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The application of Vavilov's approaches to the phylogeny and evolution of cultivated species of the genus *Avena* L.

I.G. Loskutov[1, 2] ✉, A.A. Gnutikov[1], E.V. Blinova[1], A.V. Rodionov[2, 3]

[1] Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
[2] Saint Petersburg State University, St. Petersburg, Russia
[3] Komarov Botanical Institute of the Russian Academy of Sciences, St. Petersburg, Russia
✉ i.loskutov@vir.nw.ru

Abstract. The central problem that Vavilov was investigating was the overall concept of global plant genetic resources. The theoretical basis of this concept consisted of the law of homologous series in variation, research on the problem of species as a system, botanical and geographical bases of plant breeding, and the key theory of the centers of origin of cultivated plants. The VIR global collection of plant genetic resources collected by Vavilov and his associates from all over the world reflects the fullness of botanical, morphological and genetic diversity, and can be used for historical, evolutionary, phylogenetic and applied breeding research aimed at unlocking the potential of all the collection material. The whole diversity of cultivated oats, as was proved by Vavilov, had originated from segetal weeds. This process can be clearly traced in Spain on the example of the cultivated diploid species *A. strigosa*, *A. abyssinica* in Ethiopia, *A. byzantina* in Turkey and Iran, and on segetal forms of *A. sativa*. The studies of the morphological features as a whole do not yield a complete picture of the evolutionary and systematic status of some oat species and forms. The methods and approaches that use DNA markers and genomic technologies, and are promising for the study of oat polymorphism and phylogeny have been actively researched recently. A number of works devoted to the molecular aspects of the evolution and phylogeny of the genus *Avena* have recently appeared. The research uses various markers of genes, gene regions, intergenic spacers (internal and external), both nuclear and chloroplast and mitochondrial, genomic approaches and other modern methods. On the basis of a comprehensive study of the complete intraspecific diversity from different zones of the distribution range of cultivated oat species as well as on the basis of an analysis of data on the geography of forms and species distribution ranges, it was established that the process of hexaploid species formation also took place in the western part of the Mediterranean, and subsequently, when moving eastward, these forms started occupying all the vast spaces in the region of the Southwest Asian center, forming a large intraspecific diversity of wild forms and weedy ones in transit to cultivated hexaploid oat species. An analysis of the intraspecific diversity of landraces has specified the centers of morphogenesis of all cultivated oat species. The phylogenetic analysis of the representative intraspecific diversity of cultivated and wild *Avena* species carried out using next generation sequencing (NGS) showed that diploid species with A-genome variants are in fact not primary diploids, but a peculiar Mediterranean introgressive hybridization complex of species that sporadically enter into interspecific hybridization. It was established that the tetraploid cultivated species *A. abyssinica* had most likely originated from the wild *A. vaviloviana*. An analysis of the ways of *A. sativa* and *A. byzantina* domestication showed that the most widespread ribotype of the *A. sativa* hexaploid was inherited from *A. ludoviciana*, and the second most widespread one, from *A. magna*, while *A. byzantina* has two unique ribotype families, most likely inherited from an extinct oat species or a still undiscovered cryptospecies.

Key words: *Avena* species; center of origin; itraspecific diversity; law of homologous series; NGS methods; sequences; VIR global collection.

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Использование подходов Н.И. Вавилова к филогении и эволюции культурных видов рода *Avena* L.

И.Г. Лоскутов[1, 2] ✉, А.А. Гнутиков[1], Е.В. Блинова[1], А.В. Родионов[2, 3]

[1] Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
[2] Санкт-Петербургский государственный университет, Санкт-Петербург, Россия
[3] Ботанический институт им. В.Л. Комарова Российской академии наук, Санкт-Петербург, Россия
✉ i.loskutov@vir.nw.ru

Аннотация. Центральной проблемой, которую исследовал Н.И. Вавилов, было учение о мировом генофонде культурных растений. Теоретическую основу этого учения составили: закон гомологических рядов в наследственной изменчивости, разработка проблемы вида как системы, ботанико-географические основы селекции и теория центров происхождения культурных растений. Собранная Н.И. Вавиловым и его соратниками со всех уголков мира коллекция генетических ресурсов растений ВИР, представляющая всю полноту ботанического, морфологического и генетического разнообразия, позволяет проводить исторические, эволюционные, филогенетические и прикладные селекционные исследования, направленные на раскрытие потенциала всего коллекционного материала. Положения Н.И. Вавилова по комплексному анализу всего видового и внутривидового разнообразия культурных и диких видов дают возможность сделать верные выводы при изучении сложных экологически дифференцированных видовых систем, связанных в своем формировании с определенной средой и воздействием отбора. Все разнообразие видов культурного овса, как было доказано Н.И. Вавиловым, имеет сорно-полевое происхождение. Этот процесс можно наглядно проследить в Испании – на примере культурного диплоидного вида *Avena strigosa*, в Эфиопии – *A. abyssinica*, в Турции и Иране – *A. byzantina* и на сорно-полевых формах *A. sativa*. Изучение комплекса морфологических признаков не дает полного представления об эволюционном и систематическом положении некоторых видов и форм овса. Для исследования полиморфизма, филогении и эволюции овса перспективны активно разрабатываемые в настоящее время методы и подходы с использованием ДНК-маркеров и геномных технологий. Появился ряд работ, затрагивающих молекулярные аспекты эволюции и филогении рода *Avena*. В исследованиях используют различные маркеры генов, участков генов, межгенных спейсеров (внутренних и внешних), как ядерных, так и хлоропластных и митохондриальных, геномные подходы и другие современные методы. На основе комплексного изучения полного внутривидового разнообразия из разных зон ареала культурных видов овса и анализа данных по географическому распределению ареалов форм и видов установлено, что процесс формирования гексаплоидных видов шел также в западной части Средиземноморья, и затем при продвижении на восток эти формы стали занимать значительные пространства в районе Юго-Западного Азиатского центра, образуя большое внутривидовое разнообразие диких и переходных сорных форм к культурным видам гексаплоидного овса. В результате анализа внутривидового разнообразия староместных сортов были уточнены центры формообразования всех культурных видов овса. Осуществленный с помощью метода секвенирования следующего поколения (NGS) филогенетический анализ представительного внутривидового разнообразия культурных и диких видов рода *Avena* показал, что диплоидные виды с вариантами генома А в действительности являются не первичными диплоидами, а своеобразным средиземноморским интрогрессивно-гибридизационным комплексом видов, спорадически вступающих в межвидовые скрещивания. Установлено, что тетраплоидный культурный вид *A. abyssinica*, вероятнее всего, происходит от дикого вида *A. vaviloviana*. Анализ путей одомашнивания культурных видов овса *A. sativa* и *A. byzantina* показал, что наиболее массовый риботип гексаплоида *A. sativa* унаследован от *A. ludoviciana*, а второй по массовости – от *A. magna*, в то же время *A. byzantina* обладает двумя уникальными семействами риботипов, скорее всего, унаследованными от вымершего вида овса или криптовида, до сегодняшнего дня не обнаруженного.
Ключевые слова: виды овса; центры происхождения; внутривидовое разнообразие; закон гомологических рядов; NGS секвенирование; мировая коллекция ВИР.

Introduction

When considering the scientific heritage of Nikolai Ivanovich Vavilov, it is notable how his studies as a plant grower, breeder, botanist and ethnographer are intertwined and complement each other. It is impossible to draw boundaries between his works on breeding, plant growing and genetics. This feature of his scientific style is of great importance, as it marks a turn in theory and research methods. He always took new paths and regarded the world of plants he was studying from a new, still unknown point of view.

The central problem N.I. Vavilov was investigating was the overall concept of the global diversity of plant genetic resources. It included a number of his major theoretical generalizations, which determined new paths in the theory of introduction and applied botany, brought world fame to Vavilov and played a prominent role in the development of genetics and agricultural crop breeding throughout the world. The theoretical basis of this concept was the law of homologous series in variation, developments of the problem of species as a system, botanical and geographical foundations of breeding, and the theory of the centers of origin of cultivated plants (Loskutov, 1999, 2009).

The main ideas that were dominant in N.I. Vavilov's works were the idea of plant world evolution, and the idea of botanical geography and the sequence of variability stages in space and time, characteristic of cultivated and wild plant species (Vavilov, 1997).

The sources of N.I. Vavilov's special approach to the study of vast plant material are found in the creative work of his great predecessors, namely Alphonse De Candolle and Charles Darwin. It is noteworthy that Vavilov's book "Studies on the Origin of Cultivated Plants" (1926) began with the words "Dedicated to the memory of Alphonse de Candolle, author of "Géographie botanique raisonnée", 1855, "La phytographie ou l'art de décrire les végétaux considérés sous différents points de vue", 1880, "Origine des plantes cultivées", 1882".

In his article "The theory of the origin of cultivated plants after Darwin" (1940), N.I. Vavilov noted that in his approach to the variability and evolution of cultivated plants, Darwin relied primarily on the works of A. De Candolle, but unlike him, Darwin was interested in the evolution of species, in hereditary changes that a species introduced into cultivation had undergone, while De Candolle was interested in establishing the homeland of cultivated plants.

Unlike De Candolle, Vavilov, like Darwin, paid great attention to both the main areas of the species origin and the evolutionary stages the species were passing during their spreading influenced by cultivation, environmental conditions, and natural and artificial selection. Based on the main provisions of the theories of Darwin and De Candolle, N.I. Vavilov formulated tasks for research designed for a long period of time. N.I. Vavilov conceived a systematic study of the genetic diversity and origin of the most important crops, encompassing all the evolutionary stages, from the primary areas where connections with wild forms can still be traced and where phylogenetic relationships between various wild species and cultivated forms can be established, tracing further historical distribution of species, up to the final aspects of modern breeding (Vavilov, 1992).

Nikolai I. Vavilov noted that evolution proceeded in space and time; which means that only by closely approaching the geographical centers of morphogenesis, having discovered all the links connecting the species, one can search for ways to master the synthesis of Linnaean species, with the understanding of the latter as systems of forms that have a huge intraspecific diversity of alleles. The problem of speciation itself was considered by Vavilov not as a problem of the formation of separate races, which, according to Darwin, were separating into specific species, but as a process of the origin of complex, genetically and phenotypically diverse populations, representing true Linnaean species, for each of which and for each related group of which its own spectrum of morphological and physiological variability is characteristic (Vavilov, 1992).

The discovery of the centers of origin of cultivated plants by Vavilov in 1926 is so significant, as it opens a possibility of finding in these areas valuable genetic diversity of plant forms that are most adaptive to various environmental conditions and are represented by heterogeneous populations (Vavilov, 1992).

In the primary centers, diverse and sometimes opposite genetic processes can take place simultaneously and independently of each other, leading to a mismatch between the centers of plant origin and the centers of the greatest intraspecific genetic diversity. These are the centers where the majority of dominant alleles of genes are concentrated. The zones of recessive forms concentration are the areas of intense mutational morphogenesis, which are located on the periphery of the centers of origin. An analysis of dominant and recessive forms ratio within species in a certain geographical area can reveal the level of morphogenesis, the rate and stage of species evolution (Vavilov, 1992).

Summing up his fruitful work on speciation, N.I. Vavilov published the work entitled "The Linnean species as a system", the main provisions of which were reported in 1930 at the 5th International Botanical Congress in Cambridge (Great Britain) (Vavilov, 1931). Here, the concept of the Linnean species as a regular system of forms, phenotypically, physiologically and genetically variable within certain limits, is very significant both for the practical purposes of studying cultivated plants and for studying the main issues of the evolutionary process. It was possible to come close to studying this process only with the understanding of the Linnaean species in its diversity, and not as a monotypic species described from a few specimens, in the way it was customary to describe species. The main problems of evolution could not be resolved without taking into account the species as a complex system of forms (genotypes). The genetics of individual species gives an idea of the hereditary nature of a species only when it is based not on a few random specimens or crop varieties, but on the definitely and carefully chosen, though it may be selective, material (Vavilov, 1992).

The global collection of plant genetic resources collected by N.I. Vavilov and his associates from all over the world reflects the fullness of botanical, morphological and genetic diversity. It has been preserved by the VIR staff in the most difficult periods of history, and now it makes it possible to conduct historical, evolutionary, phylogenetic and applied breeding research aimed at unlocking the potential of all the material in the collection (Loskutov, 2009).

According to Nikolai I. Vavilov, a species is a complex, mobile, isolated morpho-physiological system associated in its genesis with a certain environment and distribution range, subject to the law of homologous series in variation (Vavilov, 1992). To determine the system of a species, it is necessary to study the complete intraspecific diversity from different parts of the distribution area and establish the range of variability of characters in different environmental conditions. These provisions constitute a theoretical basis that makes it possible to predict the discovery of various plant forms and explains how the system of hereditary forms of a species evolves according to growing conditions. The law of homologous series helps to establish solid foundations for the taxonomy of cultivated plants, gives an idea of the place of each systematic unit in the vast wealth of the plant world. Therefore, a real intraspecific classification should be based on an integrated approach to the concept of the rank of a botanical variety as an objective unit of complex polymorphic species systems. N.I. Vavilov emphasized the complexity of the species system as a whole, consisting of connected and mutually penetrating parts, forms and genotypes, in which he points to the facts associated with the genus *Avena* L. (Vavilov, 1951).

N.I. Vavilov about evolution and phylogeny of the genus *Avena*

In his works, Vavilov paid great attention to the evolution and phylogeny of the entire genus *Avena* L. In 1927, he definitely spoke of four main genetic groups of cultivated oats related by origin: *A. sativa* L. – *A. fatua* L.; *A. byzantina* K. Koch – *A. sterilis* L.; *A. strigosa* Schreb. – *A. barbata* Pott; and *A. abyssinica* Hochst. Particularly intricate was the first, extremely polymorphic group of *A. sativa*, the origin of which is associated with Asia (Vavilov, 1992). This point of view began to dominate in all studies, in contrast to the opinion about the European origin of cultivated oats (Ladizinsky, 1989).

From the genetic point of view, oats (*Avena* L.) have not been sufficiently studied compared to other cereal crops. A systematic study of varietal diversity and individual species of the genus provides general information on the localization of the centers of their morphogenesis, evolution, and domestication (Loskutov, 2007). *Avena* species are characterized by great morphological and eco-geographical diversity, and landraces are highly adaptable. Since the early 20th century, the world literature has accumulated a significant amount of data on numerous forms and species of the entire genus, and on the centers of their greatest diversity and origin (Malzew, 1930; Baum, 1977; Vavilov, 1992; Rodionova et al., 1994; Loskutov, Rines, 2011; Ladizinsky, 2012).

Landraces, including Mediterranean ones, collected during the expeditions of N.I. Vavilov and his associates, were researched in the 1930–1950s and have not been studied in detail by now (Mordvinkina, 1960). Nowadays, many problems remain completely unresolved; there is no consensus on the origin, systematic status, relationships and ways of cultivation of oat species. Comparative studies of (landraces and segetal) varieties and wild species of oats from the evolutionary, taxonomic and breeding points of view are caused by the great interest of breeders in their practical use.

The determination of the areas of origin and morphogenesis in oat species employs the differential botanical-geographical method investigated and widely used by N.I. Vavilov. The essence of this method is in the determination of a wide intraspecific diversity when analyzing the differentiation of some plant species into botanical varieties and genetic groups, in elucidating the nature of the distribution of the hereditary diversity of forms of a given species within the distribution range, with the establishment of geographical centers of accumulation of this diversity and geographical localization of the morphogenetic process (Vavilov, 1992). The analysis of collection accessions showed that all the considered forms of oats belonging to individual species were characterized by morphological features and certain distribution ranges.

According to N.I. Vavilov, it is impossible to reduce the origin of cultivated oat species to a single geographical center. Cultivated oat species (diploid and polyploid) are undoubtedly of polyphyletic origin. Some species, in all likelihood, entered cultivation independently. In any case, it would be erroneous to consider cultivated oats definitely associated only with Europe. The presence of endemic hulled and naked groups of *A. sativa* in China, wide distribution of wild and weedy *A. fatua* and *A. ludoviciana* Durieu in Turkestan, Bukhara, Afghanistan, Persia, the Transcaucasus, and Armenia, the presence of many original groups of cultivated and wild oats there testify to the participation of Asia in the formation of the *A. fatua*–*A. ludoviciana*–*A. sativa* group of hulled and naked forms (Vavilov, 1992).

The whole diversity of cultivated oats, as shown by Vavilov (Vavilov, 1992), had originated from segetal weeds. With the spread of the species northward or to the highlands, to more harsh and humid growing conditions, oats eventually replaced the main crops (among which it had originally been only a weed plant), and itself became a proper cultivated plant. This process can be clearly traced in Spain on the example of the cultivated diploid species *A. strigosa*, on *A. abyssinica* in Ethiopia, *A. byzantina* in Turkey and Iran, and on segetal forms of *A. sativa* convar. *asiatica* (Vavilov) Rodionova et Soldatov and *A. sativa* convar. *volgensis* (Vavilov) Rodionova et Soldatov (Loskutov, 2007).

Intraspecific diversity of cultivated oats species

According to the classification of N.A. Rodionova et al. (1994), the cultivated diploid *A. strigosa* is divided into three subspecies, i. e. *A. strigosa* (Schreb.) subsp. *strigosa*, *A. strigosa* subsp. *brevis* (Roth) Husn., and *A. strigosa* subsp. *nudibrevis* (Vavilov) Kobyl. et Rodionova, which are clearly geographically differentiated. Of the 15 identified botanical varieties in the entire *A. strigosa* species, 8 were found among native specimens from Spain and 11 among specimens from Portugal. In total, 13 botanical varieties are found on the Iberian Peninsula, most of which are endemic to this region. The greater part of diverse forms of this species was distributed in Spain, Portugal, Germany and Great Britain; besides, individual forms originated from a number of other European countries. Thus, the center of origin and diversity of the diploid cultivated species *A. strigosa* is the Iberian Peninsula, where both its wild relatives and probable progenitors, the diploid species *A. hirtula* Lag. and *A. wiestii* Steud., are widespread (Loskutov, Rines, 2011). According to archaeological data collected by A.I. Malzew (1930), *A. strigosa* was the first oat species that was cultivated in Europe already in the Neolithic era, i. e. about 1500 BC.

The tetraploid cultivated species *A. abyssinica* that infests barley and wheat fields is currently cultivated to a limited extent, although it has a cultural type of caryopses articulation that prevents their shattering when ripe. In addition to this endemic species, only tetraploid species *A. vaviloviana* (Malzew) Mordv. and *A. barbata* of the wild ones grow in Ethiopia. *A. abyssinica* shares many features with *A. vaviloviana* and is considered to be its cultural counterpart.

All of the small intraspecific diversity of six forms in the rank of botanical varieties of *A. abyssinica* is found only in the present-day Ethiopia (Rodionova et al., 1994). According to A.I. Malzew (Malzew, 1930), the Ethiopian center of diversity of tetraploid oat species is a secondary one, and the forms distributed in it had links with the Mediterranean center of origin in the early historical epoch. The secondary status of this center is also proved by the fact that two related species, *A. vaviloviana* and *A. abyssinica*, have a purely spring type of growing, which is secondary to the winter type of growing. Apparently, these two species, having found the most favorable climatic and soil conditions in Ethiopia, south of the Mediterranean center, spread there and could not advance further due to more

harsh arid climatic conditions in the countries adjacent to Ethiopia (Loskutov, Rines, 2011).

The hexaploid cultivated species *A. byzantina*, according to N.A. Rodionova et al. (1994), numbers 15 botanical varieties, 9 of which were found among landraces from Algeria, 8 from Morocco and Turkey each, 7 from Greece, 6 from Israel, and 5 from Spain and Italy each; the rest of the countries where this species was distributed had from one to three botanical varieties. It was noted by N.I. Vavilov that the main area of diversity of this species is concentrated on the Mediterranean coast of North Africa (Vavilov, 1992). Therefore, the primary center of morphogenesis in *A. byzantina* is the territory of Algeria and Morocco, where its greatest local botanical diversity is concentrated, while the presence of a large number of intermediate forms in Turkey indicates that this region is a secondary center of diversity for this species (Loskutov, 2007).

A study of the intraspecific diversity of the collection of the hulled forms of the hexaploid cultivated species *A. sativa* L. showed that segetal forms of this group of botanical varieties, numbering about 130 landraces in the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) collection, are localized on the territory of Iran, Georgia and the Russian Federation (Dagestan, Tatarstan, Bashkortostan and Chuvashia). This group of forms weeding crops was characterized by primitive or transitional features and had a clear confinement to certain distribution ranges. An analysis of the data on the composition of botanical variety of landraces in the collection showed that the forms of *A. sativa* subsp. *sativa* convar. *asiatica* (Rodionova et al., 1994) demonstrated the greatest diversity only in Iran and Georgia, where all three botanical varieties characterizing this group were identified, while in the Russian Federation (Dagestan), only one botanical variety from this group was identified.

In addition, a form belonging to a botanical variety from the group *A. sativa* subsp. *sativa* convar. *volgensis* (Rodionova et al., 1994) and representing a link between the two groups of varieties was found here. The group of *A. sativa* subsp. *sativa* convar. *volgensis* itself has four botanical varieties, the greatest diversity of which is confined to the Russian Federation. All four botanical varieties were found in Tatarstan, three were found in Bashkortostan, Chuvashia and Ulyanovsk province, two in Udmurtia, and only one in Kirov and Saratov provinces, and Mordovia each. In other regions of distribution of hulled oats, these forms are not found. Apparently, it was from the South-Western Asiatic center via Iran and further through Georgia into the Russian Federation (Dagestan, Saratov and Ulyanovsk provinces, Tatarstan, Chuvashia, and Bashkortostan) that the hulled forms of *A. sativa* subsp. *sativa* started first weeding crops, then entering cultivation and spreading in all directions (Loskutov, 2007).

Another subspecies, *A. sativa* subsp. *nudisativa* (Husn.) Rodionova et Soldatov, or naked forms of hexaploid oats (Rodionova et al., 1994), originated from China, as stated by Vavilov (Vavilov, 1992). It is known from the literary sources that naked oats were widespread in China already in the 5th century AD (Zukovskij, 1962). Eastward from the main center of diversity, the growing conditions changed, resulting in the appearance of naked mutations of *A. sativa*, which settled in new habitats. Thus, a cycle of transition of forms of the wild, cultivated hulled and naked types is observed here for both *A. sativa* and *A. strigosa*. The last type, being a recessive mutation, appears at a distance from the territory of the main diversity of the closely related hulled forms.

An analysis of data on intraspecific diversity of landraces of naked cultivated hexaploid species *A. sativa*, numbering over 40 accessions, showed that out of four botanical varieties identified in the VIR collection (Rodionova et al., 1994), all four were identified among accessions from Mongolia, three among those from China and two among those from the adjacent Krasnoyarsk Territory in the Russian Federation (Loskutov, 1999). Two most common botanical varieties *A. sativa* subsp. *nudisativa* var. *inermis* Koern. and *A. sativa* subsp. *nudisativa* var. *chinensis* Doell. are characteristic of accessions from other regions. Consequently, the center of diversity of naked hexaploid oat forms is a region in Mongolia and northwestern China.

DNA markers and genomic technologies in evolution studies of *Avena* species

Studies of a complex of morphological features do not yield a complete picture of the evolutionary and systematic status of some species and forms of oats. The methods and approaches that use DNA markers and genomic technologies and are currently undergoing active development are promising for the study of polymorphism and phylogeny of oats.

Recently, there has been a number of works dealing with the molecular aspects of the evolution and phylogeny of the genus *Avena* (Fu, 2018; Peng et al., 2018, 2022; Latta et al., 2019; Ahmad et al., 2020; Liu et al., 2020; Fominaya et al., 2021; Jiang et al., 2021; Yan et al., 2021). These studies use various markers, such as the ITS1-5.8S rRNA-ITS2 sequences (Rodionov et al., 2005; Nikoloudakis et al., 2008; Nikoloudakis, Katsiotis, 2008; Tyupa et al., 2009), and external transcribed spacers (Rodrigues et al., 2017). These works have clarified a number of relationships between *Avena* species with different genomes (see the Table).

When studying the relationships of hexaploid *A. sativa*, *A. sterilis* and diploid *A. strigosa*, retrotransposons and ITS sequences were used. An analysis of the ITS sequences showed very high homology in all three species, but FISH (fluorescent *in situ* hybridization) revealed differences in the position of nucleolar organizers (containing rDNA). According to the pattern of retrotransposon polymorphism, the hexaploid *A. sativa* turned out to be closer to *A. sterilis* than to the diploid *A. strigosa* (Tomas et al., 2016). Diploid wild species with their greatest diversity of forms in the western Mediterranean, presumably gave rise to the cultivated species *A. strigosa*, which is most widely distributed in the Iberian Peninsula. The wild species *A. hirtula* and

Speciation in the genus *Avena* L. (Loskutov, 2007)

Section	Species			Genome	2*n*
	Wild		Cultivated		
	floret disarticulation	spikelet disarticulation			
Aristulatae (Malzew) Losk. comb. nova	*A. clauda* Durieu	*A. pilosa* M. B.		Cp	14
	A. longiglumis Durieu			Al	
	A. damascena Rajhathy & B.R. Baum			Ad	
	A. prostrata Ladiz.			Ap	
	A. wiestii Steud.	*A. atlantica* B.R. Baum & Fedak		As	
	A. hirtula Lag.		*A. strigosa* Schreb.		
	A. barbata Pott ex Link			AB	28
	A. vaviloviana (Malzew) Mordv.		*A. abyssinica* Hochst.		
Avenae		*A. ventricosa* Balansa		Cv	14
		A. bruhnsiana Gruner			
		A. canariensis B.R. Baum, Rajhathy & D.R. Sampson		Ac	
		A. agadiriana B.R. Baum & Fedak		AB	28
		A. magna H.C. Murphy & Terrell		AC	
		A. murphyi Ladiz.			
		A. insularis Ladiz.		AC?	
	A. fatua L.	*A. sterilis* L.	*A. byzantina* K. Koch	ACD	42
	A. occidentalis Durieu	*A. ludoviciana* Durieu	*A. sativa* L.		

