasticity" explains rather adaptive processes and characterizes the increased variability. The term "ecological canalization", as an attribute of development homeostasis, has the meaning of stabilizing phenotypic variability (Debat, David, 2001). Such a dialectical opposition seems to us to be a source of microadaptation of the small-leaved lime tree. In our case, first of all – to the climatic conditions. The nature of the unexplained share of the developmental stability variance remains unclear, as stated by many authors (Houle, 2003; Sultan, 2003; Lajus, Alekseev, 2004; Scheiner, 2014; Tuci et al., 2018). The results of studies conducted in 2004–2007 showed an increased fluctuating asymmetry in the area of the Karbolit on the fourth trait (distance between the bases of the first and second veins of the 1st order), which indicates a high functional variability of traits exhibiting developmental stability (Baranov, 2014).

Conclusion

The fluctuating asymmetry, associated by negative correlation with the size of the trait, manifested itself as an ontogenetic form of variability and depended on local and climatic factors.

The authors believe that long-term phenogenetic monitoring of natural populations using a set of additional environmental factors and bilaterally symmetrical features will allow a more complete analysis of the quantitative components of plastic and fluctuation variability.

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Acknowledgements. The authors are grateful to everyone who assisted in the collection and preparation of herbarium material.

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

Received May 8, 2018. Revised April 9, 2019. Accepted April 9, 2019.