A. wiestii most likely gave rise to the autotetraploid species *A. barbata* (Holden, 1979; Thomas, 1995).

Studies of the origin of polyploid oat species by the comparative analysis of the characteristics of the genome and DNA markers do not make it possible to draw unambiguous conclusions. For instance, C. Li et al. (2000) examined the occurrence of the species-specific satellite DNA ASS49 in 40 microsatellites and 4 minisatellites in diploids and polyploids in order to determine the species that was the diploid and tetraploid ancestor of the hexaploid oat. This comparison showed that the Ac genome of the diploid *A. canariensis* B.R. Baum, Rajhathy & D.R. Sampson is a more probable ancestral genome for A subgenomes of hexaploids rather than *A. strigosa*, which is usually regarded as such.

However, studies of other polymorphic markers give different results. For instance, the AFLP patterns of diploid, tetraploid, and hexaploid oat species show that it is not *A. canariensis*, but *A. wiestii* that is a more probable donor of A genomes for hexaploids with an ACD genomic constitution (Fu, Williams, 2008).

DNA samples were used to study the order of nucleotide sequences in species with different chromosome sets. The pAs102 probe obtained from *A. strigosa* (As) during *in situ* hybridization showed that sequences complementary to this probe are found in diploids with the A and C genomes, in tetraploids with the AC genome, and in hexaploids with the ACD genome. On the other hand, homologous sequences of the pAs102 probe were found in *A. strigosa*, *A. longiglumis* Durieu and *A. sativa*. A not very precise sequence is present in *A. murphyi* Ladiz. and is completely absent in other diploid species with the A and C genome variants (Linares et al., 1996, 1998).

It is assumed that the tetraploid wild species *A. vaviloviana* (*A. abyssinica* being its cultivated analog) is similar in some morphological features to the hexaploid wild species *A. occidentalis* Durieu originally found in Algeria. It has been established that, according to some morphological features, *A. vaviloviana* and *A. abyssinica* may be relics of the ancient African flora (Baum, 1971). In addition, B. Baum (1972) notes that morphological similarity was found between three species, namely *A. vaviloviana*, *A. occidentalis* and questionable from our point of view species *A. septentrionalis*, which A.I. Malzew (1930) attributed to the subspecies *A. fatua* growing in Siberia. These species are currently distributed on the territory in the form of broken (disjunctive) relict distribution ranges, and thus confirm the point of view, according to which the species

of the genus *Avena* occupied entire (rather than fragmented) and diverse distribution ranges in the recent geological past, in comparison with the ranges of modern species (Baum, 1971; Rajhathy, 1971).

The genetic unity and interfertility of *A. barbata* and *A. vaviloviana* with *A. abyssinica* was confirmed by a genetic study of hybrids. It is assumed that the weedy species *A. barbata*, brought to Ethiopia together with barley grain, gave rise to the cultivated species *A. abyssinica*, which infests barley crops to this day (Thomas, 1995). On the basis of the material for the study and analysis of interspecific crossings, chromosome structure, morphological, biochemical characters and geographical distribution of species, it was concluded that diploid species with the As genome (*hirtula-wiestii*) were the ancestors of the group of tetraploid species with the AB genome (*barbata-vaviloviana-abyssinica*), or AA′. In turn, the last group, evolutionarily unrelated to any other group of oat species, is a lateral branch of the genus *Avena* (Rajhathy, Thomas, 1974).

According to F.A. Coffman (1977), the ancestor of the diversity of cultivated hexaploid forms is *A. sterilis*, originating from the Asian continent. Apparently, the cultivated species *A. byzantina* originated from this species, and then *A. fatua*, a malicious weed that infests cultivated crops, appeared. The further consideration of the hexaploid species evolution showed that when studying translocations in oat chromosomes and the ratio of the geographical distribution of different forms using data cluster analysis, a high degree of genetic relationship was noted between *A. byzantina* accessions and forms of *A. sterilis* from northern Mesopotamia, on the one hand, and *A. sativa* accessions and forms of *A. sterilis* from eastern Anatolia, on the other hand (Zhou et al., 1999).

Further studies of all hexaploid species showed that translocations (97 %) were characteristic of *A. sativa*, in contrast to *A. byzantina* (11 %). As a result, it was suggested that two cultivated species, *A. sativa* and *A. byzantina*, were independently introduced into cultivation. A study of *A. fatua* and *A. occidentalis* showed that most forms of these species have the same translocations as *A. sativa* and, therefore, are regarded as side branches of oat evolution (Jellen, Beard, 2000).

Differences in genome size between species with different ploidy levels were significant and depended on genomic duplication, while differences in genome size within a certain ploidy level were mainly due to different genomic composition. The flow cytometry method made it possible to diagnose individual species and, in some cases, to establish intergenomic relationships between them (Yan et al., 2016).

By using 12 primer pairs of microsatellite markers of the chloroplast genome, 70 accessions of 25 *Avena* species from the VIR collection were analyzed. From 2 to 9 alleles were identified, and the average value of genetic diversity (H) amounted to 0.479. The differences in the length of alleles allowed the identification of 45 haplotypes. The most polymorphic were the diploid species *A. eriantha* Durieu (*A. pilosa* M. B.) and *A. ventricosa* Balansa with the C genome, one of the diploid species with the As genome (*A. atlantica* B.R. Baum & Fedak) and tetraploid species *A. insularis* Ladiz. (AC genome) and *A. agadiriana* B.R. Baum & Fedak (AaBa genome). *A. insularis*, which is often regarded as the species closest to the hexaploid ones, is probably the most primitive among the tetraploid species with the AC genome, and cannot be the direct ancestor of the hexaploid species. This study identified new informative markers for the analysis of the chloroplast genome of the genus *Avena* and refined data on the phylogenetic relationships of oat species (Yan et al., 2016).

Based on the sequenced and annotated reference oat genome, quantitative trait loci (QTLs), economically valuable traits and those associated with grain quality in populations of cultivated *A. sativa* have been found and characterized. Strong and significant associations have been found between the positions of candidate genes and QTLs that affect heading date, as well as those that influence the concentrations of oil and β-glucan in the grain (Tinker et al., 2022).

In 2022, the genomes of three species of the genus *Avena* L. were completely sequenced; these were an allohexaploid cultivated oat species (*Avena sativa*, AACCDD, $2n=6x=42$) and two of its close wild relatives: diploid *A. longiglumis* (AA, $2n=14$) and tetraploid *A. insularis* (CCDD, $2n=4x=28$).

The publication of the results of the whole genome sequencing (Kamal et al., 2022) showed that the reference genome of cultivated oats *A. sativa* has a mosaic structure that differs sharply from the genomes of other members of the Poaceae family. This study showed that during the formation of a hexaploid oat species, at least 226 Mb of gene-rich regions from the C subgenome were translocated into subgenomes A and D, which is associated not with the loss of individual genes, but with a large number of translocations in the latter. In contrast to hexaploid common wheat, crosses between species with different ploidy and alien introgressions were extremely complex in the genus *Avena*, suggesting the presence of an incompatible genome architecture, which is an additional barrier preventing genetic improvement in *A. sativa*. Average expression values across transcriptome samples from six tissues showed that C subgenome genes were slightly less expressed (32.32 %) than those in the D (33.53 %) and A (33.76 %) subgenomes. A network approach revealed that genes from the C subgenome were found in divergent expression modules more frequently than their A and D subgenome homoeologues (Kamal et al., 2022).

Based on the sequenced and annotated reference oat genome, genome-wide recombination profiles were examined to confirm the presence of a large unbalanced translocation from chromosome 1C to chromosome 1A and a possible inversion on chromosome 7D, which are typical for oats (Tinker et al., 2022).

Subsequently, the time of divergence of three oat subgenomes was calculated. The divergence time of the

A subgenome was ~47.3 thousand years ago, the C subgenome, ~47.0 thousand years ago, and the D subgenome, ~53.3 thousand years ago (Nan et al., 2023).

A comprehensive evaluation of the diversity of species of the genus *Avena* using next generation sequencing (NGS) methods

A comprehensive study of a representative set of accessions from the collection of the VIR, demonstrating a wide ecological and geographical diversity of all four cultivated and 21 wild species of the genus *Avena* L. with different ploidy levels, showed that the diploid species *A. bruhnsiana* Gruner has a hybrid origin, i. e. is a notospecies, one of the ancestors of which was *A. ventricosa*, and the second, apparently, *A. clauda*. The karyotype of *A. bruhnsiana* is diploid ($2n = 14$) (Loskutov, Abramova, 2006), therefore it can be assumed that this is a homoploid hybrid. Judging by the diversity of rDNA sequences, *A. clauda* itself is probably also a homoploid hybrid: one of its main ribotypes is identical to that of *A. pilosa*, while the other is isolated (Gnutikov et al., 2022b).

Therefore, out of the four studied diploid oat species with the C genome, two are homoploid hybrids. It has also been found that species with two nucleolar organizers in the genome on different chromosomes often have at least two ribotypes, while *A. ventricosa*, which has one nucleolar organizer (NOR), has only one ribotype. This may indicate that rDNA homogenization proceeds within one NOR more effectively than that at loci located on different chromosomes. Perhaps this is due to the fact that one of the mechanisms of rDNA homogenization is associated with the conjugation of homologous chromosomes and, therefore, proceeds more effectively within one NOR than between NORs located on different chromosomes (Eickbush T.H., Eickbush D.G., 2007; Sochorová et al., 2018).

Among the studied C genome species of oats, there is one autotetraploid ($2n = 28$), perennial, cross-pollinated, narrowly endemic species from Algeria, *A. macrostachya* Balansa & Durieu. This species is considered the most ancient species of the genus *Avena* (Nikoloudakis, Katsiotis, 2008; Peng et al., 2008, 2010). Morphologically, this perennial species is a primitive representative of the genus *Avena* (Malzew, 1930). Some researchers even assigned it to the genus *Helictotrichon* Besser (Holub, 1958). *Avena macrostachya* differs from diploid oat species with the C genome by a symmetrical karyotype with the predominance of equal-armed chromosomes, an absence of diffuse heterochromatin, a predominantly pericentromeric location of C-positive bands, as well as the size and morphology of satellite chromosomes (Badaeva et al., 2010). As it turned out, a symmetrical karyotype is not characteristic of diploid species with the C genome. At the same time, large blocks of C-heterochromatin in the pericentromeric regions of chromosomes of this species indicate its relationship with the C genome species. This confirms that the C genome of *A. macrostachya* is of a special type, hence its designation CmCm (Rodionov et al., 2005). It was also believed that *A. macrostachya* could have a previously undescribed EE genome (Loskutov, 2007). Our analysis of NGS data for 18S-ITS1-5.8S rDNA sequences showed that *A. macrostachya* ribotypes are comparatively far from other existing C genome oats.

An analysis of the intragenomic rDNA polymorphism of diploid oat species with different variants of the A genome showed significant differences in the number of ribotypes, haplotypes, nucleotide diversity indices, genetic distance, and genetic differentiation (Rodionov et al., 2005).

The evaluation employed accessions with a high ecological and geographical diversity, which represented all variants of the A genome, i. e. the As (*A. atlantica*, *A. hirtula*, *A. wiestii*), Ac genome (*A. canariensis*), Ad (*A. damascena* Rajhathy & B.R. Baum), Al (*A. longiglumis*), and Ar (*A. prostrata* Ladiz.). Also, one species with the C genome, *A. clauda*, was taken into analysis. Sequences of 169 accessions revealed 156 haplotypes, of which seven haplotypes are common for two to five species. Sixteen ribotypes were identified, which consisted of a unique sequence with a characteristic set of single nucleotide polymorphisms and deletions. The number of ribotypes per species varied from one in *A. longiglumis* to four in *A. wiestii*. Although most of the ribotypes were species-specific, two ribotypes were found to be common for three species (one for *A. damascena*, *A. hirtula*, and *A. wiestii*, and the other one for *A. longiglumis*, *A. atlantica*, and *A. wiestii*), while a third ribotype was common for *A. atlantica* and *A. wiestii*. A characteristic feature of the ribotype of *A. clauda*, a species with the diploid C genome, is that two different ribotype families were found in this species. Some of these ribotypes are characteristic of species with the Cp genome, while others are closely related to ribotypes of the As genome. It means that *A. clauda* may be a hybrid of oats with the As and C genomes.

Despite the fact that the studied species of the genus *Avena* were diploids, it turned out that most of them contained several different rDNA families. A comparative study of rDNA patterns in individual species showed that an rDNA pattern, as a rule, is mosaic and species-specific in all cases. At the same time, oat species with the A genome can reflect hybridization events that took place in their evolutionary past as a way of their speciation (Loskutov et al., 2021; Gnutikov et al., 2022a).

A large set of landraces with unique, so-called segetal botanical varieties of cultivated oats was subjected to study. These forms are specialized weeds of emmer wheat and barley, which spread together with the grain of cultivated plants and weed crops. All these botanical varieties form a separate clade with a good level of support, while their differences are small (p-distance from 0.003 to 0.02). All of them are hexaploids with the ACD genome (Loskutov, Rines, 2011), however, it should be remembered that Sanger sequencing reveals only the most massive subgenome variant in the polyploid genomic set.

The NGS results revealed two ribotype families most represented in terms of the number of sequences in the

polyploid genomic set, which are common for almost all studied botanical varieties related to *A. fatua* and *A. sativa*. These two ribotype families correspond to the sequences of the A genome and the D (A′) genome, which was previously assumed to be a variant of the A genome (Loskutov, 2007). At the same time, most of the ribotypes in these genomes were common for all the studied accessions.

It is also of great interest that the C genome sequences were not found in the general pool of hexaploid sequences; they were located separately as a very small fraction, probably strongly altered by the processes of post-hybridization transformation. Similar data are confirmed by cytogenetic studies. The FISH method showed that the C subgenomes of polyploid oat species have lost most of the rDNA, and only very weak 35S rDNA-positive signals can be detected on them (Badaeva et al., 2010).

Using sequence-tagged DNA sequencing on the Roche 454 platform, intragenomic polymorphism of one of the 35S rRNA regions (18S rDNA fragment–ITS1–fragment of 5.8S rDNA) in three hexaploid *Avena* species with karyotypes AACCDD and the tetraploid species *A. insularis* (AACC or CCDD) has been studied (Rodionov et al., 2020). Instead of the expected 50 % of C-variant ITS1 in *A. insularis* and 33 % of C-variant ITS1 in hexaploids *A. fatua*, *A. ludoviciana*, and *A. sterilis*, the actual rate of C subgenome specific ITSs comprised around 3.3 % of rDNA in *A. insularis* and 1.4–2.4 % of rDNA in hexaploid genomes. The 18S rDNA, ITS1 and 5.8S rDNA of the C subgenome origin were 10 times more variable than the same sequences from the A genome. Some of the C subgenome sequences contained deletions, including deletions in the 18S rRNA coding region.

The results of FISH hybridization with pTa71 and pTa794 confirm the fact that polyploids lost a significant part of the 35S rDNA and 5S rDNA obtained from a diploid ancestor with the CC karyotype. The sequences of the ITS1 C subgenomes of polyploid species are diverse, but among them it is possible to single out the main (core) variant approximately equidistant from the ITS diploids carrying the Cv and Cp genomes. The results show that the loss of 35S rDNA C subgenomes occurs against the background of the accumulation of many single nucleotide substitutions (SNPs) and deletions accumulation in these sequences. In the "repressed" 35S rRNA loci of C subgenomes, multiple mutations were apparently not accompanied by homogenization of rDNA. Hence, there is a reason to believe that the processes of rDNA isogenization and the process of transcription/silencing are related phenomena (Rodionov et al., 2020).

Obtaining results using the method of locus-specific next generation sequencing (NGS) on the Illumina platform allowed for a phylogenetic analysis of representative intraspecific diversity of cultivated and wild species of the genus *Avena*. It has been established that diploid species with the A genome (variants of Al, Ap and As genomes) are in fact not primary diploids, but a kind of Mediterranean introgressive hybridization complex of species that sporadically enter into interspecific hybridization. It has been determined that the contribution of *A. canariensis* (considered to be a donor of the A genome for hexaploids) to the genomic constitution of hexaploids (ACD) is insignificant, and according to our data, it is of hybrid origin, as two ancestral species with close, but not identical ribotypes, took part in its formation. It has been established that the tetraploid cultivated species *A. abyssinica* most likely originated from the wild *A. vaviloviana*. At the same time, *A. agadiriana*, previously considered as an ancestor to *A. abyssinica* and its group of relatives, forms separate unique subgenomes (ribotype families). An analysis of the ways of domestication of three oat species *A. abyssinica*, *A. sativa*, and *A. byzantina* showed that the most widespread ribotype of the *A. sativa* hexaploid was inherited from *A. ludoviciana*, and the second most widespread, from *A. magna* H.C. Murphy & Terrell. The cultivated species *A. byzantina* has two unique ribotype families, most likely inherited from an extinct oat species or cryptospecies, which has not been discovered until now (Gnutikov et al., 2021, 2022c).

On a representative set of oat species (*Avena* L.), the origin of wild polyploid species was analyzed. The 18S–ITS1–5.8S rDNA region was used for NGS analysis. In polyploid oats, 15 major ribotypes were found (more than 1000 reads per rDNA pool). Pools of marker sequences of polyploid oat species were compared with sequences of putative diploid ancestors: *A. atlantica* (As genome), *A. hirtula* (As), *A. canariensis* (Ac), *A. ventricosa* (Cv), and *A. clauda* (paleopolyploid with the Cp genome and rDNA sequences related to the A genome). The results confirmed some earlier hypotheses about the origin of the polyploid species of the genus *Avena*. Tetraploid oats, which were previously identified as species with the AC genome, do have this genomic constitution. The data obtained do not support the hypothesis of CD genome recruitment in the tetraploid species *A. magna*, *A. murphyi*, and *A. insularis*. At the same time, D genome sequences were not found in tetraploid oats with the AC genome related to oats with the ACD genome.

The sequences associated with the A genome may have been inherited from the As genomic species *A. atlantica*, while the sequences associated with the D genome were already formed in the hexaploid oat or were taken from an unknown ancestor related to *A. clauda*. It was found that AB tetraploid oats may have inherited their A genome ribotypes from *A. atlantica* (As1 ribotype), whereas their B genome ribotype is specific and may be derived from the A genome family. The A genome sequences in the ACD genome species of the genus *Avena* were probably inherited from *A. murphyi* (AC). The sequences associated with the C genome could be derived from the diploid species *A. ventricosa*. All hexaploid species show a different ribotype pattern from tetraploids; the main ribotypes of *A. fatua*, *A. ludoviciana*, and *A. sterilis* probably belong to the D group and are also common with one of the main ribotypes of the diploid species *A. clauda* (Gnutikov et al., 2023).

Conclusion

Thus, on the basis of a comprehensive study of the complete intraspecific diversity of cultivated oat species from different zones of the distribution range and analysis of data on the geographical location of distribution ranges of forms and species, it was confirmed that the place of the greatest distribution and species morphogenesis in the genus *Avena* is located in the western part of the Mediterranean center of origin of cultivated plants, namely, on the territory of the northwestern part of the African continent and partly on the southwestern tip of Europe. It has been established that the process of hexaploid species formation also proceeded in the western part of the Mediterranean, and then, moving eastward, these forms began to occupy more and more areas in the South-West Asian center, forming a large intraspecific diversity of wild and weedy forms in transit to cultivated hexaploid oat species. Based on the analysis of the intraspecific diversity of landraces, the centers of morphogenesis of all cultivated oat species were specified.

The phylogenetic analysis of the representative intraspecific diversity of cultivated and wild *Avena* species, carried out using NGS methods, showed that diploid species with A genome variants are in fact not primary diploids, but a peculiar Mediterranean introgressive hybridization complex of species that sporadically enter into interspecific hybridization. It was established that the tetraploid cultivated species *A. abyssinica* had most likely originated from the wild *A. vaviloviana*. An analysis of the ways of *A. sativa* and *A. byzantina* domestication showed that the most widespread ribotype of the *A. sativa* hexaploid was inherited from *A. ludoviciana*, and the second most widespread one – from *A. magna*, while *A. byzantina* has two unique ribotype families, most likely inherited from an extinct oat species or a still undiscovered cryptospecies. Hexaploid wild species show a different pattern of ribotypes than tetraploids; the main ribotypes of *A. fatua*, *A. ludoviciana*, and *A. sterilis* probably belong to the D group and are also common with one of the main ribotypes of the diploid species *A. clauda*.

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ORCID

I.G. Loskutov orcid.org/0000-0002-9250-7225
A.A. Gnutikov orcid.org/0000-0002-5264-5594
E.V. Blinova orcid.org/0000-0002-8898-4926
A.V. Rodionov orcid.org/0000-0003-1146-1622

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Allelic diversity of the *Vrn* genes and the control of growth habit and earliness in wheat

S.E. Smolenskaya, N.P. Goncharov ✉

Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
✉ gonch@bionet.nsc.ru

Abstract. Wheat is one of three main food crops around the world, which has the largest distribution area due to its adaptation to the different environments. This review considers polymorphisms and allelic variation of the vernalization response genes *Vrn* controlling the major adaptation traits in wheats (the genus *Triticum* L.): growth habit (spring *vs.* winter) and length of vegetative period (earliness). The review summarizes available information on the allelic diversity of the *Vrn* genes and discusses molecular-level relationships between *Vrn* polymorphisms and their effect on growth habit (spring *vs.* winter) and earliness (length vegetative period in spring plants) in di-, tetra- and hexaploid wheat species. A unique attempt has been made to relate information on mutations (polymorphisms) in dominant *Vrn* alleles to the values of the commercially most important trait "length of plant vegetative period (earliness)". The effects of mutations (polymorphisms) in the recessive *vrn* genes on vernalization requirement in winter wheats are considered, and this trait was formalized. The evolution of the winter/spring growth habit in the genus *Triticum* species is discussed. A scheme of phylogenetic interactions between *Vrn* alleles was constructed on the basis of these polymorphisms; the paper considers the possibilities to enhance the diversity of polymorphisms for the dominant *Vrn* genes and their alleles using wheat related species and rarely used alleles and discusses the prospects of breeding for improved earliness for concrete agroecological zones.
Key words: wheat; *Vrn* genes; winter/spring growth habit; length of plant vegetative period; earliness.

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Аллельное разнообразие генов *Vrn* и контроль типа и скорости развития у пшениц

С.Э. Смоленская, Н.П. Гончаров ✉

Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
✉ gonch@bionet.nsc.ru

Аннотация. Пшеница, являясь одной из трех основных продовольственных культур мира, занимает самый широкий ареал за счет адаптивности к разнообразным условиям возделывания. В обзоре рассматриваются полиморфизм и аллельная изменчивость генов *Vrn* (от англ. *response to vernalization*), контролирующих важнейшие адаптационные признаки пшениц – тип (яровость *vs.* озимость) и скорость развития у диких и возделываемых видов пшениц (род *Triticum* L.). Суммируется информация об аллельном разнообразии генов *Vrn* и обсуждается связь полиморфизмов этих генов на молекулярном уровне с их влиянием не только на признак «тип развития (яровость *vs.* озимость)», но и на признак «скороспелость (длина вегетационного периода яровых растений, ДВП)» у ди-, тетра- и гексаплоидных видов. Предпринята попытка связать полученную информацию о мутациях (полиморфизмах) доминантных аллелей генов *Vrn* с выраженностью наиболее важного с хозяйственной точки зрения признака «продолжительность ДВП (скороспелость)», которая ранее в обзорах не предпринималась. Рассматривается влияние мутаций (полиморфизмов) в последовательностях рецессивных генов *vrn* на признак «потребность в яровизации» у озимых форм растений пшениц и выполнена его формализация. Обсуждается эволюция озимости/яровости в роде *Triticum*. На основе выявленных полиморфизмов построена схема филогенетических взаимодействий аллелей генов *Vrn* и рассматриваются возможности расширения полиморфизма по доминантным генам *Vrn* и их аллелям за счет видов-сородичей и редко используемых алелей и перспективы селекции на изменение ДВП (скороспелости) для конкретных зон возделывания.
Ключевые слова: пшеница; гены *Vrn*; яровость; озимость; длина вегетационного периода; скороспелость.

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Introduction

Many of the cultivated and wild herbaceous plant species growing in temperate climates have developed the spring growth habit (cryophobic plants) or the winter growth habit (cryophilic plants) as adaptations to natural environments (Gupalo, Skripchinsky, 1971). The spring plants complete their entire development cycle during a single vegetation season, while the winter ones do not proceed to reproduction unless they have been exposed to low temperatures.

In wild and cultivated wheat species, delays in transition from vegetative to reproductive development are controlled by the vernalization response genes *Vrn* regulating growth habit (spring *vs*. winter) and earliness, the vernalization requirement duration genes *Vrd* controlling duration of vernalization treatment in winter wheats, and the photoperiod sensitivity/insensitivity gene *Ppd* for response to photoperiod. Any of the dominant genes *Vrn*: *Vrn-1* (Yan et al., 2003), *Vrn-3* (Yan et al., 2006) and *Vrn-D4* (Kippes et al., 2016), controls spring growth habit and is epistatic over the recessive alleles of these genes. An exception is the dominant gene *Vrn2* described in *Triticum monococcum* L. (Yan et al., 2004a) and controlling winter growth habit: this gene expression are destroyed in polyploid wheat species.

An any dominant allele of the gene *Vrn*, except *Vrn2*, is enough for a plant to become a spring (Pugsley, 1971; Yan et al., 2004b; Fu et al., 2005; Knippes et al., 2018). Winter hexaploid wheat varieties are homozygous for the recessive alleles of all the three *Vrn-1* genes at once (Stelmakh, 1987); while winter tetraploids, for two genes, *Vrn-A1* and *Vrn-B1*, because the dominant genes *Vrn-3* and *Vrn-D4* have no recessive alleles, and so all winter varieties carry their null-alleles. No interaction between *Vrd* and *Vrn* has been described. A number of investigations have proposed schemes for the interaction between the *Vrn* with *Ppd* (Chen A. et al., 2014). However, the mechanisms underlying the interactions between these genes are not yet fully understood (Goncharov, 2012; Kiseleva, Salina, 2018). The *Vrn* genes are estimated to account for about 75 % of the control of variability of the trait duration of the vegetative period (DVP), and the *Ppd* genes, for about 20 % (Stelmakh, 1981). The third group of loci, EPS (earliness *per se*), controlling earliness *per se*, is under polygenic control (van Beem et al., 2005; Royo et al., 2020) and explains only about 5 % of DVP variation (Stelmakh, 1981).

The *Vrn-1* controlling the adaptability of wheat to environments (the traits spring/winter growth habit and earliness) are transcription factor genes (Trevaskis et al., 2003; Yan et al., 2003) that determine the expression of many genes involved in response to environmental stresses. Mutations in such genes not only disrupt their function, but also cause remarkable phenotypic changes. In wheat, DVP (earliness) is one of the important traits allowing the wild and cultivated species to take full advantage of the spring-summer season. At the same time, the *Vrn* genes have direct effects on plant productivity, yield and resistance to stresses, such as drought, low temperatures, pests and diseases, to mention a few (Zotova et al., 2019).

This paper considers the results of the modern molecular and genetic studies concerning spring/winter growth habit control and the effect of the allelic diversity of the *Vrn* genes on DVP in spring plants.

How many VRN loci does wheat have?

To date, six dominant *Vrn* genes (three *Vrn-1*: *Vrn-A1*, *Vrn-B1*, and *Vrn-D1*) (Yan et al., 2003), two *Vrn-3* (*Vrn-A3*, and *Vrn-B3*) (Nishimura et al., 2018), one *Vrn-D4* (Kippes et al., 2016)) and one recessive gene, *vrn-2* (Yan et al., 2004a) have been described as the ones controlling spring growth habit. Let us consider their main features.

VRN-1 locus. In di-, tetra- and hexaploid wheats, the spring growth habit is most commonly controlled by the *Vrn-1* genes (Genotypes…, 1985; Catalogue…, 1987; Goncharov, 1998; Lysenko et al., 2014; among others). These genes are located in distal part of long arms of the homeologous group 5 chromosomes: *Vrn-A1* on 5AL (Law et al., 1976; Galiba et al., 1995; Dubcovsky et al., 1998), *Vrn-B1* on 5BL (Barrett et al., 2002; Iwaki et al., 2002) and *Vrn-D1* on 5DL (Law et al., 1976). It has been shown that the *Vrn-1* genes are orthologous to the *Arabidopsis thaliana* (L.) Heynh. closely related *CAULIFLOWER* (*CAL*), *APETALA1* (*AP1*) and *FURITFULL* (*FUL*) meristem identity genes controlling the reproductive/flowering meristem transition (Ferrándiz et al., 2000; Yan et al., 2003; Preston, Kellogg, 2006; Dhillon et al., 2010). As was found, in *Arabidopsis*, *FUL* controls not only the development of carpels and fruits, but also flowering time (Ferrándiz et al., 2000). Later, another gene, *WAP1* (*Wheat APETALA1*) were characterized as AP1-like MADS-box gene in common wheat), was found and characterized as an activator of the transition from vegetative to reproductive development (Yan et al., 2003). It was shown that *WAP1* in wheat corresponds to *Vrn-1* (Trevaskis et al., 2003). *WAP1* expression begins before the transition to reproductive phases and continues until maturity (Murai et al., 2003).

The dominant *Vrn-A1* alleles have insertions and/or deletions in the promoter regions as well insertions and/or deletions and single nucleotide polymorphisms (SNPs) in the first intron, which the native recessive gene *vrn-A1* does not (Supplementary Material)[1]. Deletions in the first intron is what differentiates most of the dominant *Vrn-B1* alleles differ from the recessive *vrn-B1* allele. Additionally, deletions or insertions within the first intron are features of the dominant *Vrn-D1* alleles.

Although molecular biological methods allowed a large number of alleles of the dominant *Vrn* genes to be described (Yan et al., 2004a, b; Fu et al., 2005; Liu et al., 2012; Milec et al., 2023), the effects of these alleles on the duration of

[1] Supplementary Material is available
https://vavilov.elpub.ru/jour/manager/files/Suppl_Smolen_Engl_27_8.pdf

vernalization treatment and flowering time were not always identified (see Supplementary Material).

VRN-2 locus. The *Vrn2* gene (*Vrn-A^{m}2*) has been revealed only in the diploidic wheat *T. monococcum* (Dubcovsky et al., 1998). This gene was mapped to the distal part of the long arm of chromosome 5A^m within the segment translocated from 4A^{m}S (Dubcovsky et al., 2006). The VRN-2 locus includes two completely linked zinc finger-CCT domain genes *ZCCT1* and *ZCCT2* that act as flowering repressors down-regulated by vernalization (Yan et al., 2004a). However, it was established that the main determinant for *Vrn-2* expression in diploid wheat *T. monococcum* and *T. boeoticum* Boiss. and barley *Hordeum vulgare* L is day length (Dubcovsky et al., 2006; Trevaskis et al., 2006).

The sequence of the *Vrn-2* genes was revealed in the winter common wheat Jagger and 2174. No allelic variants of *Vrn-A2* in the A genome or *Vrn-D2* in the D genome were found (Chen Y. et al., 2009). Two duplicated copies of *Vrn-B2* were found in 2174. The *Vrn-B2* allele was not found in Jagger, suggesting this variety carries a null allele of this gene. The null-allele had no effect on flowering time in a segregated population. Mapping of *Vrn-B2* showed that both of its copies in 2174 were closely associated with a SNP on chromosome 4BL, suggesting that the *Vrn-B2* duplicates were located in a tandem-like manner at the same locus. Identical *Vrn-B2* sequences have been found in contig sequences of chromosomes 4BS, 2BS and 5DL in Chinese Spring (CS) (International Wheat Genome Sequencing Consortium..., 2018). In *Aegilops squarrosa* L. (=syn. *Ae. tauschii* Coss.), the sequence of the gene *Vrn-D2* was not found (Chepurnov et al., 2023). Thus, the gene *Vrn-2* in tetra- and hexaploid wheats is inactivated (Tan, Yan, 2016).

VRN-3 locus. The dominant gene *Vrn-B3* (formerly *Vrn 5* or *Vrn-B4*) was mapped to the short arm of chromosome 7B using 82 recombinants obtained from crosses between CS and the substitution line CS/Hope 7B (Yan et al., 2006). The gene is activated by vernalization and long day; it has been identified as an orthologue of the gene *FLOWERING LOCUS T* (*FT*) in *Arabidopsis* (Yan et al., 2006; Cockram et al., 2007). It is not easy to understand the role of *TaFT* in flowering regulation, because both common wheat and barley each possesses a 78 % identical paralogous copy of *FT2* (*TaFT2* and *HvFT2*, respectively) (Yan et al., 2006; Faure et al., 2007). As the *TaFT*/*TaFT2* duplication event took place after these cereals and *Arabidopsis* split off, this event is unrelated to the duplication of *FT*/*TSF*, the twin sister of *FT* found in *Arabidopsis* (Li, Dubcovsky, 2008).

The dominant gene *Vrn-A3* (homologous to *Vrn-B3*) has only been revealed in tetraploid wheats and mapped on the short arm of chromosome 7A (Nishimura et al., 2018). It is unlikely that it has homologs in common wheat, and it must be inactive as is *vrn-2* in *T. monococcum*.

VRN-4 locus. The dominant gene *Vrn-D4* was discovered in the line Gabo-2 (Knott, 1959; Pugsley, 1972; Goncharov, 2003) selected from the Australian commercial common wheat cv. Gabo. This gene was localized on chromosome 5D (Kato, 1993) and mapped to the centromeric region of the same chromosome (Kippes et al., 2015). The most current hypothesis is that the dominant gene *Vrn-D4* can have emerged in polyploid wheats due to a translocation of a ~290 kb-fragment of the long arm of chromosomes 5A to the proximal part of the short arm of chromosome 5D (Kippes et al., 2015). The translocated segment includes a *Vrn-A1* copy that carries mutations in the coding and regulatory regions (Kippes et al., 2015).

The gene is expressed at earlier stages of spring plants[2], and its sequence does not contain any of mutations that were previously described for the dominant gene *Vrn-A1* and that endow common wheat with spring growth habit (Yan et al., 2003, 2004b). The dominant gene *Vrn-D4* has instead three SNPs in the first intron, where the binding site for the TaGRP2 protein described as a negative regulator for *Vrn-A1* is located (Fu et al., 2005).

At present, no B-genome genes homologous to *Vrn-4* are known. As the dominant gene *Vrn-D4* has not been found in *Ae. tauschii*, the D-genome donor to hexaploid wheat (Chepurnov et al., 2023), it can be concluded that this mutation occurred in polyploids.

Thus, spring wheat carry mutations in the promoter or the first intron of the *Vrn* genes (Yan et al., 2004b; Fu et al., 2005). At the same time, most of the dominant alleles of the *Vrn-1* genes described to date (*Vrn-A1a*, *Vrn-A^{m}1a*, *Vrn-A1b*, *Vrn-A1d*, *Vrn-A1e*, *Vrn-A^{m}1g*, *Vrn-A1h* and *Vrn-A1i*) carry mutations in the promoter regions, within the VRN box, including SNPs, indels or its full elimination (Shcherban, Salina, 2017). The mutations found in the *Vrn* genes are presented in Supplementary Material. Chromosomal locations of the *Vrn* genes are detailed in Table 1. They were confirmed by molecular biological studies (Kiseleva, Salina, 2018).

The fact that the dominant alleles of the *Vrn-1* genes carry insertions and deletions that the recessive (intact) alleles do not may be an indication that they are evolutionarily younger (Milec et al., 2023). This allows their phylogenetic relationships to be inferred (see the Figure).

Vernalization of winter and spring wheats and its molecular and genetic network

Vernalization is the need of winter plants adapted to temperate climates for exposure to low temperatures, ensuring the transition of them from vegetative to reproductive development. A requirement for vernalization is an adaptive trait that helps prevent flowering before winter and permits flowering in the favorable conditions of spring. Winter plants are assumed to carry recessive (native) alleles of the

[2] See scales for growth and development in cereals (Efremova, Chumanova, 2023).

Table 1. Designation and localization in chromosomes of genes for growth habit in spring and winter wheat (after Goncharov (2012) with addition)

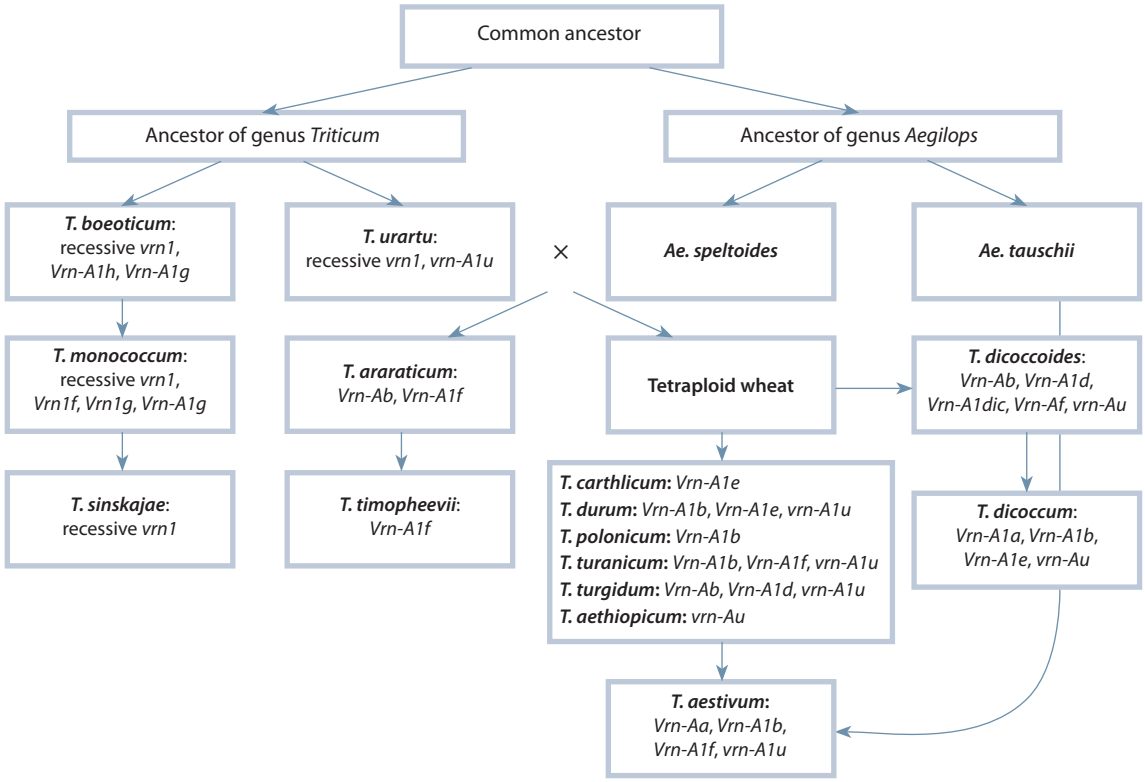
Phenotype	Haploid genotype	Allelism and chromosomal localization	Reference
	Tetra- and hexaploid wheats according to hypothesis and data of K. Tsunewaki, B.S. Jenkins (1961) and K. Tsunewaki (1962)		
Spring	*Sg1Sg2Sg3**	*Sg1–Sg1^c–sg1* (5D)	Tsunewaki, Jenkins, 1961; Tsunewaki, 1962
	sg1sg2sg3		
	*Sg1sg2Sg3**		
	*Sg1^{c}Sg2^{c}Sg3**	*Sg2–Sg2^c–sg2* (5A)	
	*Sg1^{c}Sg2Sg3**	*Sg3–sg3* (2B)	
	*Sg1^{c}Sg2Sg3**		
	*sg1sg2sg3Sg5**	*Sg5–sg5* (5B)	Singh, 1967
Winter	*sg1sg2Sg3*		Tsunewaki, 1962
	According to A.T. Pugsley (1972) hypothesis and to a number of author data		
Spring	*Vrn-A1vrn-B12vrn-D1*	*Vrn-A1–vrn-A1* (5AL)	Law et al., 1976
	vrn-A1Vrn-B12vrn-D1	*Vrn-B1–vrn-B1* (5BL)	Barrett et al., 2002
	vrn-A1vrn-B12Vrn-D1	*Vrn-D1–vrn-D1* (5DL)	Law et al., 1976
	vrn-A1vrn-B12vrn-D1Vrn-B3	*Vrn-B3* (7BS)**	Yan et al., 2006
	vrn-A1vrn-B12vrn-D1Vrn-A3	*Vrn-A3* (7AS)**	Nishimura et al., 2018
	vrn-A1vrn-B12vrn-D1Vrn-D4	*Vrn-D4* (5DS)**	Kippes et al., 2014
	and an any combination of dominant genes		
Winter	*vrn-A1vrn-B1vrn-D1*		Pugsley, 1972
	Diploid *T. boeoticum* and *T. monococcum* according to J. Dubcovsky et al. (1998) hypothesis and to a number of author data		
Spring	*Vrn-A1vrn-A2*	*Vrn-A1-vrn-A1* (5AL)	Dubcovsky et al., 1998
	vrn-A1vrn-A2	*Vrn-A2–vrn-A2* (4AL)	Yan et al., 2004b
Winter	*vrn-A1Vrn-A2*		Dubcovsky et al., 1998

* Spring growth habit is observed for any allelic state of the gene *Sg3*.
** The gene does not have recessive alleles.

vrn genes, with mutations in any of them leading to partial or complete inhibition of response to vernalization (Fu et al., 2005; Milec et al., 2023) and to a conversion of winter growth habit to spring ones. Spring plants form ears without vernalization, even though late-ripening spring forms, including the facultative growth ones[3], may respond to vernalization by promoting earliness and a reducing DVP. Vernalization in the late-ripening spring plants is poorly studied. In southern latitudes, vernalization is believed to provide autumn-sown late-ripening spring plants protection against damage from early-autumn light frosts.

[3] Facultative growth habit is an agrotechnological characteristic. Facultative growth habit plants can be both autumn-sowing and spring-sowing as reserve crops. At present, the State Register of RF includes three facultative growth habit cultivars produced in the Lukyanenko National Grain Center (Krasnodar, Russia) (State Register…, 2023).

A major obstacle to the study of the transition from vegetative to reproductive development is misidentification of the functions performed by the alleles of the *Vrn* genes. The misidentification arose from a terminological confusion started by Australian scientists A.T. Pugsley (1968) and R.A. McIntosh (1973), who were unfortunate to replace "spring growth" (Tsunewaki, 1962) with "response to vernalization" (Pugsley, 1971) (see Table 1). This term replacement were certain reasons (Pugsley, 1968); however, they were rather speculative. Years went by, but even so J. Dubcovsky, a molecular biologist, overlooked the issue and allowed this terminological mess to become part of the subsequent editions of "Catalogue of Gene Symbols for Wheat" (McIntosh et al., 2013). Note that the gene symbol *Sg* (spring growth) has immediate relevance to the



Scheme of *Triticum* and *Aegilops* genera evolution (according to Goncharov, 2011, with additions).

Different alleles of *Vrn-A1* gene among wheat species are presented in appropriate boxes next to the species names. Section *Timopheeevii* is presented in grey boxes, while section *Monococcon*, *Dicoccoides* and *Triticum* are in white ones (after Konopatskaia et al., 2016, with additions).

trait spring growth habit *vs.* winter growth habit and allows this trait to be explicitly formalized (Goncharov, 2004). In this case, the classification of the trait is genotype-based, not phenotype-based (Steinfort et al., 2017).

Need to pay attention that genotyping and phenotyping data may be inconsistent (see Table 2 in M. Makhoul (2022)). This relates to autumn-sown spring cultivars in the southern regions of the Eastern Hemisphere (Makhoul et al., 2022). Unfortunately, it is becoming more and more popular to state (postulated) the phenotypes depending on the sowing season (Steinfort et al., 2017). While, the phenotyping has to base solely on growth habit of plant (spring *vs*. winter). A.T. Pugsley (1983) begins his terminology-related considerations with "winter growth habit", that is, the physiological condition of a wheat plant requiring treatment to low temperatures (vernalization) and, consequently, having "response to vernalization" before it can come to reproductive phase.

And only the next step (question) is about phenotyping based on growth habit (spring *vs*. winter). The trait has to phenotyped as a qualitative morphological one (Goncharov, 2004). Plant phenotypes differ in that some plants switch to reproductive growth within a single spring-summer season and some do not. Wheat varieties are phenotyped with respect to this trait in the summer, at high positive temperatures, during ontogenesis after planting in the field or a greenhouse.

Response to vernalization is a quantitative trait, and so the accessions should be phenotyped using low temperatures (vernalization). In this case, the ultimate question is one about the duration of vernalization treatment (Dolgushin, 1935). Spring plants, even late-ripening ones, do not require vernalization to proceed to reproductive growth. Producing a unified approach for phenotyping spring/winter will make it possible to correctly compare all available research results.

In the database Wheat Trait Ontology, the traits plant growth habit (vernalization) and earliness are in the same subclass Development of class Trai and are associated with plant phenotype (Nédellec et al., 2020). The trait response to vernalization is not there, it is in the subclass Response to environmental conditions, meaning are the response of plants to the influence of the external environment (to a stress factor).

An important part of a unified approach to defining and phenotyping a trait is not only the terminology, but also the symbols of wheat genes. After the power to decide was shifted from one group of researchers (Ausemus et al., 1946) to another (McIntosh et al., 1973), the misleading terms "response to vernalization" became "legalized".

Table 2. Polymorphism of recessive alleles of the *Vrn* genes in winter wheat

Gene/locus	Allele	Mutation	Accession	Reference
		Diploid wheat ($2n = 2x = 14$)		
Vrn-A1	*vrn-A1u*	Identical to sequences of polyploid wheats	*T. urartu* Thum. ex Gandil. IG 44829	Golovnina et al., 2010
VRN-2 (*ZCCT1*)	*vrn2*	Point mutation at position 35 in the coding region of the CCT domain	*T. monococcum* DV92	Yan et al., 2004a
	vrn2	Complete deletion of *ZCCT1*	*T. monococcum*	
	vrn2	Data not present	*T. monococcum* TRI 17025	Shcherban et al., 2015b
		Tetraploid wheat ($2n = 4x = 28$)		
Vrn-A1	*vrn-A1b.3*	Deletion of the "T" upstream and the 20 bp deletion downstream of the VRN-box	*T. turgidum* PI 223173, *T. durum* PI 655432	Muterko et al., 2016
	vrn-A1b.4	»	*T. dicoccoides* PI 466941	
		Hexaploid wheat ($2n = 6x = 42$)		
Vrn-A1	*vrn-1a/vrn-A1b*	SNP in exon 4	Jagger (PI 593688)/2174 (PI 602595)	Chen Y. et al., 2009
	vrn-A1b.3	Deletion of the "T" upstream and the 20 bp deletion downstream of the VRN-box	*T. spelta* L. PI 168680 *T. vavilovii* Jakubz. PI 428342	Muterko et al., 2016
Vrn-B1	*vrn-B1*	5′ UTR 1-bp deletion + 8 bp start of a 2nd deletion	i: Triple Dirk C	Yan et al., 2004b
Vrn-D1	*vrn-D1*	5′ UTR 1-bp deletion + 15 bp start of a 2nd deletion	i: Triple Dirk C	
Vrn-D1	*vrn-D1r*	SNP polymorphism CArG-boxes of the *vrn-A1* promoter		Strejčková et al., 2021; Makhoul et al., 2022
Vrn-B3		Null-allele	cv. Yanzhan 4110	Chen F. et al., 2013

Note. Mutations in the recessive alleles of the gene *vrn-A1* in hexaploid wheat (Chen Y. et al., 2009) and the gene *Vrn-2* in diploidic wheat (Yan et al., 2004b) are in the coding regions.

That is why, although *Vrn* is a legal abbreviation and late-ripening spring and facultative (intermediate) growth habit varieties have response to vernalization, we will be using a more relevant term "growth habit (spring *vs*. winter)" throughout. We suggest the term "vernalization response" be left only for winter wheat (Fayt et al., 2018). Whether or not the recessive genes *vrn* really control vernalization response in winter varieties is still a question. Let us have a closer look at the matter.

Polymorphism of the recessive alleles of *Vrn* genes in winter wheat

All dominant *Vrn* genes known to date that control the qualitative difference between spring and winter wheats have been cloned. Two mutually exclusive hypotheses have been proposed: one stating that the duration of vernalization treatment in winter plants depends on the variability of the recessive alleles *vrn-A1* for winter growth habit (Pugsley, 1971; Chen Y. et al., 2009, 2010) and the other stating that it depends on a system of genes independent of them (Gotoh, 1979; Bulavka, 1984; Fayt, 2003, 2006a, b; Stelmakh et al., 2005) and unrelated to the expression of the recessive *vrn* genes.

This process has been poorly studied genetically and not studied at all at the molecular and biological level. Now it is obvious that the polymorphisms for the recessive genes *vrn* in winter wheat varieties do not explain differences in the duration of vernalization treatment between these varieties (Table 2). Not a single exception invalidating this genetic model has been reported in the studies, in which a large number of cultivars/germplasms from wheat species with different ploidy levels were screened using molecular markers for the recessive alleles of the each of *Vrn-1* genes (Yan et al., 2003, 2004a, b; Fu et al., 2005; Bonnin et al., 2008; Zhang X.K. et al., 2008; Santra et al., 2009; Chen Y. et al., 2010). The polymorphism for the *Vrn-3B* and *Vrn-4D* genes, in which the recessive allele is represented only by only as a null allele, makes an exception.

The alleles that have SNPs in exon 4 of the recessive gene *vrn-A1* are associated with the regulation of the de-

velopment of winter plants and are designated *vrn-A1a* in Jagger (PI 593688) and *vrn-A1b* in 2174 (PI 602595). In a field assessment of a population of 96 recombinant inbred lines from crosses between Jagger and 2174, Y. Chen et al. (2009) showed that the *vrn-A1a* plants had an earlier onset of shooting. At the same time, the effects of the alleles on the duration of vernalization treatment have not been checked experimentally.

Plants with the 3_SNPs haplotype showed higher transcription levels of the gene *Vrn-A1* than 1_SNP plants (Kippes et al., 2018). An assumption was made that the single nucleotide polymorphism in the regulatory region of the first intron should probably be associated with differences in the duration of vernalization treatment in the winter wheat. However, the attempt made by N. Kippes et al. (2018) to associate the SNPs in the recessive gene *vrn-A1* with the duration of vernalization treatment cannot be recognized successful: the authors used the winter near-isogenic line Triple Dirk C, a derivative of the spring cv. Triple Dirk, and in our experiments, Triple Dirk C plants, in the field, progressed into shooting (and some came to ear) within four months without vernalization (Goncharov, 2012).

It can be concluded that none of the known changes (point mutations) in the sequences of the recessive genes *vrn* has any effect on the duration of vernalization treatment in the winter wheat accessions (see Table 2).

Note that isogenic lines for the *Vrd* genes controlling variation in the duration of vernalization treatment in winter common wheat have long since been created (Fayt, 2006b) and can now be used in molecular and biological experiments. These genes reside on the winter common wheat's chromosomes: *Vrd1* on 4A and *Vrd2* on 5D (Fayt et al., 2007).

Allelic variability at the VRN locus and winter growth habit. Mutations in the regulatory regions of the *Vrn-1* gene are associated with prevalent spring growth habit, while the point mutations of a gene (or genes) at the VRN-2 locus (the *vrn-2a* allele) or the deletion of an entire gene (the *vrn-2b* allele) are also associated with spring growth as a recessive trait in diploid wheat *T. monococcum* and barley *H. vulgare* (Yan et al., 2004b; Dubcovsky et al., 2005). No multiple allelism of the dominant gene *Vrn-2* controlling winter growth habit has been revealed. This offers indirect evidence that this gene is not associated with the duration of vernalization treatment in the diploid *T. monococcum* or *T. boeoticum*. What genes control it at barley is not known either.

Variability of dominant alleles of the *Vrn* genes in spring accessions of di-, tetra- and hexaploid wheats and their effect on duration of the vegetative period

The number of works analyzing the distribution of the dominant genes *Vrn* and their alleles in the main wheat cultivation areas is impressive (Catalogue…, 1987; Goncharov, 1998; Fu et al., 2005; Zhang X.K. et al., 2008; Lysenko et al., 2014; Smolenskaya et al., 2022; and others). Differences of the regions by alleles is shown (Genotypes…, 1987; Stelmakh, 1990; Goncharov, 1998). As far as modern spring common wheat are concerned, *Vrn-A1a* is prevalent in cold-winter areas where spring wheat are sown only in the spring. By contrast, the dominant alleles of the homologous genes *Vrn-B1a* and *Vrn-D1a* are highly frequent in the varieties cultivated in the Mediterranean climate, where spring wheats are sown in the autumn (Stelmakh, 1990; Zhang X.K. et al., 2008; Shcherban, et al., 2015a). Noteworthy, *Vrn-D1a* emerged in Southern Europe in the 1930s together with photoperiod-insensitivity and reduced height genes coming from Japanese common wheat (Goncharov, 2012). The question as to whether the dominant gene *Vrn-B3* can be widely used outside China (Bonnin et al., 2008) requires special close consideration. This gene has not been found in Russia's cultivars (Lysenko et al., 2014), nor has it been found in the progeny of the variety Hope (Goncharov, Gaidalenok, 2005), the gene *Vrn-B3* donor for the isogenic line CS/Hope 7B.

Facultative growth habit plants. In English-language literature, facultative growth plants are known as "intermediate" (Flood, Halloran, 1986). According to B.V. Rigin and the colleagues, the spring growth habit in them should be determined by the dominant *Vrn-A1* gene (Genotypes…, 1985), while in A.F. Stelmakh' opinion, exclusively by the dominant gene *Vrn-B1* (Stelmakh, 1981). In Chinese wheat, the facultative growth habit plants possess the dominant allele *Vrn-D1b* (Zhang X.K. et al., 2008).

Because facultative growth habit plants (sometimes called semi-spring) play an important role in wheat production in some areas (Fayt et al., 2018), 689 Chinese varieties were studied for the frequency and distribution of the allele *Vrn-D1b* in them. The results showed that allele *Vrn-D1a*, *Vrn-D1b* and *vrn-D1* were present in 27.3, 20.6 and 52.1 % of the specimens, respectively. Pedigree analysis indicates that *Vrn-D1b* originated from Chinese landraces (Guo et al., 2015).

A study of F_2 hybrid segregating for *Vrn-D1b* and *Vrn-D1a* in greenhouse long-day conditions without vernalization showed that the *Vrn-D1b* homozygote plants would heading 32 days later than *Vrn-D1a* homozygotes. Because *Vrn-D1b* has the same deletion in the first intron as does *Vrn-D1a* and a single nucleotide mutation in the promoter region and is associated with facultative growth habit, the authors proposed that the mutation in the promoter can change the basal activity level of gene *Vrn-D1*, which is already active due to the deletion in the first intron (Zhang J. et al., 2012).

Copy number of the *Vrn* genes. Change in the copy number (CNV) of the *Vrn-1* genes is one of the sources of genetic variability in hexaploid wheat (Díaz et al., 2012; Würschum et al., 2015). In most cases, CNV is associated with changes in gene *Vrn* expression (Muterko,

2023); however, data on their effect on the DVP are inconsistent.

Hexaploid wheat species (2*n* = 6*x* = 42)

The most economically important point in the study of allelism of the dominant genes *Vrn* is the search for their functional association with the DVP. Data on DVP (earliness) in spring wheat are quite inconsistent. According to K.A. Flaksberger (1938), it is in a range between 76 and 140 or more days. Other authors report variations from 70–80 to 120–130 days (Kumakov, 1980). Opinions differ as to how to classify commercial common wheat varieties by maturity (Goncharov N.P., Goncharov P.L., 2018), as this classification has a clear-cut region-specific flavor. At the same time, earliness can be associated with different combinations of the dominant alleles of the *Vrn* genes (see Supplementary Material).

VRN-A1 allele. The distribution of spring common wheat into ripeness groups revealed that this trait is influenced by a combinations of certain dominant genes *Vrn* and their alleles (Stelmakh, 1993; Likhenko et al., 2014; Smolenskaya et al., 2022). Spring varieties with the dominant gene *Vrn-A1* are usually more early-ripening than the varieties with dominant genes *Vrn-B1* and *Vrn-D1* (Stelmakh, 1993). It has been demonstrated the main contributor to the reduction in duration between emergence of plant seedlings and heading is the dominant allele *Vrn-A1a*, while *Vrn-A1b*, in contrast, accounts for later heading (Efremova et al., 2016). Additionally, the varieties with the dominant allele *Vrn-A1b* is rare in Siberia, 8 % (Smolenskaya et al., 2022). The *Vrn-A1a* has an insertion in promoter region and *Vrn-A1b*, in contrast, a deletion (Yan et al., 2004b).

B.V. Rigin and the colleagues (2021) stated that the ultra-ripening lines Rico (K-65588) and Rimax (K-67257) had the shortest time from emergence plant shootings to heading among all spring common wheat accessions in the VIR collection. Their genotypes revealed dominant alleles for three *Vrn* genes at once, *Vrn-A1*, *Vrn-B1* (respectively *Vrn-B1a* or *Vrn-B1c*), and *Vrn-D1*.

Any of the dominant alleles, *Vrn-A1a* or *Vrn-A1b*, disables response to vernalization, while any of the dominant alleles of the *Vrn-B1* or *Vrn-D1* genes induces a residual response and leads to later flowering (Stelmakh, 1993). These data were confirmed by studies showing that the dominant alleles *Vrn-A1a* and *Vrn-A1b* in combination with the dominant gene *Vrn-B1* can provide optimum flowering time and potentially high yield in the Pacific Northwest region of the USA, while spring wheat varieties with the dominant gene *Vrn-D1* may have advantage in Idaho and Oregon, where the vegetation periods are longer (Santra et al., 2009).

VRN-B1 allele. A novel allele, *Vrn-B1c*, probably associated with earlier ripening in late-ripening spring varieties was revealed using near-isogenic lines with different alleles of the *Vrn-B1* gene (Shcherban et al., 2012a). Its prevalence among common wheat varieties in Western Siberia and the North Kazakhstan, when spring growth habit being under monogenic control, was demonstrated (Shcherban et al., 2012b). In the absence of epistatic effects of the dominant *Vrn-A1* gene, this allele causes earlier heading than does *Vrn-B1a* (Shcherban et al., 2013). The effect of *Vrn-B1f* on heading time is similar to that of *Vrn-B1c*, but the mechanism of its regulation most likely appears to be different (Strejčková et al., 2021).

VRN-D1 allele. The dominant gene *Vrn-D1* occurs only in hexaploid wheat cultivars in the Asian region and some Italian varieties (Stelmakh, 1993; Goncharov, 1998). K. Iwaki and the colleagues (2000, 2001) found the dominant allele *Vrn-D4* in a large number of common wheat cultivars from different regions worldwide (55 cultivars out of 272 studied). The highest frequency of occurrence was observed in accessions from India and the bordering countries (Iwaki et al., 2000, 2001). This dominant gene had previously been found in most accessions of the Indian hexaploid endemic species *T. sphaerococcum* Perciv. (Goncharov, Shitova, 1999).

The dominant allele *Vrn-D^{t}1* with a 5.4-kb deletion in the first intron was found in spring plants of *Ae. tauschii* from the Middle East (Takumi et al., 2011). One more dominant allele was described later (Chepurnov et al., 2023). This allele has effect on heading time.

All the variants identified in three *Vrn-1* homeologs in wheat were designated as separate alleles, but not all of them were experimentally confirmed to have any effect on DVP (earliness) (see Supplementary Material).

VRN-B3 allele. The nucleotide substitutions or insertions/deletions in the copies of the *FT* gene (*Vrn-B3*) in the A and D genomes in 239 local, old local and modern commercial cultivars from different regions cause DVP polymorphisms (Bonnin et al., 2008). In contrast to *Vrn-1*, the homeologous copies of the *FT* gene showed no evidence of epistatic interactions (Bonnin et al., 2008). *TaFT* overexpression in transgenic *T. aestivum* plants considerably accelerated flowering compared to the non-transgenic control (Yan et al., 2006).

The absence of isogenic lines does not allow its different alleles to be compared for the strength of their phenotypic manifestation. Note that, line 620 with *Vrn-B3* had much later heading (Goncharov, 2012). Later heading was also observed in cultivars carrying various *Vrn-B3* alleles, *Vrn-B3a* and *Vrn-B3b* (Chen F. et al., 2013), and *Vrn-B3d* and *Vrn-B3e* (Berezhnaya et al., 2021).

Two hundred and seventy eight Chinese spring common wheat cultivars were characterized using molecular markers of the *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Vrn-B3* genes. The varieties possessing from three to four dominant *Vrn* genes, including the rare dominant gene *Vrn-B3*, were the earliest (30–31 days to heading on average), and one-, two-, or three-gene combinations, including the dominant gene *Vrn-A1*, but not *Vrn-B3*, followed (38 days to heading on

average). On the basis of these data, the dominant *Vrn-1* genes were ranked according to the amount of their influence on DVP reduction in the Chinese cultivars: *Vrn-A1* > *Vrn-B1* > *Vrn-D1* (Zhang X.K. et al., 2008). This ranking is not the same as those reported elsewhere (Gotoh, 1979; Goncharov, 2003).

VRN-D4 allele. The dominant gene *Vrn-D4* has a weaker effect on DVP than have the *Vrn-A1*, *Vrn-D1* or *Vrn-B3* genes (Kippes et al., 2014), but stronger than *Vrn-B1* (Gotoh, 1979; Goncharov, 1998).

Tetraploid wheat species (2*n* = 4*x* = 28)

Based on the analysis of *Vrn-A3* expression using sister lines, earlier flowering in accession TN26 of *T. dicoccum* Schrank ex Schuebl. than in accession TN28 of *T. pyramidale* Perciv. is due to a 7-bp insert in the promoter region of the gene which, this insert including a *cis*-element of the GATA box (Nishimura et al., 2018). The analysis revealed the presence of the early-flowering alleles of *Vrn-A3* in spelt wheat from Ethiopia and India and their absence in the accessions of *T. durum* Desf. and common wheats. These results led the authors to the conclusion that the *Vrn-A3a-h1* and *Vrn-A3a-h2* alleles should be useful in breeding for earliness in durum and common wheat (Nishimura et al., 2021).

T. carthlicum Nevski and *T. dicoccum* accessions possessing the *Vrn-B1c* allele with a retrotransposon insertion passed to flower without vernalization. Transcripts in the winter DH-lines possessing the recessive *vrn-B1* allele were observed no sooner than after vernalization (Chu et al., 2011).

Two spring accessions, PI 208912 (Iraq) of *T. turgidum* and PI 74830 (China) of *T. durum* and one winter accession PI 221422 (Serbia) of *T. turgidum* started to flower without vernalization. However, they did so substantially later than plants with the dominant *Vrn-A1* or *Vrn-B1* genes. Interestingly, winter accession PI 221422 started to flower 25 days later than did spring accessions PI 208912 and PI 74830. All of them have recessive *vrn-B1* alleles and null alleles *vrn-B3*. It is proposed that their late flowering is due to the *Vrn-A1i* allele (Muterko et al., 2016).

The combination of the dominant *Vrn-A1* and *Vrn-B1s* alleles was associated with early flowering the tetraploid species *T. dicoccum* and *T. dicoccoides* (Körn. ex Asch. et Graebn.) Schweinf. (Muterko et al., 2016). *Vrn-A1* allelism is a possibility in *T. dicoccum* (Rigin et al., 1994).

The gene's allelic variant coming from *T. militinae* Zhir. et Migusch. was designated *Vrn-A1f*-like. QTL analysis showed that it caused a 1.9–18.6-day delay in the flowering time of Tähti and Mooni, depending on cultivation conditions (Ivaničová et al., 2016).

In all *T. timopheevii* (Zhuk.) Zhuk. accessions studied, the spring growth habit was associated with the dominant *Vrn-A1f-ins* and *Vrn-A1f-del/ins* alleles (Golovnina et al., 2010; Shcherban et al., 2016). The same allele was found in wild *T. araraticum* Jakubz. (Golovnina et al., 2010). Noteworthy, this species has an extremely limited number of spring forms (Goncharov, 1998).

Diploid wheat species (2*n* = 2*x* = 14)

It is possible that the pattern of inheritance in diploid wheats is more sophisticated than it used to be thought before, as spring growth habit in the wild *T. boeoticum* has recently been shown to be under digenic control (Fu Hao, Boguslavskyi, 2023). Similar results obtained for the *T. monococcum* by L. Smith (1939) have remained unnoticed. Spring accessions of *T. urartu*, the A^u-genome donor for polyploid wheat species, were found to have a *Vrn-A1* mutation typical for the section *Triticum* species (Golovnina et al., 2009). However, as few as four *T. urartu* accessions from among 400 studied were spring (Goncharov, 1998), of which two were "odd" in that they were *T. urartu* phenotypically (with velvety pubescence of leaves), but *T. boeoticum* karyotypically (Adonina et al., 2015) and, therefore, *Vrn-A1* polymorphism is most likely to have emerged no sooner than in polyploid wheats.

In field conditions, *T. monococcum* with various deletions in the promoter region of the *Vrn-A1f* and *Vrn-A1g* alleles showed 59–60 days to heading on average and did not differ significantly from each other in terms of this measure ($p = 0.842$) (Chepurnov, Blinov, 2022).

Enhancing the diversity of polymorphisms in the *Vrn* genes and prospects in breeding for reduced duration of the vegetative period

The polymorphism in dominant *Vrn* genes controlling spring growth habit in varieties of Siberia and the European part of Russian Federation is extremely low (Lysenko et al., 2014; Smolenskaya et al., 2022). In 75 % of the cultivars in Siberia, this trait is under digenic control exerted by the dominant *Vrn-A1* and *Vrn-B1* genes; in 25 %, under monogenic control exerted by dominant genes (among 24 cultivars, 19 are controlled by a single dominant gene *Vrn-A1* and 5, by a single dominant gene *Vrn-B1*). Trigenic control was discovered for one cultivar, Tulun 15, (Likhenko et al., 2014). The conclusion made by E.A. Moiseeva and N.P. Goncharov (2007) that spring growth habit in the of Western and Eastern Siberian wheat cultivars is controlled by two dominant *Vrn* genes has been confirmed. An increased prevalence of the allele *Vrn-B1c* in West Siberian cultivars and of the allele *Vrn-B1a* in East Siberian cultivars has been observed, suggesting their selectivity to environments of these regions (Smolenskaya et al., 2022). Other regions of the Russian Federation have not yet been considered with this amount of scrutiny (Lysenko et al., 2014).

Our assumption is that, the level of DVP-related polymorphism in spring wheat cultivars in Siberia in particular and Russia in general can be enhanced by introgression of the dominant alleles of the *Vrn* genes from their wild

ancestor (Goncharov, Chikida, 1995; Goncharov, 1998) or by using rare alleles that are present in their gene pool (Stelmakh, Avsenin, 1996; Koval, Goncharov, 1998) but have not been studied by a molecular genetic methods and are rarely used in the breeding. Note that *T. urartu* Thum. ex Gandil. – the donor of the A^u genome of polyploid wheats does not carry any mutation that could be new to spring common wheat (Golovnina et al., 2010). The use of the diploid species *T. monococcum* carrying the A^b genome (Goncharov et al., 2007; Nishiura et al., 2018) appears to be impracticable either, due to its evolutionary unrelatedness to cultivated wheat. Consequently, the model based on *T. monococcum* is not successful, as it leads modern plant cultivation nowhere.

The aim of the future efforts is to develop a simple model predicting wheat phenology, with effects of vernalization and photoperiods taken into account. New facts about the expression of the *Vrn* genes, their allelic composition, and interaction with other genes will allow us to learn more about the associations known to date (Distelfeld et al., 2009; Jin, Wei, 2016; Krasileva et al., 2017; Kiseleva, Salina, 2018; Milec et al., 2023). This knowledge will undoubtedly contribute to increasing the efficiency of next generation breeding.

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Comparative assessment of the copy number of satellite repeats in the genome of Triticeae species

P.Yu. Kroupin, A.I. Yurkina, A.A. Kocheshkova, D.S. Ulyanov, G.I. Karlov, M.G. Divashuk

All-Russia Research Institute of Agricultural Biotechnology, Moscow, Russia
pavel-krupin@yandex.ru

Abstract. Satellite repeats are a significant component of the genome of Triticeae and play a crucial role in the speciation. They are a valuable tool for studying these processes. *Pseudoroegneria* species play a special role among grasses, as they are considered putative donors of the St-genome in many polyploid species. The aim of this study was to compare the copy number of satellite repeats in the genomes of Triticeae species. Quantitative real-time PCR was applied to determine the copy numbers of 22 newly discovered satellite repeats revealed in the whole-genome sequences of *Pseudoroegneria* species and one additional repeat previously identified in the genome of *Aegilops crassa*. The study focused on seven species of *Pseudoroegneria*, three species of *Thinopyrum*, *Elymus pendulinus*, *Ae. tauschii*, *Secale cereale*, and *Triticum aestivum*. Based on the copy number level and coefficients of variation, we identified three groups of repeats: those with low variability between species (medium-copy CL82), those with medium variability (low- and medium-copy CL67, CL3, CL185, CL119, CL192, CL89, CL115, CL95, CL168), and those with high coefficients of variation (CL190, CL184, CL300, CL128, CL207, CL69, CL220, CL101, CL262, CL186, CL134, CL251, CL244). CL69 exhibited a specific high copy number in all *Pseudoroegneria* species, while CL101 was found in both *Pseudoroegneria* and *Th. junceum*, CL244 in *Th. bessarabicum*, CL184 in *P. cognata* and *S. cereale*. CL95, CL128, CL168, CL186, CL207, and CL300 exhibited higher copy numbers in *P. cognata* compared to other species; CL3, CL95, CL115, CL119, CL190, CL220, CL207, and CL300 in *P. kosaninii*; CL89 in *P. libanotica*; CL134 in *P. geniculata*. Our assessment of the copy number of new satellite repeats in the St-genome and the analysis of their amplification specificity between species can contribute to the molecular-genetic and chromosome markers used for evolutionary, phylogenetic, and population studies of Triticeae species.
Key words: Triticeae; satellite repeats; qPCR; whole-genome sequencing.

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Сравнительная оценка копийности сателлитных повторов в геноме видов Triticeae

П.Ю. Крупин, А.И. Юркина, А.А. Кочешкова, Д.С. Ульянов, Г.И. Карлов, М.Г. Дивашук

Всероссийский научно-исследовательский институт сельскохозяйственной биотехнологии, Москва, Россия
pavel-krupin@yandex.ru

Аннотация. Сателлитные повторы составляют значительную часть генома Пшеницевых, играя важную роль в видообразовании, что делает их ценным инструментом для изучения этих процессов. Особое место среди злаков занимают виды *Pseudoroegneria* – наиболее вероятные доноры St-генома у многих полиплоидов. Цель настоящего исследования состояла в сравнительной оценке копийности сателлитных повторов в геномах Triticeae. С помощью количественной полимеразной цепной реакции в реальном времени была установлена копийность 22 сателлитных повторов, выявленных в полногеномных нуклеотидных последовательностях видов *Pseudoroegneria*, и одного ранее опубликованного повтора, обнаруженного в геноме *Aegilops crassa*. Объектами анализа стали семь видов *Pseudoroegneria*, три вида *Thinopyrum*, *Elymus pendulinus*, *Ae. tauschii*, *Secale cereale* и *Triticum aestivum*. По уровню копийности и коэффициентам вариации нами выделено три группы повторов: с низким уровнем вариативности между видами (среднекопийный CL82), средним уровнем вариативности (низко- и среднекопийные CL67, CL3, CL185, CL119, CL192, CL89, CL115, CL95, CL168) и с высокими значениями коэффициента вариации (CL190, CL184, CL300, CL128, CL207, CL69, CL220, CL101, CL262, CL186, CL134, CL251, CL244). Повтор CL69 показал специфическую высокую копийность для всех видов *Pseudoroegneria*, CL101 – у *Pseudoroegneria* и *Th. junceum*, CL244 – у *Th. bessarabicum*, CL184 – у *P. cognata* и *S. cereale*. У *P. cognata* более высокую копийность, по сравнению с остальными видами, проявили повторы CL95, CL128, CL168,

CL186, CL207, CL300; у *P. kosaninii* – CL3, CL95, CL115, CL119, CL190, CL220, CL207 и CL300; у *P. libanotica* – CL89; у *P. geniculata* – CL134. Проведенные нами оценка копийности сателлитных повторов, найденных в St-геноме, и анализ специфичности их амплификации между видами могут пополнить арсенал молекулярно-генетических и цитогенетических маркеров, используемых для эволюционных, филогенетических и популяционных исследований представителей трибы Пшеницевых.

Ключевые слова: Triticeae; сателлитные повторы; qPCR; полногеномное секвенирование.

Introduction

Triticeae is an economically important tribe of the Poaceae family, comprising approximately 500 species of annual and perennial herbaceous plants (NCBI database: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). Wheat, rye, barley, and fodder grasses are among the species of this tribe that play a significant role in providing food for humanity and have also become an integral part of animal diets (Hodkinson, 2018).

The interest in studying phylogenetic relationships within the Triticeae tribe is largely driven by the potential of wild wheat species to serve as valuable sources of economically important genes for the improvement of cultivated cereals. For example, *Thinopyrum* and *Dasypyrum* serve as gene donors for resistance to various diseases (Yang et al., 2005; Luo P.G. et al., 2009; Salina et al., 2015; Wang S. et al., 2019; Li L.F. et al., 2022; Guo et al., 2023). By crossing wheat and *Agropyron*, it is possible to increase head productivity (Zhang J. et al., 2016). Representatives of *Pseudoroegneria* are drought-resistant and are used as pasture grasses (Wu et al., 2023b).

The Triticeae tribe consists of approximately 100 annual and 400 perennial species, which carry one (in diploids) or several (in polyploids) of 13 genomes (Wang, Lu, 2014). Representatives of *Pseudoroegneria* carry the St-genome, and it is believed that this genus was the donor of the St-genome for *Elymus* and some *Thinopyrum* species (Mahelka et al., 2011; Dobryakova, 2017; Linc et al., 2017; Lei et al., 2018; Chen N. et al., 2020; Agafonov et al., 2021). Plants of the genus *Agropyron*, at all ploidy levels ($2n = 2x/4x/6x$), are distinguished by the presence of a P-genome (Zhang Y. et al., 2015). Genome J (=E) is mainly composed of diploids *Thinopyrum bessarabicum* (genome J = J^b) and *Th. elongatum* (genome E = J^e). The J-genome is evolutionarily close to the D-subgenome of common wheat, and the most likely donor of the D-subgenome is *Aegilops tauschii* (Baker et al., 2020). This may explain why the chromosomes of the D-subgenome in the introgressive lines of common wheat, developed with the aim of improving it through hybridization with *Thinopyrum*, show the highest frequency of introgressions from the J-genome (Chen Q. et al., 2001; Liu Z. et al., 2007; Cui et al., 2018).

At present, the origin, relationships, and proximity of genomes within the Triticeae tribe remain controversial and uncertain. The challenges associated with studying Triticeae genomes are primarily due to the significant differences between the polyploid subgenome and the ancestral genome of the diploid parent organism. These differences arise from the modifications that occur during evolution. Additionally, the diploid ancestor of the donor organism may have become extinct or has not yet been found (Jakob, Blattner, 2010; Liu Q.-L. et al., 2020; Sha et al., 2022). Perennial polyploid species, for example, *Th. intermedium* and *Th. ponticum*, may have an unbalanced genome or chromosomal translocations (Kruppa, Molnar-Lang, 2016; Liu Y. et al., 2023). This could be associated with the transition to vegetative reproduction, which does not involve sexual processes for seed formation and therefore does not require stable meiosis (Comai et al., 2005; Husband et al., 2013). The same species are characterized by recombinant subgenomes, the origin of which is still unclear (Wang R.R.C. et al., 2015; Liu Y. et al., 2023). Discussions continue regarding potential donors of the Y-subgenomes in *Elymus* and *Roegneria* (Yan et al., 2011; Liu Q.-L. et al., 2020; Wu et al., 2021), as well as the maternal form in the occurrence of *Thinopyrum*, *Roegneria*, *Elymus*, *Kengyilia*, and other polyploids (Mahelka et al., 2011; Luo X. et al., 2012; Zeng et al., 2012; Lei et al., 2018; Chen N. et al., 2020).

In addition, there is a problem of correlation between the species identification of a particular specimen based on botanical traits (often influenced by the environment) and that based on molecular genetic and cytogenetic characteristics (Wang, Lu, 2014; Al-Saghir, 2016; Rodionov, 2022). For example, this issue arises in *Elymus*, as demonstrated by studies conducted by A.V. Rodionov et al. (2019), V. Lucia et al. (2019), and L. Tan et al. (2021). Another example is the relationship between *Th. elongatum* and *Th. bessarabicum* that bear fairly similar genomes, but differ in botanical characteristics (Grewal et al., 2018; Dai et al., 2021; Chen C. et al., 2023). The problem is compounded by the fact that natural spontaneous hybrids are often found (Chen C. et al., 2022; Luo Y.C. et al., 2022; Wu et al., 2023a). The study of phylogenetic relationships deepens our understanding of evolutionary processes in plants and speciation, and helps to improve biosystematics. The acquired knowledge will enhance the efficiency of utilizing genetic resources from wild species by improving the understanding of their proximity to the genomes of cultivated crops and the potential for obtaining valuable introgressions.

The genome of Triticeae grasses is characterized by a large size, which complicates whole-genome deep sequencing and makes assembly difficult (Rabanus-Wallace, Stein, 2019). A significant portion of the Triticeae genome is composed of repetitive DNA, known as repeats. The repeatome include mobile elements, gene clusters (specifically the 5S and 45S rRNA genes), and satellite repeats (Dvořák, 2009; Shcherban et al., 2015; Gao et al., 2023; Vershinin et al., 2023).

Satellite repeats are tandem repeating non-coding sequences that exist as arrays of varying lengths in genetically silent heterochromatin regions (Badaeva, Salina, 2013). Satellite repeats are considered to be the most variable and rapidly evolving components. They can be species-specific or common to closely related species (Belyayev et al., 2019; Garrido-Ramos, 2021; Thakur et al., 2021). Comparative analysis of copy number, nucleotide sequences, and localization on the chromosomes serves as a tool for basic phylogenetic and evolutionary studies of plants, including Triticeae species (Anamthawat-Jónsson, Heslop-Harrison, 1993; Vershinin et

al., 1994; Kishii et al., 1999; Anamthawat-Jónsson et al., 2009; Han et al., 2017; Linc et al., 2017; Ruban, Badaeva, 2018; Said et al., 2018; Salina, Adonina, 2019; Dai et al., 2021; Wu et al., 2021; Chen C. et al., 2022; Kroupin et al., 2023; Shi et al., 2023). Satellite repeats have found practical use as PCR markers and chromosomal markers for identifying introgressions of alien genetic material containing valuable economic traits in the genomes of cultivated cereals (Li G. et al., 2016; Han et al., 2017; Liu L. et al., 2018; Chen J. et al., 2019).

Tangible progress has been made in the study of Triticeae genomes, owing to the invention of whole-genome sequencing methods and bioinformatic algorithms for analyzing the data obtained (Rabanus-Wallace, Stein, 2019; Gao et al., 2023). The rapid growth in the volume of information on genome-wide sequences of Triticeae has significantly accelerated and simplified the search for repetitive DNA that can be used as chromosomal markers (Du et al., 2017; Said et al., 2018; Tang et al., 2018; Chen J. et al., 2019; Kroupin et al., 2019a, 2022; Lang et al., 2019a; Liu Q.-L. et al., 2020; Wu et al., 2021, 2022). Due to the significant presence of repetitive DNA in the Triticeae genomes, information about satellite repeats can be obtained even through sequencing with low coverage. This greatly simplifies the process of searching for repeated sequences (Navajas-Perez, Paterson, 2009; Kroupin et al., 2019b; Šatovic-Vukšic, Plohl, 2023).

A well-established method for quantifying the number of copies of repetitive DNA, including satellite repeats, is quantitative real-time PCR (qPCR) (Harpke, Peterson, 2007; Navajas-Pérez et al., 2009; Baruch, Kashkush, 2012; Feliciello et al., 2015; Divashuk et al., 2016, 2019, 2022; Pereiera et al., 2018; Shams, Raskina, 2018). Compared to Southern blot or dot-blot hybridization on a membrane, or fluorescent *in situ* hybridization on chromosomes, qPCR has proven to be an easier-to-use, accurate, and effective method for assessing the copy number of the target sequence. This method allows researchers to identify the number of copies of satellite repeats in the genome and the variability between genomes (Kalendar et al., 2020; Pös et al., 2021).

In this study, the whole-genome sequencing of *Pseudoroegneria spicata*, *P. libanotica*, *P. tauri*, *P. geniculata*, *P. cognata*, and *P. kosaninii* revealed the presence of 22 satellite repeats. In order to comprehend the potential of using them as tools for evolutionary and phylogenetic studies of wild representatives of the Triticeae tribe, as well as for studying wide hybrids using molecular biology and cytogenetics methods, it is crucial to first determine the copy number of satellite repeats in the genomes of St-genome carriers. Therefore, we have chosen *Pseudoroegneria* species with different ploidy levels as our research subject.

To assess the specificity of satellite repeats for the St-genome, we included *Thinopyrum* species in the experiment, which contain the J-genome that is common among Triticeae. We also selected *Th. intermedium* with the J^rJ^{vs}St-genomic formula, serving as a carrier of the St-subgenome and St-specific repeats in the recombinant J^{vs}-genome. To explore the potential of utilizing the identified satellite repeats for the characterization of distant wheat and rye hybrids, *Triticum aestivum* and *Secale cereale* accessions were included in the study. In addition, due to the evolutionary proximity of the J- and D-genomes, we included the *Ae. tauschii* accession. *E. pendulinus* was also used, carrying both the St-subgenome targeted by our work and the Y-subgenome of unknown origin, which is common among *Elymus sensu lato* species. The experiment also included a satellite repeat of CL244, which we obtained as a result of analyzing the whole-genome nucleotide sequence of *Ae. crassa* (D^1X^{cr}), a carrier of the D-genome variant (Kroupin et al., 2022). Despite this, CL244 was not found in *Ae. tauschii*, showed a small number of hybridization sites on the chromosomes of *T. aestivum* and *Ae. crassa*, while on the chromosomes of the J^b-genome in *Th. bessarabicum*, bright signals were observed indicating a high level of its abundance.

Materials and methods

The Triticeae species with various genomic compositions, as listed in the Table, served as the material for our study.

The young leaves of the plants were frozen in liquid nitrogen. Genomic DNA was then isolated using the CTAB protocol (Rogers, Bendich, 1985). This DNA was used for subsequent sequencing and quantitative PCR (qPCR). The concentration and purity of the isolated DNA were tested using Qubit 4 (Thermo Fisher Scientific, USA) and electrophoresis in a 0.8 % agarose gel.

Shotgun sequencing libraries were synthesized using the Swift 2S Turbo DNA Library Kit (Swift Bioscience, USA) in accordance with the manufacturer's protocol. To assess the quality of the libraries, a test run was conducted on the MiSeq device (Illumina, Inc., USA). The libraries were then converted and sequenced using DNBSEQ-G400 on one track. The initial amount of DNA was 25 ng. The fragments were approximately 350 bp long and were indexed at both ends using the Swift 2S Turbo Unique Dual Indexing Kit (Swift Bioscience). Sequencing was performed on Illumina NextSeq (Illumina, Inc.), using the NextSeq 500/550 Mid Output Kit v.2.5 (Illumina, Inc.).

Bioinformatic analysis was conducted on the processing and assembly of the reads of nucleotide sequences that involved a sequence of satellite tandem repeats. The uniqueness of these sequences was evaluated in comparison to previously published methods described in P.Y. Kroupin et al. (2022). The primer sequences for the identified monomers of satellite repeats are provided in Supplementary Material 1[1].

Quantitative real-time PCR was conducted using DNA from the accessions listed in the Table as a template, with three replicates. The amplification was carried out using a CFX real-time amplifier system (Bio-Rad Laboratories, Inc., USA) and a reaction mixture of Real-Time PCR Mix containing the Eva Green fluorophore (Synthol Ltd., Russia) in accordance with the manufacturer's protocol. A single-copy gene, *VRN1*, was used as the reference gene. Primer concentration in mixtures consisted of 10 ng/µl, while DNA concentration was 0.4 ng/µl. Amplification was performed according to the following program: preincubation – 10 min at 95 °C; followed by 40 cycles: denaturation – 10 s at 95 °C; primer annealing – 30 s at 60 °C.

Statistical analysis, including the calculation of the average values of Cq, standard deviation, and the corresponding number of copies relative to the reference gene *VRN1*, was performed using Bio-Rad CFX and Manager 3.1 software. To

[1] Supplementary Materials 1 and 2 are available https://vavilov.elpub.ru/jour/manager/files/Suppl_Kroupin_Engl_27_8.xlsx

Plant material

Species	Accessions	Source	2*n*	Genome formula	Target of use (sequencing, qPCR)
P. spicata	PI 578855	GRIN[1]	14	StSt	Sequencing, qPCR
P. spicata	PI 236671	GRIN	28	StStStSt	Sequencing, qPCR
P. libanotica	PI 228389	GRIN	14	StSt	qPCR
P. libanotica	PI 330690	GRIN	14	StSt	Sequencing
P. tauri	PI 380652	GRIN	14	StSt	Sequencing, qPCR
P. geniculata	PI 670437	GRIN	28	StStStSt	Sequencing, qPCR
P. cognata	PI 670361	GRIN	28	StStStSt	Sequencing, qPCR
P. kosaninii	PI 237636	GRIN	56	StStStSt StStStSt	Sequencing, qPCR
Th. intermedium	PI 401200	GRIN	42	$J^{vs}J^{vs}J^{r}J^{r}$StSt	qPCR
Th. bessarabicum	PI 531711	GRIN	14	$J^{b}J^{b}$	qPCR
Th. junceum	PI 119604	GRIN	42	$J^{b}J^{b}J^{b}J^{b}J^{e}J^{e}$	qPCR
E. pendulinus	PI 639804	GRIN	28	StStYY	qPCR
Ae. tauschii	K-608	VIR[2]	14	DD	qPCR
S. cereale	cv. Bereginya	NGC[3]	14	RR	qPCR
T. aestivum	cv. Chinese Spring	–	42	BBAADD	qPCR

[1] GRIN – germplasm resources information network of the Agricultural Research Service of the US Department of Agriculture (USDA-ARS Germplasm Resources Information Network).
[2] VIR – N.I. Vavilov All-Russian Institute of Plant Genetic Resources.
[3] NGC – P.P. Lukyanenko National Grain Center.

assess the similarity of copy numbers among repeats, we have introduced the concept of "repeat copy number pattern", a set of copy number values for a specific repeat within the set of species being studied. To assess the similarity of copy number among the species under investigation, we have introduced the concept of "species copy number pattern", a set of values of the copy number of the satellite repeats being studied for a particular species.

Pearson's correlation coefficients (r) were calculated using Statistica 12 software (StatSoft, USA) to determine the relationship between repeat copy number patterns and species copy number patterns. The significance level was set at $p < 0.05$. Diagrams were constructed using the principal component analysis method for satellite repeats and the studied species. The diagrams were based on the data obtained from Statistica 12 software, which included information on the copy number of satellite repeats. The coefficient of variation of the satellite repeatability values between species was calculated using Microsoft Excel (USA).

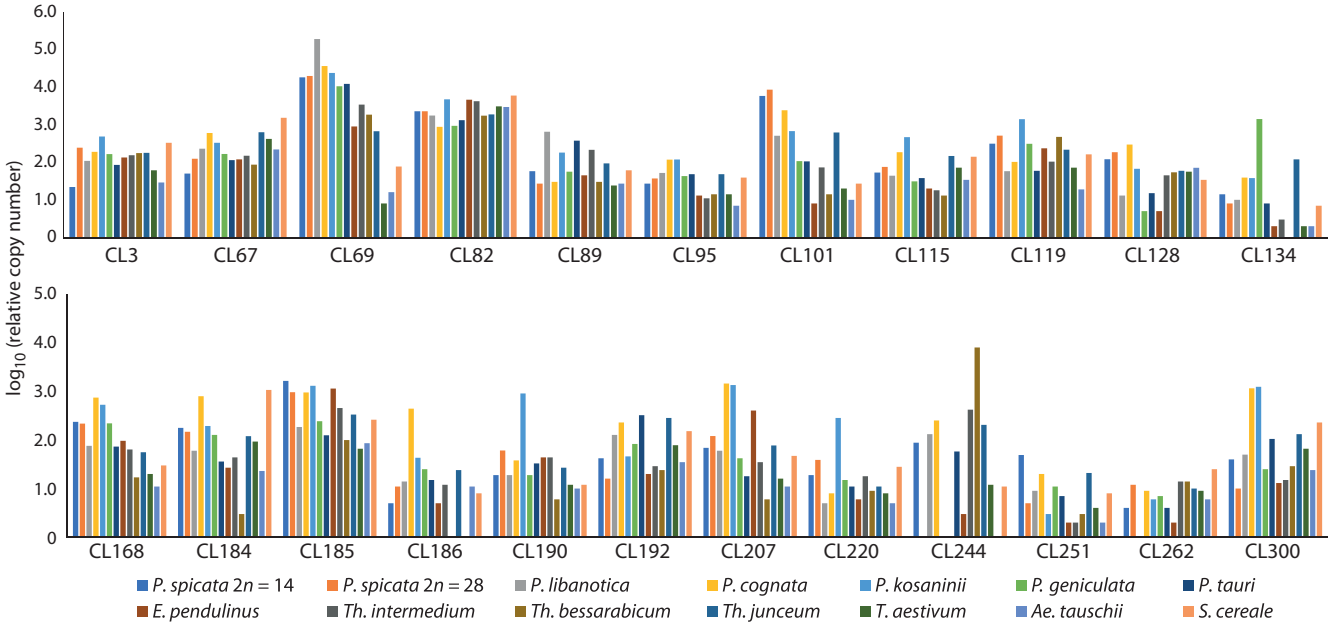
Results

Characteristics of identified satellite repeats

In the framework of the present study, a total of 22 satellite repeats were found in separate genome sequence assemblies. As a result of analyzing the nucleotide sequence of the diploid accession of the *P. spicata* (2*n* = 14) genome, 10 repeats were identified. These repeats include CL69, CL82, CL101, CL119, CL128, CL168, CL184, CL207, CL251, and CL262. Additionally, four repeats were found in the nucleotide sequence of the *P. tauri* genome (CL67, CL89, CL185, and CL192), as well as in the *P. kosaninii* genome (CL3, CL115, CL220, and CL300). Furthermore, one repeat was found in each of the genomes of *P. libanotica* (CL95), *P. geniculata* (CL134), *P. cognata* (CL186), and a tetraploid *P. spicata* (CL190).

The characteristics of the identified satellite including its length and the most similar sequences in the NCBI database are presented in Supplementary Material 1. After comparing the nucleotide sequences of the 22 repeats with those previously published in the NCBI, we did not find any matches for nine of them (CL69, CL89, CL95, CL168, CL185, CL207, CL251, CL262, CL300). For the remaining 13, the level of identity among similar published sequences ranged from 70 to 98 %. This indicates that they are different from previously published sequences (see Supplementary Material 1).

Two satellite repeats showed similarities to repeats found in common wheat: CL119 was similar to the pTa-465 clone (77 % identity), and CL101 was similar to the Spelt1-like subtelomeric repeat (80 % identity). Three repeats showed similarities to the following known satellites: CL220 to CL219, which was detected in the *Ae. crassa* genome (82 %), CL134 to CL97 from the *Th. bessarabicum* genome (71 %)



Relative copy number of the satellite repeats in the studied species of the Triticeae tribe expressed as a decimal logarithm.

and CL186 to ACRI_TR_CL80 from the *A. cristatum* genome (70 %). The other three repeats show similarities to microsatellites: CL128 has similaritiy to L15 identified in the *P. stipifolia* genome (84 %), CL190 shows similarity to P523 from the genome of *Ae. tauschii* (81 %), and CL82, to pTa-451 from common wheat (88 %). Four repeats we found showed similarity to the following mobile elements: CL184, which has a 98 % similarity to the *Cassandra* retrotransposon from the rye genome, CL67 and CL115, which have a 91 and 78 % similarity to retrotransposons from the barley genome *Cereba* and *Sandra5*, respectively, and CL192, which has a similarity to transposon XJ from the *Ae. tauschii* genome (70 %). The CL3 repeat was most similar to the E-gene-specific marker *Th. elongatum* 51-6 (79 %).

Assessment of the copy number of satellite repeats using qPCR

The data obtained on the relative copy numbers of 23 satellite repeats in 14 species, calculated in relation to the reference single-copy *VRN1* gene, are shown in Supplementary Material 2.1. All of the repeats we studied differed in terms of copy numbers and the coefficient of variation between the species. Since the order of the obtained copy numbers varied significantly, the results were presented in the form of a decimal logarithm for the convenience of comparing their abundance (see the Figure and Supplementary Material 2.2). Hereafter, we will simply refer to the decimal logarithm of relative copy number as "copy number". Since the copy number rate varied from 0 to 5, the repetitions were classified into the following groups based on their copy number: low (≤ 2), medium (> 2, < 4), and high (≥ 4). Since the coefficient of variation ranged from 0 to 0.6, we assumed that the variability was low when its value was less than 0.1, medium when it fell between 0.1 and 0.25, and high when it exceeded 0.25.

CL82 turned out to be the least variable repeat: its abundance was medium-closer to high and ranged from 2.9 to 3.8.

The medium coefficient of variation for the copy number of satellite repeats in all studied accessions (from 0.16 to 0.25) was observed in nine specific repeats, namely CL67, CL3, CL185, CL119, CL192, CL89, CL115, CL95, and CL168 (listed in ascending order based on their coefficient of variation). The average copy number values in *Pseudoroegneria* species were 2–11 % higher compared to the entire studied collection. However, CL67 had the highest copy number in the rye genome (3.2). CL89 showed the highest value in *P. libanotica* (2.8), CL3, CL119, and CL115 had the highest values in *P. kosaninii* (2.7, 3.1, and 2.7, respectively), CL95 had the highest value in both *P. cognata* and *P. kosaninii* (2.1), and CL168 had the highest value in *P. cognata* (2.9). The remaining repeats of this group were generally characterized by a low-to-medium level of copy number across all accessions. The minimum copy number was observed in CL192, ranging from 1.2 to 2.5, while the maximum copy number was found in CL185, ranging from 1.8 to 3.2.

A high level of variability was observed in the following 13 repeats: CL190, CL184, CL300, CL128, CL207, CL69, CL220, CL101, CL262, CL186, CL134, CL251, and CL244. The coefficient of variation ranged from 0.27 to 0.43. In this group, the following repeats can be distinguished: CL69 with a high copy number in *Pseudoroegneria* species (4.0–5.3), medium in *Thinopyrum* and *E. pendulinus* (2.8–3.0), and low in the rest; CL101 with a medium copy number in *Pseudoroegneria* (2.0–3.9) and *Th. junceum* (2.8), low in the rest; CL244, close-to-high in *Th. bessarabicum* (3.9), significantly varies in *Pseudoroegneria* species (0–2.4), and is medium to low in the others. CL184 is the highest in *P. cognata* (2.9) and *S. cereale* (3.0) compared to the others (0.5–2.3). Individual repeats showed the highest copy number in specific *Pseudo-*

roegneria species: CL128 and CL186 in *P. cognata* (2.5 and 2.6, respectively), CL190 and CL220 in *P. kosaninii* (2.5 and 3.0, respectively), CL134 in *P. geniculata* (3.1), CL207 and CL300 in *P. cognata* and *P. kosaninii* (ranging from 3.1 to 3.2). CL251 and CL262 were characterized by an overall low copy rate, ranging between 0.3–1.7 and 0–1.4, respectively.

Correlation analysis (see Supplementary Material 2.3) and principal component analysis (see Supplementary Material 2.4) were used to identify the following groups with similar repeat copy number patterns: 1) CL3, CL115, CL119, CL190, and CL220 ($r \geq 0.77$); 2) CL95, CL207, and CL300 ($r \geq 0.87$); 3) CL128, CL168, and CL186 ($r \geq 0.72$). Correlation analysis (see Supplementary Material 2.5) and principal component analysis (see Supplementary Material 2.6) revealed a high level of similarity in the species copy number patterns among the following groups: 1) *Pseudoroegneria* accessions ($r > 0.9$); 2) *E. pendulinus*, *Th. intermedium* and *Th. junceum* ($r > 0.8$); 3) rye, common wheat, *E. pendulinus*, *Th. junceum*, and *Ae. tauschii* ($r \geq 0.89$). The medium level of similarity in the species copy number pattern was observed between *Th. intermedium* and *Pseudoroegneria* ($r > 0.6$). The species copy number pattern in *Th. bessarabicum*, on average, showed the least similarity with other species.

Discussion

Satellite repeats constitute a significant portion of the Triticeae genome and play a crucial role in the formation and evolution of new species. As a consequence, they serve as valuable tools for analyzing these processes (Shcherban, 2015; Salina, Adonina, 2019; Vershinin et al., 2023). The search for new satellite repeats is necessary to understand the phylogenetic relationships and evolution of the Triticeae tribe, which is of significant importance to humans. One of the initial steps in determining the suitability of the identified satellite repeats as tools for such studies is to conduct a comparative assessment of their copy number in related species.

Some of the satellite repeats we found in the St-genome showed a similar copy number among the studied species. Homologs have been found in the genomes of wheat and barley, suggesting their common origin. CL82 and CL119 showed similarity to pTa-451 and pTa-465, respectively, which were identified in *T. aestivum* (Komura et al., 2013). CL67 is similar to the centromeric retrotransposon *Cereba* (Hudakova et al., 2001) and is conserved in Triticeae (Dvořák, 2009). Although CL3 is 79 % identical to the E-specific repeat 51-6, it did not show any specificity for *Thinopyrum* species in our study. This suggests that we have discovered an older and less genome-specific variant.

CL69 was distinguished by a high copy number in *Pseudoroegneria* accessions and a medium copy number in *Thinopyrum* and *E. pendulinus* species. This may indicate its occurrence before the divergence of the St- and J-genomes. CL101 has a medium copy number in *Pseudoroegneria* and *Th. junceum* species and could also occur in a common ancestor of the St- and J-genomes. Since CL101 is 80 % identical to the subtelomeric Spelt1-like repeat, it is likely that it may have a common origin with Spelt-1, which is common in *Triticum* and *Aegilops*. This repeat is characterized by significant variation in copy number between species (Pestsova et al., 1998; Ruban, Badaeva, 2018). The copy number of CL69 and CL101 in individual accessions is relatively high, reaching values of up to 3.9 and 5.3, respectively. This makes them suitable candidates for use as chromosomal markers in the FISH procedure. Further experiments using the FISH method will show whether these repeats can serve as chromosomal markers for identifying the St-subgenome in polyploid species, such as *E. pendulinus*, studying recombinant J^{vs}-genomes in intermediate wheatgrass and investigating chromosomal rearrangements in wide wheat hybrids.

The highest copy number value in *P. cognata* and *S. cereale* was demonstrated by CL184, which shows similarity to the *Cassandra* retrotransposon found in the rye genome. *Cassandra* is found in the genomes of many plant species and is characterized by significant differences in copy number between them (Kalendar et al., 2020). Since one of the mechanisms by which satellite repeats are propagated throughout the genome is through the movement of retroelements (Garrido-Ramos, 2021; Šatović-Vukšić, Plohl, 2023), it is possible that we have identified a repeat that has been retained as a consequence of *Cassandra* spreading along the ancestral lineage of the St- and R-genomes.

CL244, previously identified by us in the *Ae. crassa* genome, was characterized by a higher copy number in *Th. bessarabicum* than in common wheat, *Ae. crassa* and *Ae. tauschii* (Kroupin et al., 2022). In this study, the results were confirmed. However, at the same time, there was a significant variation in copy number between *Pseudoreogneria* species, which could be attributed to the elimination or accumulation of CL244 during speciation and subsequent evolution. CL244 has terminal localization on chromosome *Th. bessarabicum* (Kroupin et al., 2022), and can presumably accumulate or be eliminated in various species, similar to the terminal repeats of Spelt-1 and Spelt-52 in *Aegilops* and *Triticum* (Raskina et al., 2011; Ruban, Badaeva, 2018) or pSc200 and pSc250 in rye (Evtushenko et al., 2016).

CL220, which is specific to *P. kosaninii*, exhibited similarities with CL219, which we had previously identified in the *Ae. crassa* genome (Kroupin et al., 2022). CL186, specific to *P. cognata*, showed similarity to ACRI_CL80, which was identified in *A. cristatum* (P-genome) (Said et al., 2018). Both repeats probably arose before the divergence of Triticeae genomes from a common ancestor and accumulated in separate species at certain periods. Since CL219 and ACRI_CL80 were localized on separate chromosomes, it can be inferred that CL220 and CL186 will also exhibit chromosome-specific localization on the chromosomes of *P. kosaninii* and *P. cognata*, respectively.

We have identified repeats that vary in copy number between *Pseudoroegneria* species with varying levels of ploidy. For example, the octaploid *P. kosaninii* is characterized by a high copy number of CL115, CL190, and CL220, while the tetraploid *P. geniculata* has a high copy number of CL134. The observed differences in the abundance of the repeats may be attributed to polyploidization processes. This is because tandem repeats in the centromeric and terminal regions have a significant impact on chromosome recognition and divergence

during cell division. This is particularly relevant for homeological genomes in polyploid plants (March, 2019; Aguilar, Prieto, 2021). Such subgenome- and even chromosome-specific repetitive elements have been detected in polyploid species such as wheat, oats, and intermedium wheatgrass. These elements are apparently necessary for the differentiation of subgenomes during cell division (Shrama, Raina, 2005; Liu Z. et al., 2008; Divashuk et al., 2016; Lang et al., 2019b; Su et al., 2019).

Comparison of the repeat copy number patterns helped determine which of them have similar copy numbers among the studied accessions (see Supplementary Materials 2.3 and 2.4). CL3, CL115, CL119, CL190, and CL220 were grouped together because they exhibited the highest levels of copy number in *P. kosaninii*. CL95, CL207, and CL300 are more specific to *P. cognata* and *P. kosaninii*. In CL128, CL168, and CL186, the maximum copy number was observed in *P. cognata*. A comparison of the species copy number patterns allowed for a general understanding of which accessions are characterized by similar repeat copy numbers.

The overall clustering of *Pseudoroegneria* species (see Supplementary Materials 2.5 and 2.6) indicates that, in general, they exhibit similar copy numbers of repeats that are different from those of other species. The copy number pattern of *E. pendulinus* ($2n = 28$, StStYY) differed from that of *Pseudoroegneria*, suggesting that the St-specific repeats we discovered could be valuable for analyzing the St-subgenome of *E. pendulinus* and other *Elymus sensu lato* accessions. *Thinopyrum* and common wheat exhibited different copy number patterns. CL244 and CL69 can be utilized to identify wheat-wheatgrass hybrids and detect introgressions from all three studied wheatgrass species into the wheat genome. Similarly, CL134 and CL251 can be used for this purpose in *Th. junceum*.

L. Wang et al. (2017) found a repeat of St_2-80 in the genome of *P. libanotica*, hybridizing along the entire length of the St-(sub)genome chromosomes and only in the terminal regions of the E-, H-, P-, and Y-(sub)genomes. Q.-L. Liu et al. (2020) identified mobile elements in the genome of *P. stipifolia*, including dispersed repeats S13, S158, and S21, which showed varying intensity between the chromosomes of the St- and Y-subgenomes. They also found S5, which had a specific point localization and differed between the chromosomes of the St- and Y-subgenomes. D. Wu et al. (2022) created chromosomal markers STlib_96, STlib_98, and STlib_117 based on satellite repeats of *P. libanotica*. However, the possibility of using these markers for the analysis of allopolyploids with the St-genome is not reported.

Our assessment of the copy number of satellite repeats found in the St-genome and the determination of their amplification specificity between species can enhance the range of molecular genetic and cytogenetic markers utilized in studying the Triticeae tribe. The copy number of satellite repeats can vary significantly between species, populations, and even within them (Wang Q. et al., 2010; Belyaev, Raskina, 2013; Pollak et al., 2018; Tao et al., 2021). The satellite repeats identified in the present study can be useful, among other purposes, for population studies of *Pseudoroegneria* and Triticeae species.

Conclusion

Based on the data from whole-genome sequencing of *Pseudoroegneria* accessions, we identified 22 satellite repeats. In the genomes of 14 representatives of the Triticeae tribe, we determined their copy number, including CL244, which was previously discovered in *Ae. crassa*. As a result of the evaluation, the studied repeats were classified according to the level of abundance and variability between species. The satellite repeats identified in the present study can be used as molecular genetic markers for evolutionary, phylogenetic, and population studies of Triticeae. They also have the potential to serve as cytogenetic markers for *in situ* hybridization.

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ORCID

P.Yu. Kroupin orcid.org/0000-0001-6858-3941
A.I. Yurkina orcid.org/0000-0002-1527-0658
A.A. Kocheshkova orcid.org/0000-0003-1924-6708
D.S. Ulyanov orcid.org/0000-0002-5880-5931
G.I. Karlov orcid.org/0000-0002-9016-103X
M.G. Divashuk orcid.org/0000-0001-6221-3659

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Molecular-genetic and cytogenetic analyses of cotton chromosome introgression from *Gossypium barbadense* L. into the genome of *G. hirsutum* L. in BC_2F_1 hybrids

M.F. Sanamyan[1] ✉, Sh.U. Bobokhujayev[1], Sh.S. Abdukarimov[2], O.G. Silkova[3]

[1] National University of Uzbekistan named after Mirzo Ulugbek, Tashkent, Uzbekistan
[2] Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan, Tashkent, Uzbekistan
[3] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
✉ sanam_marina@rambler.ru

Abstract. Substitution lines of the cotton *Gossypium hirsutum* L. involving chromosomes of the tetraploid species *G. barbadense* L., *G. tomentosum* Nutt. ex Seem., and *G. mustelinum* Miers ex Watt. are a valuable source for breeding, increasing the genetic diversity of *G. hirsutum*. The substitution of certain *G. hirsutum* L. chromosomes with *G. barbadense* chromosomes affect fibre elongation, fibre yield, fibre strength, and micronaire. To increase the efficiency of creating lines, it is necessary to study the nature of the introgression of alien chromosomes into the *G. hirsutum* L. genome. As a result of molecular genetic analysis of BC_2F_1 hybrids obtained from crossing monosomic lines of the cotton *G. hirsutum* from the cytogenetic collection of Uzbekistan with monosomic backcross hybrids BC_1F_1 *G. hirsutum* × *G. barbadense* on the same chromosomes, genetic differences between the hybrids in the profile of chromosome-specific microsatellite SSR markers were found. The predominant introgression of chromosomes 4, 6 and 12 of the A_t-subgenome and 22 of the D_t-subgenome of *G. barbadense* was revealed, while chromosomes 2 and 7 of the A_t-subgenome and 18 of the D_t-subgenome of *G. barbadense* were characterized by elimination. Among them, chromosomes 7 of the A_t-subgenome and 18 of the D_t-subgenome of *G. barbadense* were eliminated in the first backcross generation. In this work, two lines, CS-B06 and CS-B07, from the American cytogenetic collection with a putative substitution involving chromosomes 6 and 7 of the A_t-subgenome were analysed. The presence of only polymorphic alleles from the species *G. hirsutum* and the absence of polymorphic alleles from the species *G. barbadense* were revealed, which showed the absence of substitution involving these chromosomes. BC_2F_1 hybrids with monosomy for both *G. barbadense* and *G. hirsutum* chromosomes were characterized by regular pairing of chromosomes and high meiotic indexes. However, many hybrids were characterized by a decrease in pollen fertility. Two hybrids with monosomy for chromosome 7 of the A_t-subgenome of *G. hirsutum* and chromosome 6 of the A_t-subgenome of *G. barbadense* had the greatest reduction in pollen viability (70.09 ± 1.57 and 75.00 ± 1.66 %, respectively). Thus, this work shows a specific feature in the introgression of individual chromosomes of the cotton species *G. barbadense* into the cotton *G. hirsutum* genome.
Key words: cotton; *Gossypium hirsutum*; *G. barbadense*; monosomic lines; chromosome-substituted hybrids; molecular genetic analysis.

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Молекулярно-генетический и цитогенетический анализ интрогрессии хромосом хлопчатника *Gossypium barbadense* L. в геном *G. hirsutum* L. у гибридов BC_2F_1

М.Ф. Санамьян[1] ✉, Ш.У. Бобохужаев[1], Ш.С. Абдукаримов[2], О.Г. Силкова[3]

[1] Национальный университет Узбекистана им. Мирзо Улугбека, Ташкент, Узбекистан
[2] Центр геномики и биоинформатики Академии наук Республики Узбекистан, Ташкент, Узбекистан
[3] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
✉ sanam_marina@rambler.ru

Аннотация. Линии хлопчатника *Gossypium hirsutum* L. с чужеродным замещением хромосом тетраплоидных видов *G. barbadense* L., *G. tomentosum* Nutt. ex Seem., *G. mustelinum* Miers ex Watt. являются ценным источником для селекции, увеличивающим генетическое разнообразие *G. hirsutum*. Замещение определенных хромосом хлопчатника вида *G. hirsutum* L. хромосомами вида *G. barbadense* оказывает влияние на удлинение, выход и прочность волокна, микронейр. Для повышения эффективности процесса создания линий необходимо изучение характера

интрогрессии чужеродных хромосом в геном *G. hirsutum* L. В результате молекулярно-генетического анализа гибридов BC_2F_1, полученных от скрещиваний моносомных линий хлопчатника *G. hirsutum* цитогенетической коллекции Узбекистана с моносомными беккроссными гибридами BC_1F_1 *G. hirsutum* × *G. barbadense* по одинаковым хромосомам, обнаружены генетические различия по профилю хромосом-специфичных микросателлитных SSR-маркеров между гибридами. Выявлена преимущественная интрогрессия хромосом 4, 6, 12 A_t-субгенома и 22 D_t-субгенома *G. barbadense*, тогда как хромосомы 2, 7 A_t-субгенома и 18 D_t-субгенома *G. barbadense* характеризовались элиминацией, среди них хромосомы 7 A_t-субгенома и 18 D_t-субгенома *G. barbadense* элиминировали уже в первом беккроссном поколении. В настоящей работе проанализированы две линии, CS-B06 и CS-B07, американской цитогенетической коллекции с предполагаемым замещением по хромосомам 6 и 7 A_t-субгенома. Обнаружены присутствие только полиморфных аллелей вида *G. hirsutum* и отсутствие полиморфных аллелей вида *G. barbadense*, что показало отсутствие замещения по этим хромосомам. Гибриды BC_2F_1 с моносомией как по хромосомам *G. barbadense*, так и по хромосомам *G. hirsutum* характеризовались регулярной конъюгацией хромосом и высоким мейотическим индексом. Однако многие гибриды отличались снижением фертильности пыльцы. Два гибрида с моносомией по хромосоме 7 A_t-субгенома *G. hirsutum* и хромосоме 6 A_t-субгенома *G. barbadense* имели наибольшую редукцию в жизнеспособности пыльцы (70.09 ± 1.57 и 75.00 ± 1.66 % соответственно). Таким образом, в этой работе показана особенность в интрогрессии индивидуальных хромосом хлопчатника вида *G. barbadense* в геном хлопчатника *G. hirsutum*.

Ключевые слова: хлопчатник; *Gossypium hirsutum*; *G. barbadense*; моносомные линии; хромосомно-замещенные гибриды; молекулярно-генетический анализ.

Introduction

Currently, four species of cotton are grown commercially worldwide, of which two species, *Gossypium herbaceum* L. (A_1-genome) and *G. arboreum* L. (A_2-genome), are diploids, and the other two species, *G. hirsutum* L. (AD_1-genome) and *G. barbadense* L. (AD_2-genome), are tetraploids (Wendel et al., 2009). The cotton plant *G. hirsutum* is a major crop that accounts for more than 90 % of the world's cotton crop (International Cotton Advisory Committee-ICAC-2019).

Global cotton consumption has shown a steady increase of 80 % between 1980/1981 and 2020/2021 (International Cotton Advisory Committee-ICAC-2021), requiring improvements in cotton yields and fibre quality. An increase in cotton yield was achieved through the creation of transgenic varieties, traditional selection, and intervarietal crossing. However, most of these varieties were obtained through selection from a narrow genotypic environment and adapted to certain soil and climatic conditions (International Cotton Advisory Committee-ICAC-2021). Thus, today, there is a reduction in genetic diversity in cultivated cotton, which causes a decrease in fibre quality and increased vulnerability to stress factors due to the close relatedness of high-yielding varieties.

Enrichment of the *G. hirsutum* genome with alleles of economically valuable genes from other cotton species is very important (Grover et al., 2022). For example, *G. tomentosum* is characterized by heat resistance, and *G. mustelinum* and *G. stocksii* are resistant to pests and diseases. It is known that fine-fibre cotton of the *G. barbadense* species is less productive and has less adaptability to growing conditions but has fibre properties that are significantly superior in quality (length, strength and fibre fineness) to the cultivated *G. hirsutum* varieties, although the latter is more productive. Given their complementary economically valuable traits, numerous attempts have been made to hybridize these two species through traditional breeding (Anwar et al., 2022). However, the interspecific hybrids had poor agronomically valuable traits, and the hybrids were characterized by limited recombination due to genomic incompatibility caused by large inversions on different chromosomes of the two subgenomes of the tetraploid species. Typically, F_1 hybrids of *G. hirsutum* × *G. barbadense* are fertile, but the phenotypes of F_2 and subsequent generations are biased towards one of their parents due to pollen sterility, suppression of crossing over, selective gene elimination and segregation failure (Zhang et al., 2014; Si et al., 2017; Fang et al., 2023).

Obtaining forms with chromosome substitution (CS) in various plant species allows for targeted introgression of specific chromosomes or arms of individual chromosomes, which represent a valuable source of new alleles of useful genes. Previously, such forms were created in many crops, which made it possible to improve some agronomic traits (Shchapova, Kravtsova, 1982; Silkova et al., 2006, 2007; Schneider et al., 2008; Tiwari et al., 2010; Rawat et al., 2011).

For a number of years, in cotton in the USA, research has been carried out to obtain lines with alien chromosome substitutions involving three tetraploid species (*G. barbadense*, *G. tomentosum*, *G. mustelinum*), and with the participation of the *G. barbadense* species, 20 lines with substitutions of individual chromosomes have already been obtained (Saha et al., 2006, 2013, 2015). The obtained lines made it possible to determine that the substitution of certain chromosomes of the cotton species *G. hirsutum* L. with chromosomes of the species *G. barbadense* L. (CS-B02, CS-B04, CS-B16, CS-B17, CS-B22Lo, CS-B22sh, CS-B25) has an effect on fibre elongation, fibre yield, fibre strength, micronaire, etc., in comparison with the original lines TM-1 and Pima 3-79 (Saha et al., 2004). Such lines have been shown to be an important breeding source that increases the genetic diversity of *G. hirsutum* L. (Jenkins et al., 2006, 2007).

Previously, monosomic lines of the Cytogenetic Collection of Cotton of Uzbekistan (CCCU), created in the genotypic environment of the highly inbred line L-458 of the species *G. hirsutum* L. (Sanamyan et al., 2014), with identified monosomy on chromosomes 2, 4, 6, 7, 12 of the A_t-subgenome and 17, 18, 21, 22 of the D_t-subgenome, as well as two lines with monosomy on telocentrics 6 and 11 of the A_t-subgenome

(Sanamyan et al., 2016a, b; Sanamyan, Bobokhujayev, 2019), were used in crossings with the Pima 3-79 line of the *G. barbadense* species, as well as in crossings with F_1 hybrids, to obtain aneuploid hybrids BC_1F_1 and subsequently to create cotton lines with chromosome substitution. The work used double screening of hybrids at all stages of backcrossing using molecular genetic markers and cytogenetic analysis (Sanamyan et al., 2022). The first stage of the study consisted of a molecular genetic analysis of hybrid plants at the seedling stage to quickly identify aneuploid forms with or without chromosome substitutions or their arms. At the second stage, a cytogenetic analysis of meiosis in hybrids at the stages of metaphase I and telophase II was carried out, and pollen fertility when stained with acetocarmine was studied to confirm the monosomic status of backcross hybrid plants and identify their peculiarities in the behavior of chromosomes.

The purpose of this work was to conduct a molecular genetic and cytogenetic study of BC_2F_1 hybrids from crosses of monosomic cotton lines of the CCCU with monosomic backcross hybrids BC_1F_1 and to elucidate the features of introgression of individual chromosomes of the cotton species *G. barbadense* into the genome of the cotton species *G. hirsutum*. In the course of this work, at the seedling stage, using molecular genetic markers (SSR), aneuploid forms were identified among BC_2F_1 hybrids, in which the substitution of chromosomes 4, 6, and 12 of the A_t-subgenome and chromosome 22 of the D_t-subgenome and the elimination of chromosomes 2 and 7 of the A_t-subgenome and 18 D_t-subgenome with *G. barbadense* were confirmed. In aneuploids BC_2F_1, the behavior of individual chromosomes of *G. hirsutum* and *G. barbadense* in meiosis was studied, and the meiotic index and pollen fertility were assessed. The promise of using molecular genetic markers at the seedling stage for accelerated selection of plants with alien substitution of individual *G. hirsutum*/*G. barbadense* chromosomes in the BC_2F_1 generation has been shown.

Materials and methods

Plant material. Monosomic and monotelosomics lines of CCCU were created in a single genotypic environment of the highly inbred line L-458 of *G. hirsutum*, obtained by M.F. Abzalov and G.N. Fatkhullaeva as a result of long-term self-pollination (F_{20}) based on variety 108-F. To create the collection, various methods were used to irradiate seeds and pollen, as well as the progeny of plants with translocations and desynapsis (Table 1) (Sanamyan, 2020). The Pima 3-79 line of the *G. barbadense* species is not sensitive to photoperiod and is highly homozygous, as it originates from a doubled haploid (Endrizzi et al., 1985). This line is the genetic standard for the species *G. barbadense* L. in the USA (Hulse-Kemp et al., 2015) and has therefore been used as the donor parent of the substituted chromosome (CS) or chromosome segments from *G. barbadense*, both in the USA and in Uzbekistan.

To obtain backcross hybrids BC_2F_1, monosomic lines on chromosomes 2, 4, 6, 7, and 12 of the A_t-subgenome and 18 and 22 of the D_t-subgenome were backcrossed with monosomic hybrids BC_1F_1(Mo × F_1(Mo × Pima 3-79)), and a monotelosomic line lacking one of the arms of chromosome 11 was backcrossed with the monotelosomal hybrid BC_1F_1(Telo × F_1(Telo × Pima 3-79)), in which monosomy and monotelosomy were on the same chromosomes as in the original aneuploids of *G. hirsutum*. All plants of the original lines and hybrids of different generations were kept year-round in the greenhouse of the National University of Uzbekistan.

Cytological tests. The behavior of chromosomes was studied in the pollen mother cells (PMCs) at the stage of metaphase I (MI) and tetrads of meiosis. For this, 2–3 mm buds in the ethyl-acetic acid mixture (7:3) were fixed. Then, the PMCs were painted with iron-acetocarmine. At temporary squashed slides at the MI stage, the nature of the pairing of chromosomes was taken into account. To analyse the stage of the tetrads, three buds were analysed from each plant, and the percentage of normal tetrads was calculated from their total number. To analyse the fertility of pollen, in the morning on the day of flowering, the opened flowers were collected, and temporary acetocarmine slides were prepared, which were laid in Petri's cups and left in the refrigerator for a day to better paint the pollen grains. Then, 10 fields of vision from each flower were analysed.

All cytological observations were carried out using microscopes, AxioScopeA1, Laboval (Carl Zeiss, Germany) and Biomed (Leica, Switzerland) with an increase in lenses of 10x, 100x, binocular nozzle of 1.6x and GF 12.5 × 120 and a 10x eyepiece. Microphotography was performed using a Mikroskopkamera AxioCamERc5s digital camera. During exhibiting, the green filter 3C-11-3 was used. Statistical processing of the received data was carried out in accordance with B.A. Dospekhov (1985).

DNA extraction and genotyping. Genomic DNA was distinguished from samples of young leaves of cytogenetically identified backcross aneuploid hybrids BC_2F_1 and young seedlings of hybrid plants (BC_2F_1) by CTAB (Saha et al., 2015). Genomic DNA was checked using electrophoresis of 0.9 % agarose, and DNA was diluted in 15 µl to a working concentration using a control solution of HindIII-extensible DNA λ-fag (25 ng/µl). The PCR amplification was carried out in 10 µl of the reaction mix containing 1.0 µl of 10-fold PCR buffer (with 25 mm $MgCl_2$), 0.2 µl BSA, 0.08 µl dNTPs (25 mm), 0.2 µl of primers 0.1 µl Taq-polymerase, and 2 µl of DNA template. PCR runs were conducted with an initial DNA denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C (step 1) for 20 s, 55 °C (step 2) for 30 s and 72 °C (step 2, step 3) for 50 s. After 35 cycles, the extension temperature of 72 °C was held for 7 min. The PCR products were visualized in a 3.5 % high-resolution agarose gel, stained with bromide ethidium and photodocumented using an Alpha Imager gel documentation system (Innotech Inc., USA).

The pairs of primers to the codominant chromosome-specific SSR markers were synthesized in accordance with genetic mapping (Dellaporta et al., 1983; Gutiérrez et al., 2009; Saha et al., 2015; Reddy et al., 2020), which are listed in Supplementary Material 1[1]. For each chromosome, an average of four loci polymorphic between L-458 (*G. hirsutum*) and Pima 3-79 (*G. barbadense*) were selected. The results of the electropherogram for the SSR were evaluated as a/b/h, where the a locus corresponded to the recipient L-458, the b locus corresponded

[1] Supplementary Materials 1–13 are available https://vavilov.elpub.ru/jour/manager/files/Suppl_Sanamyan_Engl_27_8.pdf

Table 1. Monosomic and monotelosomal lines of cotton *G. hirsutum* L. cytogenetic collection of Uzbekistan

No.	Catalog number	Monosomy symbol	Origin	Line name	Chromosome monosomy	Univalent size
1	233	Mo16	Obtained in M_1 after pollination with gamma-irradiated pollen of line L-458 (dose 25 Gy)	Monosomic line Mo16	2 A_t-subgenome	Large
2	255	Mo38	Obtained in M_2 after pollination with gamma-irradiated pollen of the L-458 line (dose 25 Gy)	Monosomic line Mo38	4 A_t-subgenome	Medium
3	275	Mo58	Obtained from the offspring of a desynaptic in M_2 after irradiation of seeds of the L-458 line with thermal neutrons (dose 15 Gy)	Monosomic line Mo58		Medium
4	276	Mo59	Obtained from the offspring of a desynaptic in M_2 after irradiation of seeds of the L-458 line with thermal neutrons (dose 15 Gy)	Monosomic line Mo59		Medium
5	277	Mo60	Obtained from the offspring of a desynaptic in M_2 after irradiation of seeds of the L-458 line with thermal neutrons (dose 15 Gy)	Monosomic line Mo60		Medium
6	292	Mo75	Obtained in M_2 after pollination with gamma-irradiated pollen of the L-458 line (dose 20 Gy)	Monosomic line Mo75		Medium
7	251	Mo34	Obtained in M_2 after pollination with gamma-irradiated pollen of the L-458 line (dose 20 Gy)	Monosomic line Mo34	6 A_t-subgenome	Large
8	309	Mo92	Obtained in M_3 after irradiation of seeds of the L-458 line with thermal neutrons (dose 27 Gy)	Monosomic line Mo92		Large
9	244	Mo27	Obtained in M_2 after pollination with gamma-irradiated pollen of the L-458 line (dose 20 Gy)	Monosomic line Mo27	7 A_t-subgenome	Medium
10	311	Mo94	Obtained in M_3 after pollination with gamma-irradiated pollen of the L-458 line (dose 20 Gy)	Monosomic line Mo94	12 A_t-subgenome	Large
11	265	Mo48	Obtained in M_1 after pollination with gamma-irradiated pollen of the L-458 line (dose 25 Gy)	Monosomic line Mo48	18 D_t-subgenome	Medium
12	234	Mo17	Obtained in M_1 after pollination with gamma-irradiated pollen of the L-458 line (dose 25 Gy)	Monosomic line Mo17	22 D_t-subgenome	Medium-small
13	238	Telo21	Isolated in the progeny of a monosomy obtained in M_1 after pollination with gamma-irradiated pollen of the L-458 line (dose 15 Gy)	Telosome line Telo21	Telo11 A_t-subgenome	Heteromorphic bivalent

to the Pima 3-79 donor line, and the h genotype corresponded to the BC_1F_1 and BC_2F_1 disomic hybrid. The elimination of the chromosomes of *G. hirsutum* in the monosomic hybrid of cotton BC_1F_1 and BC_2F_1 was determined by the lack of marker amplification by chromosomes of *G. hirsutum* (maternal) and the presence of only allele-specific products of PCR of *G. barbadense* (paternal) (Liu et al., 2000). For all types of substitutions of individual chromosomes as controls, DNA of chromosome-substitution lines of the American cytogenetic collection was used, with the exception of chromosome 2.

Results

Identification of substitutions of chromosomes *G. barbadense*/*G. hirsutum* in BC_2F_1 hybrids using chromosome-specific molecular genetic markers

According to the previously developed scheme (Sanamyan et al., 2022), the molecular genetic analysis of BC_2F_1 plants was carried out at the seedling stage before they were transplanted into the soil of the greenhouses to accelerate the release of monosomics through chromosomes of donor species to sepa-

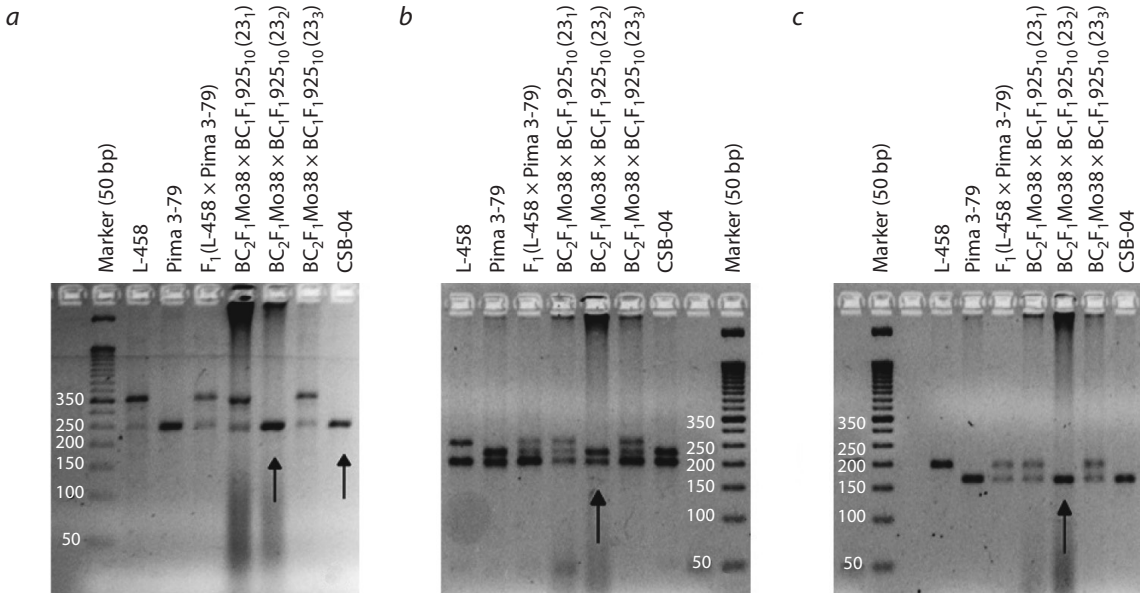

Fig. 1. Electrophoregram of the DNA amplicons of SSR markers in hybrid seedlings of BC_2F_1(Mo38 × $BC_1F_1925_{10}$) according to chromosome 4 of the A_t-subgenome: *a* – Gh107; *b* – Gh117; *c* – TMB0809.

rate their molecular markers from plants with chromosomes of the recipient species. Since most of the monosomics were identified earlier, only two crossing variants, BC_2F_1(Mo16 × $BC_1F_1(923_7)$ and BC_2F_1(Mo38 × $BC_1F_1(925_{10})$), were analysed at the seedling stage.

The results of the analysis were discovered by five monosomics (21_1, 21_2, 21_4, 21_7 and 22_1, where the numbers indicate sowing plant numbers) in two families (21*n* and 22*n*, where the numbers indicate the sowing numbers of the families), and the letter "*n*" for a different number of plants in the BC_2F_1 (Mo16 × BC_1F_1 (923_7)) variant, where there was supposed to be a substitution of chromosome 2 of the A_t-subgenome. These plants were characterized by the presence of chromosome-specific alleles only from the L-458 line *G. hirsutum*, while the *G. barbadense* alleles were absent. Since earlier the chromosome-specific SSR markers BNL834, BNL3971, TMB0471, and JESPR179 had been localized on chromosome 2 of the A_t-subgenome of cotton (Gutiérrez et al., 2009; Lacape et al., 2009) (see Supplementary Materials 1–3), the data obtained indicated the lack of chromosome 2 substitution in all five backcross seedlings in BC_2F_1(Mo16 × $BC_1F_1923_7$), which was a negative result of this study, as it made it necessary to obtain further new hybrid background seeds and study the new BC_2F_1 hybrid offspring.

One seedling (23_2) with substitution of chromosome 4 was found in the BC_2F_1(Mo38 × $BC_1F_1925_{10}$). This hybrid was characterized by the presence of alleles only from *G. barbadense*, which was revealed upon receipt of PCR products as a result of amplification with four chromosome-specific SSR markers: BNL2572, GH107, GH117, and TMB0809 (Hoffman et al., 2007; Gutiérrez et al., 2009) (see Supplementary Materials 1, 2; Fig. 1).

Confirmation of chromosomal substitutions in the other 10 variants was carried out in previously cytogenetically studied BC_2F_1 monosomic hybrids. Analysis of monosomics with a putative substitution in chromosome 4 showed amplification of five allele-specific PCR products of SSR markers TMB0809, Gh107, Gh117, CIR249, JESPR234 only for *G. barbadense* in monosomic (530_1) from the variant of BC_2F_1(Mo58 × $BC_1F_1115_1$), in two monosomics (284_1 and 284_{11}) in BC_2F_1(Mo59 × $BC_1F_11041_4$), in monosomic (494_3) from BC_2F_1(Mo60 × $BC_1F_1117_5$), and in monosomic (496_1) in BC_2F_1(Mo75 × $BC_1F_1298_2$) (see Supplementary Materials 1, 4, 5), which confirmed the substitution of the chromosomes in them.

Analysis of monosomic (497_4) in the BC_2F_1(Mo34 × BC_1F_1 (293_3)) variant and monosomic (499_2) in the BC_2F_1(Mo92 × $BC_1F_1(1040_2)$) variant with a putative substitution of chromosome 6 revealed alleles only from *G. barbadense*, while alleles of the *G. hirsutum* species were absent, based on the localization of 11 chromosome-specific SSR markers BNL1440, BNL3650, BNL2884, BNL1064, BNL3359, TMB1277, TMB0154, TMB0853, TMB1538, Gh039, and Gh082 (Gutiérrez et al., 2009) ((see Supplementary Materials 1, 5; Fig. 2), substitution of these chromosomes was confirmed.

The molecular genetic analysis of two monosomics (500_{11} and 500_{12}) from BC_2F_1(Mo27 × $BC_1F_1(111_2)$) defined only the alleles of the L-458 *G. hirsutum* line, while alleles of the *G. barbadense* species were absent. Before four chromosome-specific SSR markers, BNL1694, Gh146, TMB0180, and TMB0561, were localized on chromosome 7 of the A_t-subgenome (Hoffman et al., 2007; Guo et al., 2008; Gutiérrez et al., 2009; Saha et al., 2015) (see Supplementary Materials 5, 6), the data obtained indicated the lack of substitution of chromosome 7 in these two monosomics.

It must be emphasized that the substituted CS-B06 and CS-B07 lines of the American cytogenetic collection that served as control in our study were characterized by the lack

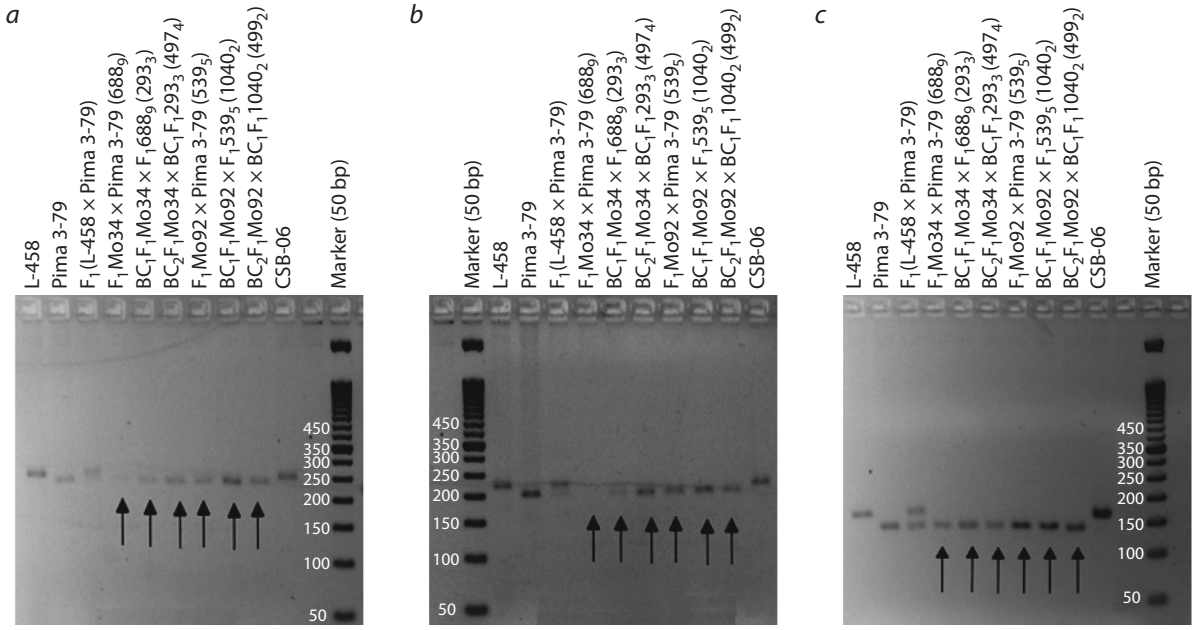

Fig. 2. Electrophoregram of SSR-marker DNA amplicons in hybrid monosomic plants BC_2F_1(Mo34 × $BC_1F_1(293_3)$) and BC_2F_1(Mo92 × $F_1BC_1(1040_2)$) according to chromosome 6 of the A_t-subgenome of cotton: *a* – TMB0853; *b* – TMB1538; *c* – Gh082.

of substitution of chromosomes 6 and 7 of cotton, since only those from the species of *G. hirsutum* were present, while those from the species *G. barbadense* were absent, as can be clearly seen in Fig. 2 and Supplementary Material 6, respectively. However, all other controls corresponded to the substitutions of the chromosomes by which the study was conducted.

In two monosomics (505_4 and 506_2) from the BC_2F_1 variant (Mo94 × $BC_1F_1299_1$), only chromosome 12 of the A_t-sub genome of *G. barbadense* was identified according to the PCR of the amplification of chromosome-specific SSR markers-BNL3261 and BNL3835 (Gutiérrez et al., 2009) (see Supplementary Materials 5, 7).

Analysis of monosomic (286_{14}) from the combination of BC_2F_1(Mo48 × $BC_1F_1114_{20}$) showed only alleles of chromosome 18 from *G. hirsutum*, while alleles of the species *G. barbadense* were absent. Since eight previously reported chromosome-specific SSR markers, namely, BNL193, BNL2544, BNL3280, BNL3479, CIR216, Gh142, TMB0114, and TMB1603, were localized on chromosome 18 of the D_t-subgenome (Reddy et al., 2020) (see Supplementary Materials 5, 8), the data indicated the lack of substitution of this chromosome.

The molecular-genetic SSR analysis of monosomic (288_1) from BC_2F_1(Mo17 × $BC_1F_1110_1$) showed the presence of only the allele from *G. barbadense*, while the allele of the *G. hirsutum* species was not found based on the localization of the chromosome-specific SSR marker BNL673. Since this marker was previously localized on chromosome 22 of the D_t-subgenome (Gutiérrez et al., 2009), the substitution of chromosome 22 was confirmed in the studied monosomic (see Supplementary Materials 5, 9).

The molecular genetic analysis of two telocentrics (790_2 and 791_1) from BC_2F_1(Telo21 × $BC_1F_1(292_1)$) showed conflicting data, possibly due to the localization of markers on different arms of chromosome 11. Therefore, the study of these monotelocentrics will be continued with the help of labelled primers since they show their more accurate localization.

Study of meiosis in BC_2F_1 hybrids with identified univalents

Analysis of the pairing of chromosomes at the MI meiosis stage revealed aneuploid plants in 12 variants of hybrid offspring obtained from the crosses of monosomic lines of the *G. hirsutum* species of the CCCU with monosomics of BC_1F_1. Therefore, two monosomics were isolated in each of the three backcrosses (with the participation of lines Mo59, Mo27 and Mo94), and one monosomic was allocated in each of the remaining nine backcross variants (with the participation of Mo16, Mo38, Mo58, Mo60, Mo75, Mo34, Mo92, Mo48 and Mo17) (Supplementary Material 10). Unfortunately, we were not able to continue research with four lines (Mo31, Mo56, Mo42 and Telo12), which were studied in the first backcross generation, due to the lack of setting of hybrid bolls.

Analysis of metaphase I meiosis in 15 BC_2F_1 monosomics, where four monosomics (21_1, 500_{11} and 500_{12}, 286_{14}) of three crossing variants with univalent chromosomes of *G. hirsutum* (2, 7 and 18) and 11 other monosomics of eight other variants with univalent chromosomes of *G. barbadense* (4, 6 and 12) found that the plants were characterized by a modal for monosomics of cotton pairing of chromosomes with 25 bivalents and one univalent (Table 2). One monosomic variant (288_1) from F_1BC_2(Mo17 × $F_1BC_1110_1$) with the substitution of chromosome of 22 of the D_t-subgenome was distinguished by the presence of additional univalents (1.94 ± 0.19 per cell), which could lead to the appearance of nullisomic gametes.

Table 2. Pairing of chromosomes at the stage of metaphase I meiosis in BC_2F_1, hybrids obtained from crossing recurrent parents with interspecific aneuploid hybrids of BC_1F_1(Mo × F_1Mo × Pima 3-79) or BC_1F_1(Telo × F_1Telo × Pima 3-79)

Chromosome	Crossing variant	Hybrid number	PMCs studied	Univalent size	Average per cell	
					univalents	bivalents
	L-458	–	11	–	0	26.00 ± 0.00
	Pima 3-79	–	12	–	0	26.00 ± 0.00
	F_1(L-458 × Pima 3-79)	680	10	–	0	26.00 ± 0.00
2	BC_2F_1(Mo16 × $BC_1F_1(923_7)$)	21_1	5	Large	1.00 ± 0.00	25.00 ± 0.00
4	BC_2F_1(Mo38 × $BC_1F_1(925_{11})$)	23_2	30	Medium	1.00 ± 0.00	25.00 ± 0.00
	BC_2F_1(Mo58 × $BC_1F_1(115_1)$)	530_1	30	Medium	1.00 ± 0.00	25.00 ± 0.00
	BC_2F_1(Mo59 × $BC_1F_1(1041_4)$)	284_1	26	Medium	1.00 ± 0.00	25.00 ± 0.00
		284_{11}	11	Medium	1.00 ± 0.00	25.00 ± 0.00
	BC_2F_1(Mo60 × $BC_1F_1(117_4)$)	494_3	15	Medium	1.00 ± 0.00	25.00 ± 0.00
	BC_2F_1(Mo75 × $BC_1F_1(298_2)$)	496_1	15	Medium	1.00 ± 0.00	25.00 ± 0.00
6	BC_2F_1(Mo34 × $BC_1F_1(293_3)$)	497_4	25	Large	1.00 ± 0.00	25.00 ± 0.00
	BC_2F_1(Mo92 × $BC_1F_1(1040_2)$)	499_2	18	Large	1.00 ± 0.00	25.00 ± 0.00
7	BC_2F_1(Mo27 × $BC_1F_1(111_2)$)	500_{11}	15	Medium	1.00 ± 0.00	25.00 ± 0.00
		500_{12}	13	Medium	1.00 ± 0.00	25.00 ± 0.00
12	BC_2F_1(Mo94 × $BC_1F_1(299_1)$)	505_4	9	Large	1.00 ± 0.00	25.00 ± 0.00
		506_2	6	Large	1.00 ± 0.00	25.00 ± 0.00
18	BC_2F_1(Mo48 × $BC_1F_1(114_1)$)	286_{14}	14	Small	1.00 ± 0.00	25.00 ± 0.00
22	BC_2F_1(Mo17 × $BC_1F_1(110_1)$)	288_1	16	Medium-small	1.94 ± 0.19	24.75 ± 0.11
Telo11	BC_2F_1(Telo21 × $BC_1F_1(292_1)$)	790_2	8	–	0.75 ± 0.37	25.63 ± 0.18
		791_1*	15	–	1.00 ± 0.41	24.75 ± 0.63

* 0.25 ± 0.25 quadrivalents on average per cell in monotelosomal plant 791_1.

In monotelosomics (790_2 and 791_1) in the BC_2F_1 variant (Telo21 × $BC_1F_1(292_1)$), paired univalents (0.75 ± 0.37 and 1.00 ± 0.41 per cell, respectively), along with heteromorphic bivalents, were found in separate PMCs. One monotelosomic (791_1) also formed one quadrivalent (0.25 ± 0.25 per cell) (see Table 2).

Analysis of the size of univalents in monosomic BC_2F_1 revealed a large size of chromosome 2 of *G. hirsutum* in one family BC_2F_1(Mo16 × $BC_1F_1(923_7)$), chromosome 6 of *G. barbadense* in two families, BC_2F_1(Mo34 × $BC_1F_1(293_3)$) and BC_2F_1(Mo92 × $BC_1F_1(1040_2)$) (see Fig. 3, *d*), and chromosome 12 of *G. barbadense* in one family, BC_2F_1(Mo94 × $BC_1F_1(299_1)$) (see Fig. 4, *b*). BC_2F_1 monosomics in five families with chromosome 4 of *G. barbadense* BC_2F_1(Mo38 × $BC_1F_1(925_{11})$), BC_2F_1(Mo58 × $BC_1F_1(115_1)$), BC_2F_1(Mo59 × $BC_1F_1(1041_4)$), BC_2F_1(Mo60 × $BC_1F_1(117_4)$) and BC_2F_1 (Mo75 × $BC_1F_1(298_2)$) (see Fig. 3, *a–c*), as well as with chromosome 7 of *G. hirsutum* BC_2F_1(Mo27 × $BC_1F_1(111_2)$) (Fig. 4, *a*), had a medium size of univalents, which confirmed that they belong to the A_t-subgenome.

A study of the size of the univalent in the plant (288_1) variant of crosses of BC_2F_1(Mo17 × $BC_1F_1(110_1)$) with chromosome 22 of *G. barbadense* revealed a medium-small size of the univalent (see Fig. 4, *d*); in a plant of another variant, BC_2F_1(Mo48 × $BC_1F_1(114_1)$) with chromosome 18 of *G. hirsutum*, it was small in size, which further confirmed that the chromosomes belong to the D_t-subgenome (Fig. 4, *c*).

Most BC_2F_1 monosomics showed a high meiotic index, which indicated that their univalent chromosomes underwent regular segregation (Supplementary Material 11). However, one monosomic variant, BC_2F_1(Mo34 × $BC_1F_1(293_3)$), with a substitution of chromosome 6, demonstrated a decrease in the meiotic index (83.66 ± 0.62) and an increase in the number of tetrads with micronuclei (9.23 ± 0.77 %) (Fig. 5). This indicated disturbances in the divergence of chromosomes and the formation of unbalanced gametes, which could lead to "a univalent shift" in the offspring. Five monosomics in the BC_2F_1(Mo60 × $BC_1F_1(117_4)$), BC_2F_1(Mo92 × $BC_1F_1(1040_2)$), BC_2F_1(Mo94 × $BC_1F_1(299_1)$) and BC_2F_1(Mo17 × $BC_1F_1(110_1)$) variants also showed a slight increase in the number of tetrads with

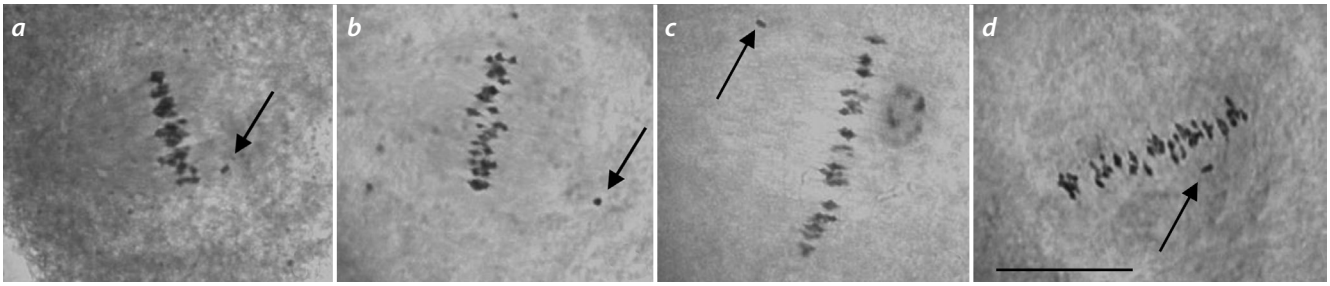

Fig. 3. Chromosome configurations in metaphase I of meiosis in hybrid BC_2F_1 plants obtained from crossing monosomic lines with interspecific monosomic hybrids $BC_1F_1(25^{II}+1^{I})$.

a – BC_2F_1(Mo58 × $BC_1F_1(115_1)$) (530_1); *b* – BC_2F_1(Mo59 × $BC_1F_1(1041_4)$) (284_1); *c* – BC_2F_1(Mo60 × $BC_1F_1(117_5)$) (494_3) ($25^{II}+1^{I}$) with chromosome 4 of *G. barbadense*; *d* – BC_2F_1(Mo34 × $BC_1F_1(293_3)$) (497_4) with chromosome 6 of *G. barbadense*. Here and in Fig. 4: Arrows indicate univalents. Scale bar = 10 μm.

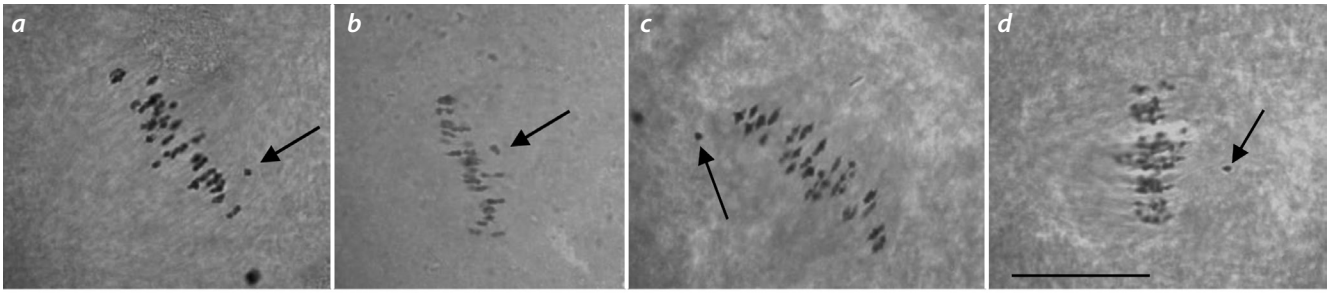

Fig. 4. Chromosome configurations in metaphase I of meiosis in hybrid BC_2F_1 plants obtained from crossing monosomic lines with interspecific monosomic hybrids $BC_1F_1(25^{II}+1^{I})$.

a – BC_2F_1(Mo27 × $BC_1F_1(111_2)$) (500_{12}) with chromosome 7 of *G. hirsutum*; *b* – BC_2F_1(Mo94 × $BC_1F_1(299_1)$) (505_4) with chromosome 12 of *G. barbadense*; *c* – BC_2F_1(Mo48 × $BC_1F_1(114_{20})$) (286_{14}) with chromosome 18 of *G. hirsutum*; *d* – BC_2F_1(Mo17 × $BC_1F_1(110_1)$) (288_1) with chromosome 22 of *G. barbadense*.

micronuclei (from 1.22 ± 0.43 up to 1.84 ± 0.37 %), which could also lead to the same consequences (Supplementary Material 12, see Fig. 5). Similar to chromosome pairing, the meiotic index showed no significant differences between backcrossed monosomics with or without single chromosome substitutions.

Two monotelosomics from the BC_2F_1 family (Telo21 × $BC_1F_1(292_1)$) showed an increase in the percentage of tetrads with micronuclei from 2.17 ± 0.30 % (791_1) to 2.32 ± 0.30 % (790_2), which could be a consequence of a disturbance in the disjunction of the telocentric and the formation of unbalanced gametes in these hybrids (see Supplementary Material 12).

Pollen viability was assessed in BC_2F_1 monosomics using acetocarmine staining. Most of them showed high pollen viability (from 90.22 ± 1.31 to 96.15 ± 0.69 %), similar to line L-458 (90.92 ± 1.15 %) (Supplementary Material 13). Specifically, two monosomics (500_{12} and 499_2) in two variants of crosses, BC_2F_1(Mo27 × $BC_1F_1(111_2)$) and BC_2F_1(Mo92 × $BC_1F_1(1040_2)$) with chromosome 7 of *G. hirsutum* and with chromosome 6 of *G. barbadense*, had the greatest reduction in pollen viability (70.09 ± 1.57 and 75.00 ± 1.66 %, respectively) (Fig. 6), but four monosomics showed a slight reduction in pollen viability (from 83.20 ± 2.39 to 87.50 ± 1.95 %). However, in one variant, BC_2F_1(Mo59 × $BC_1F_1(1041_4)$), two monosomics were characterized by differences in pollen viability of more than 17 %, and in another variant, BC_2F_1 (Mo27 × $BC_1F_1(111_2)$), these differences were more than 20 %.

Discussion

In recent years, a comprehensive analysis of alien addition and alien substitution lines, including morpho-biological, genetic, cytogenetic and molecular genetic methods, has proven itself (Schneider, 2010; Tiwari et al., 2010; Rawat et al., 2011; Garg et al., 2016).

An integrated approach using differential C-staining, fluorescence *in situ* hybridization (FISH) and gliadin analysis in analyses of introgression lines of *T. aestivum* × *Ae. columnaris* allowed to identify substitutions, addition chromosomes or fragments of individual chromosomes in 15 lines, while in five lines, the presence of alien genetic material was not detected (Shishkina et al., 2017). In a study of introgression lines obtained from backcrosses with bread wheat varieties of the synthetic form RS7 (BBAAUS), using C-staining, FISH, and DNA markers, lines with substitution of wheat chromosomes and with chromosome rearrangements were found; however, two lines were characterized by the absence of alien introgressions (Davoyan et al., 2019). It has become obvious that in studies of the genomic composition of alien substituted forms, it is extremely necessary to use a complex of cytological and molecular genetic methods.

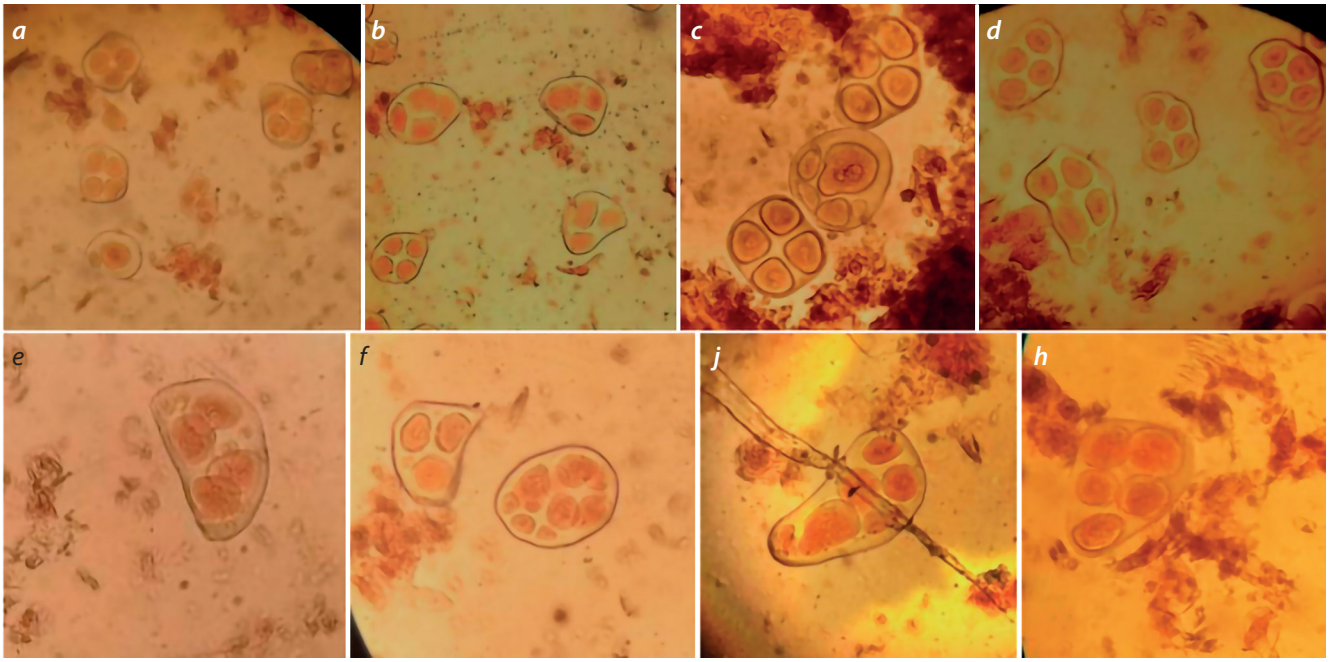

Fig. 5. Sporades in the monosomic hybrid plant BC_2F_1(Mo34 × BC_1F_1(293$_3$)) (497$_4$): *a* – monad with micronuclei; *b* – triads and tetrads; *c* – monad with micronuclei and tetrads; *d–f* – tetrads with micronuclei; *g* – pentad with micronuclei; *h* – pentad.

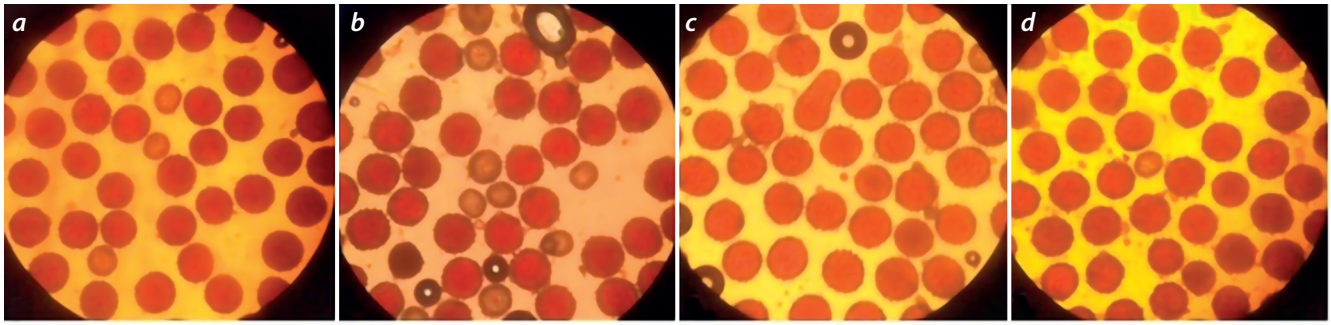

Fig. 6. Fertile (colored) and sterile (uncolored) pollen in monosomic hybrids BC_2F_1 obtained from crossing monosomic lines with monosomic hybrids BC_1F_1(Mo × F_1Mo × Pima 3-79): *a, b* – BC_2F_1(Mo75 × $BC_1F_1$298$_2$) (496$_1$); *c, d* – F_1BC_2(Mo34 × $F_1BC_1$293$_3$) (497$_4$).

In cotton, studies using SSR markers and genomic *in situ* hybridization (GISH) have also been initiated, which allowed the isolation of five monosomic alien addition lines (MAALs) in the backcross progeny of a pentaploid obtained from crosses of the species *G. hirsutum* with the Australian diploid species *G. australe* F. Muell. (Sarr et al., 2011). The use of BAC-FISH probes in five diploid cotton species allowed to successfully identify individual chromosomes and map 45S and 5S rDNA to specific chromosomes of five species (Gan et al., 2012). Comparison of the cytogenetic map of chromosome 1 of the species *G. herbaceum* L., constructed using BAC-FISH, with the genetic maps of chromosome 1 of the species *G. hirsutum*, *G. arboreum*, and *G. raimondii* showed that most of the identified BAC clones are located in the same order on different maps, with the exception of three markers indicating chromosome rearrangements (Cui et al., 2015). Unfortunately, such complex analysis methods have not yet been used to study chromosome substitution lines.

Modern genotypes of cultivated cotton are characterized by restriction of alleles for beneficial traits due to monophyletic origin and the formation of a "genetic bottleneck" that arose during domestication from a common ancestor and crosses between the same genotypes of elite forms (Saha et al., 2018). This has stimulated the search for genetic diversity among different cotton species.

The creation of 17 substituted cotton lines (CS-B), where each homologous pair of chromosomes or chromosomal arms of the species *G. hirsutum* (TM-1) was substituted by a homologous chromosome or arm of the species *G. barbadense* (Pima 3-79) (Stelly et al., 2005), made it possible to associate the most important traits of fibre quality with a single chromosome or its arm (Saha et al., 2004; Jenkins et al., 2006), to

begin the introgression of favorable genes for the improvement of cultivated cotton (Jenkins et al., 2006, 2007) and to study chromosomal effects on agronomic traits (fibre yield, boll weight, raw cotton yield) and data processing using a genetic model (ADAA) (Saha et al., 2010).

Later, some of these cotton lines did not receive molecular-genetic confirmation (Gutiérrez et al., 2009; Saha et al., 2015; Ulloa et al., 2016). In recent work, chromosome-specific markers (SSRs) were used in a MAGIC population created by crossing 18 CS-B lines with three Upland cotton cultivars. Ultimately, the same five lines (CS-B05sh, CS-B06, CS-B07, CS-B12sh and CS-B15sh) that were listed in previous articles contained "little or no introgression of the whole chromosome or chromosome region" (Fang et al., 2023). Only 13 CS-B lines contained "significant introgression" from the *G. barbadense* species, and the reasons for the lack of molecular-genetic confirmation in some chromosome substitution cotton lines remain unclear.

When creating cotton lines with *G. barbadense*/*G. hirsutum* chromosome substitution, the selection of plants with the needed genotype was accelerated thanks to molecular-genetic testing of backcross plants at the seedling stage (Sanamyan et al., 2022). This also contributed to the continuation of backcrossing of only those hybrid forms that had the desired genotype. This rapid selection of plants with the desired genotype underscored the advantages of using molecular markers (SSRs) in such studies.

In this work, using chromosome-specific SSR markers in the BC_2F_1(Mo16 × $BC_1F_1(923_7)$) variant in six seedlings with monosomy, the elimination of chromosome 2 of the *G. barbadense* A_t-subgenome and the presence of chromosome 2 of the *G. hirsutum* A_t-subgenome were detected, while in one seedling of another family BC_2F_1(Mo38 × $BC_1F_1(925_{10})$) chromosome 4 of the A_t-subgenome of *G. barbadense* was revealed, which indicates chromosome substitution in this plant. Confirmation of chromosome substitutions carried out by molecular genetic analysis in previously cytogenetically identified monosomic BC_2F_1 hybrids was established only on chromosomes 4, 6, and 12 of the A_t-subgenome and chromosome 22 of the D_t-subgenome of cotton in eight variants, while in two variants, BC_2F_1(Mo27 × $BC_1F_1(111_2)$) and BC_2F_1(Mo48 × $BC_1F_1(114_{20})$), the absence of substitution of chromosome 7 of the A_t-subgenome and 18 of the D_t-subgenome was revealed. Consequently, the lack of elimination of chromosome 4 of the A_t-subgenome of *G. barbadense* in the five studied backcross variants (involving lines Mo38, Mo58, Mo59, Mo60 and Mo75) indirectly indicates its preferential transmission through gametes, while the elimination of chromosomes 7 of the A_t-subgenome and 18 of the D_t-subgenome of *G. barbadense* already in the first backcross generation indicates their non-competitiveness in comparison with homeologues of *G. hirsutum*.

It must be emphasized that the presence of PCR products obtained as a result of amplification only with chromosome-specific SSR markers for chromosomes 6 and 7 of the A_t-subgenome of *G. hirsutum* in two lines (CS-B06 and CS-B07) of the American cytogenetic collection, which served as controls in our study, was a new confirmation of the incorrect determination of the substitution of chromosomes 6 and 7 of the A_t-subgenome, which had previously been emphasized by other researchers (Gutiérrez et al., 2009; Ulloa et al., 2016). In this regard, elucidating the reasons for the lack of introgression of donor chromosome 2 of the A_t-subgenome of *G. barbadense* during the backcrossing of hybrids is of great interest for future research.

To date, the reasons for the elimination of donor chromosomes in backcross hybrids remain unclear; however, it is known that in wheat-rye lines, the frequency of introgression of an alien chromosome depends both on the genotype of the line and on the genotype of the variety used in the crossing (Krasilova et al., 2011). Analysis of introgression lines of hybrid wheat with *Aegilops columnaris* Zhuk. showed that introgression processes depend on the parental wheat genotype and the level of divergence of homeologous chromosomes of the parent species (Badaeva et al., 2018). The chromosomes of those species that are taxonomically diverged from bread wheat to a greater extent are characterized by a low compensatory ability, which could be caused by structural rearrangements. Since no studies have yet been carried out in cotton to elucidate the factors influencing the frequency of introgression of an alien chromosome, studies of introgressive lines of wheat can contribute to the understanding of similar processes in other plant species.

All of the above can further clarify the processes causing the elimination of the donor chromosome of *G. barbadense* to occur during backcrossing in some types of crosses, but today it is known that the chromosomes of the D_t-subgenome of cotton have fewer small inversions than the chromosomes of the A_t-subgenome (Chen et al., 2020). In addition, tetraploid cotton has two reciprocal translocations, Chr.4/Chr.5 and Chr.2/Chr.3, which arose after polyploidization, and were confirmed by the presence of homologous loci (Wang et al., 2016). Additionally, inversions were found on many chromosomes, excluding chromosomes Chr.1, Chr.6, Chr.10, Chr.11, Chr.14, Chr.16, Chr.21, Chr.22 and Chr.24. All of the above structural changes in the chromosomes of tetraploid cotton could contribute to the difficulties that arose during the introgression of homeologous chromosomes.

A comparative analysis of chromosome pairing in backcross monosomics of different crossing variants revealed only single monosomics with additional univalents BC_2F_1(Mo17 × $BC_1F_1(110_1)$), which theoretically could lead to a "univalent shift" in the offspring. However, as the study showed, the elimination of the *G. barbadense* chromosome during the process of backcrossing was observed in the offspring of other backcrossing hybrids with modal pairing of chromosomes, which indicated the existence of a mechanism for eliminating an alien chromosome, independent of the pairing of chromosomes and their subsequent disjunction.

However, it was expected that in one variant of crosses BC_2F_1(Mo34 × $BC_1F_1(293_3)$) in hybrid monosomic (497_4) with modal chromosome pairing, any disturbances in the genotype of the offspring could occur due to the formation of partially unbalanced gametes due to a reduced meiotic index (83.66 ± 0.62) and an increased percentage of tetrads with micronuclei (up to 9.23 ± 0.77 %). Therefore, the discovery

in the next backcross generation BC_3F_1(Mo34 × $BC_2F_1497_4$) of five seedlings without substitution of chromosome 6 of the A_t-subgenome of cotton was predictable and indicated the exclusivity of the predicted event (Sanamyan, unpublished).

Assessment of pollen fertility after staining with acetocarmine in aneuploid backcross cotton plants revealed a decrease in different variants, which indicated the abortion of nullisomal gametes. Often, in the same crossings, monosomic hybrids were characterized by differences in the number of viable pollen. On the other hand, it is not possible to explain differences in the genotypes of monosomic hybrids only by differences in pollen fertility. It was previously shown that the assessment of pollen fertility after staining with acetocarmine in the progeny of monosomic cotton plants is not entirely convincing as a method for separating monosomic and disomic plants due to the abortion of unbalanced microspores in early development (Brown, Endrizzi, 1964). This assessment indicates the structural variability of genomes of interspecific monosomic hybrids with and without alien chromosome substitution. This variability at the level of chromosome behavior in the first division of meiosis is not detected using routine staining methods, but at the level of pollen viability, it is clearly visible.

Conclusion

This work shows a peculiarity in the introgression of individual chromosomes of the cotton plant *G. barbadense* into the genome of the cotton plant *G. hirsutum*. Chromosomes 4, 6, and 12 of the A_t-subgenome and 22 of the D_t-subgenome of *G. barbadense* showed predominant introgression; BC_2F_1 hybrids with monosomic *G. barbadense/G. hirsutum* substitution were obtained on these chromosomes. Chromosomes 2, 7 of the A_t-subgenome and 18 of the D_t-subgenome of *G. barbadense* were characterized by elimination; among them, chromosomes 7 of the A_t-subgenome and 18 of the D_t-subgenome of *G. barbadense* were eliminated in the first backcross generation.

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ORCID

O.G. Silkova orcid.org/0000-0003-3299-2975

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Inactivation of the TIM complex components leads to a decrease in the level of DNA import into *Arabidopsis* mitochondria

T.A. Tarasenko[1], K.D. Elizova[1], V.I. Tarasenko[1] ✉, M.V. Koulintchenko[1, 2], Yu.M. Konstantinov[1]

[1] Siberian Institute of Plant Physiology and Biochemistry of the Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia
[2] Kazan Institute of Biochemistry and Biophysics of Kazan Scientific Center of the Russian Academy of Sciences, Kazan, Russia
✉ vslav@inbox.ru

Abstract. The phenomenon of DNA import into mitochondria has been shown for all major groups of eukaryotes. In plants and animals, DNA import seems to occur in different ways. It has been known that nucleic acids enter plant organelles through alternative channels, depending on the size of the imported molecules. Mitochondrial import of small DNA (up to 300 bp) partially overlaps with the mechanism of tRNA import, at least at the level of the outer membrane. It is noteworthy that, in plants, tRNA import involves components of the protein import apparatus, whose role in DNA transport has not yet been studied. In this work, we studied the role of individual components of the TIM inner membrane translocase in the process of DNA import into isolated *Arabidopsis* mitochondria and their possible association with the porin VDAC1. Using knockout mutants for the genes encoding Tim17 or Tim23 protein isoforms, we demonstrated for the first time the involvement of these proteins in the import of DNA fragments of different lengths. In addition, inhibition of transport channels with specific antibodies to VDAC1 led to a decrease in the level of DNA import into wild-type mitochondria, which made it possible to establish the specific involvement of this porin isoform in DNA import. In the *tim17-1* knockout mutant, there was an additional decrease in the efficiency of DNA import in the presence of antibodies to VDAC1 compared to the wild type line. The results obtained indicate the involvement of the Tim17-1 and Tim23-2 proteins in the mechanism of DNA import into plant mitochondria. At the same time, Tim23-2 may be part of the channel formed with the participation of VDAC1, while Tim17-1, apparently, is involved in an alternative DNA import pathway independent of VDAC1. The identification of membrane carrier proteins involved in various DNA import pathways will make it possible to use the natural ability of mitochondria to import DNA as a convenient biotechnological tool for transforming the mitochondrial genome.
Key words: mitochondria; DNA import; Tim17; Tim23; VDAC1; transport channel; knock-out mutant; *Arabidopsis thaliana*.

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Инактивация компонентов комплекса TIM приводит к снижению уровня импорта ДНК в митохондрии арабидопсиса

Т.А. Тарасенко[1], К.Д. Елизова[1], В.И. Тарасенко[1] ✉, М.В. Кулинченко[1, 2], Ю.М. Константинов[1]

[1] Сибирский институт физиологии и биохимии растений Сибирского отделения Российской академии наук, Иркутск, Россия
[2] Казанский институт биохимии и биофизики Казанского научного центра Российской академии наук, Казань, Россия
✉ vslav@inbox.ru

Аннотация. Феномен импорта ДНК в митохондрии показан для всех основных групп эукариот. В растениях и животных импорт ДНК, по-видимому, происходит различными путями. Известно, что в растительные органеллы нуклеиновые кислоты попадают по альтернативным каналам в зависимости от размера импортируемых молекул. Импорт ДНК небольшого размера (до 300 п. н.) частично перекрывается с механизмом импорта тРНК, по крайней мере, на уровне внешней мембраны. Примечательно, что у растений в импорт тРНК вовлечены компоненты аппарата импорта белков, чья роль в транспорте ДНК до настоящего времени оставалась неизученной. В настоящей работе мы провели исследование роли отдельных компонентов транслоказы внутренней мембраны TIM в процессе импорта ДНК в изолированные митохондрии арабидопсиса и их возможной связи с порином VDAC1. С использованием нокаут-мутантов по генам, кодирующим изоформы белков Tim17 или Tim23, мы впервые показали участие этих белков в импорте фрагментов ДНК разной длины. Кроме того, ингибирование транспортных каналов специфическими антителами к VDAC1 приводило к снижению уровня импорта ДНК в митохондрии дикого типа, что позволило установить специфическое участие этой изоформы порина в импорте ДНК. В нокаут-мутанте *tim17-1* происходило дополнительное снижение эффективности импорта ДНК в присутствии антител к VDAC1 в сравнении с линией дикого типа. Полученные результаты указывают на участие белков Tim17-1 и Tim23-2 в аппарате импорта ДНК в растительные митохондрии. При этом Tim23-2 может быть частью

канала, формируемого при участии VDAC1, в то время как Tim17-1, по-видимому, вовлечен в альтернативный, независимый от VDAC1, путь импорта ДНК. Выявление мембранных белков-переносчиков, участвующих в различных путях импорта ДНК, позволит использовать природную способность митохондрий к поглощению ДНК в качестве удобного биотехнологического инструмента для трансформации митохондриального генома.
Ключевые слова: митохондрии; импорт ДНК; Tim17; Tim23; VDAC1; транспортный канал; нокаут-мутант; *Arabidopsis thaliana*.

Introduction

Mitochondria are double-membrane organelles of aerobic eukaryotes that are responsible for providing energy to the cell and have their own genetic system. Mitochondrial DNA (mtDNA), a legacy of the endosymbiotic event (Martin et al., 2015), encodes rRNA, tRNA, ribosomal proteins, and oxidative phosphorylation proteins (Morley, Nielsen, 2017). The ability to modify the mitochondrial genome can become a convenient tool for making targeted changes in mtDNA in order to obtain plants with valuable agricultural characteristics and solve issues of gene therapy treatment of human mitochondrial diseases.

The methodology for transforming mitochondria with exogenous DNA is at the initial stages of its development (Larosa, Remacle, 2013), since effective methods for manipulating the mitochondrial genome using targeted delivery of nucleic acid molecules have not yet been established. One promising approach to transforming the mitochondrial genome could be manipulation of the DNA import process – the natural ability of mitochondria to uptake DNA from the cytoplasm.

The phenomenon of DNA import was initially demonstrated for plants (Koulintchenko et al., 2003; Konstantinov et al., 2016), but was subsequently described for mammalian mitochondria (Koulintchenko et al., 2006) and yeast (Weber-Lotfi et al., 2009). It should be noted that currently there is no complete understanding of how the transmembrane transfer of DNA into the mitochondrial matrix occurs. Apparently, DNA import involves different pathways in plants and mammals (Koulintchenko et al., 2006). Moreover, in plant mitochondria, DNA transfer can occur through several alternative mechanisms involving a variety of protein complexes (Weber-Lotfi et al., 2015; Tarasenko et al., 2021).

DNA import into plant mitochondria throughout the outer membrane occurs with the participation of porin (VDAC, voltage-dependent anion channel) (Koulintchenko et al., 2003). The role of VDAC has also been shown in tRNA import (Salinas-Giegé et al., 2015), a cellular process that ensures the functioning of the genetic system of these organelles (Morley, Nielsen, 2017). Several VDAC isoforms are present in plant cells (Tateda et al., 2011); in particular, there are four functional isoforms in *Arabidopsis thaliana* (Tateda et al., 2011) that perform different roles. VDAC1 is more important for plant growth and disease resistance (Tateda et al., 2011), while VDAC3 appears to be involved in the stress response (Hemono et al., 2020). Based on the tRNA binding intensity of four VDAC isoforms, it was suggested that VDAC4 is involved in the import of tRNA into mitochondria (Hemono et al., 2020).

Differential interaction of mitochondrial porins with tRNA is also characteristic of other plants. Thus, only VDAC34 appears to be involved in tRNA import in potato, since this isoform shows strong binding to the tRNA molecule (Salinas et al., 2014). *Arabidopsis* VDAC isoforms could potentially also specialize in DNA transport depending on the length of the imported molecules (Tarasenko et al., 2021). In *Arabidopsis* knockout lines lacking VDAC1, VDAC2, or VDAC4, there was an increase in DNA import accompanied by induction of VDAC3 expression, which could be part of a cellular mechanism aimed at compensating for the absence of a porin isoform.

Import of small DNA (up to 300 bp) through the inner mitochondrial membrane may occur with the participation of adenine nucleotide transporters (ADNT1), ATP-Mg/Pi (APC) (Tarasenko et al., 2021) and/or phosphate transporter MPT (Weber-Lotfi et al., 2015). The import of medium-sized DNA (400–7000 bp) involves the AAC adenine nucleotide transporter (ADP/ATP carrier) (Koulintchenko et al., 2003). The involvement of CuBP, a subunit of respiratory complex I, in the transport of medium- and large-sized DNA (Weber-Lotfi et al., 2015) appears to be related to the stabilization of the channel through which larger molecules are transported.

The competitive inhibition method has been used to demonstrate the possible interplay of the import pathways of tRNA and small DNA (Weber-Lotfi et al., 2015), which is not surprising given the involvement of VDAC in the import of both tRNA (Salinas et al., 2006) and DNA (Koulintchenko et al., 2003; Tarasenko et al., 2021). It is noteworthy that components of the protein import apparatus are also involved in the process of tRNA import in plants (Verechshagina et al., 2018). This fact indicates the multifunctionality of some membrane transporters in plant mitochondria. Based on these data, it is logical to assume that the components of protein complexes involved in the processes of tRNA and/or protein translocation occurring in plant mitochondria may also be involved in the DNA import mechanism.

The most obvious candidate for the role of a multifunctional transporter appears to be the TIM complex, also known as TIM17:23, which is responsible for the transport of proteins into the mitochondrial matrix. This membrane complex is directly linked into a single channel with the translocase of outer mitochondrial membrane proteins TOM (translocase of the outer membrane), individual components of which are involved in tRNA import in plants (Salinas et al., 2006).

The inner membrane translocase TIM17:23, one of the largest mitochondrial protein complexes, consists of two main subunits – Tim17 and Tim23. This protein is anchored in the inner membrane by four transmembrane helices, forming a translocation channel (Ryan et al., 1998; Truscott at al., 2001). It is known that the Tim23 subunit is responsible for the formation of the pore, and Tim17 is responsible for the stabilization and regulation of this pore (Verechshagina et al., 2018). Each of these subunits has three isoforms in *Arabidopsis* plants (Murcha et al., 2007), but the degree of their participation in protein import appears to be different, indicating their potential role in other cellular processes.

It is known that the TIM17:23 complex is dominated by the Tim23-2 subunit, which is characterized by the highest level of expression in all tissues (Murcha et al., 2003). Considering the high degree of homology of Tim23 isoforms, indicating their potentially interchangeable properties, it cannot be excluded that the predominant Tim23-2 isoform may have a multifunctional role in cellular processes, similar to what was established for the main components of the TOM complex (Salinas-Giegé et al., 2015). Notably, the Tim23-2 subunit is present in respiratory complex I in addition to TIM17:23 (Murcha et al., 2005; Wang et al., 2012). The Tim23-3 subunit, in contrast, has a low level of expression and is the most divergent in sequence from the other two isoforms (Murcha et al., 2007), which may indicate that this protein performs additional functions.

The most common Tim17 isoform in *Arabidopsis* is the Tim17-2 protein, which is characterized by a consistently high level of expression throughout development. The Tim17-1 isoform has a fairly high (75 %) degree of similarity to Tim17-2 (Wang et al., 2014). It should be noted that to date there are no unambiguous data on the role of Tim17-1 in plants (Wang et al., 2014). Unlike Tim17-2, the Tim17-1 isoform is characterized by changes in expression levels depending on the developmental stage, with the most pronounced increase at the seed development stage, but a gradual decrease with development (Wang et al., 2014). Obviously, protein import is ensured by the predominant isoforms in an adult plant, while the minor Tim17-1 could potentially specialize in performing functions not related to mitochondrial biogenesis.

In our work, we showed that the Tim23-2 and Tim17-1 proteins are involved in the transmembrane transfer of DNA into the mitochondrial matrix, while Tim23-2 appears to be responsible for the import of exclusively short fragments. In addition, the VDAC1 porin isoform appears to be directly involved in the process of DNA import and is likely part of a channel formed with the participation of the Tim23-2 protein. The data obtained open up prospects for further studies of the role of TIM components in the import of nucleic acids into mitochondria.

Materials and methods

Plant material and growing conditions. We used wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) plants and GABI_689C11 (*tim23-2*, At1g72750 gene), SALK_129386 (*tim23-3*, At3g04800 gene) and SALK_092885 (*tim17-1*, At1g20350 gene) knockout lines. Seeds of these lines were provided by Monika Murcha (ARC Center of Excellence in Plant Energy Biology, Perth, Australia). Seeds were subjected to stratification for 3 days at 4 °C, and then grown at 22 °C in a KBW720 growth chamber (Binder, Germany) in pots filled with a compost/vermiculite mixture in a ratio of 2:1 at a photosynthetic photon flux density of 150 μmol $m^{-2} \cdot s^{-1}$ and 16-hour photoperiod.

Preparation of DNA import substrates. For DNA amplification, Taq polymerase (Thermo Scientific, USA) was used in accordance with the manufacturer's recommendations. The genetic construct pCK/GFP/PRmt (Koulintchenko et al., 2003), containing the *GFP* gene sequence, was used as a PCR template.

Amplification of DNA fragments of 2732 bp (Forward: 5′-CCAACCACCACATACCGAAA-3′; Reverse: 5′-ACGCT CTGTAGGATTTGAACC-3′) and 265 bp (Forward: 5′-AT GAGTAAAGGAGAAGAACTTTTCACT-3′; Reverse: 5′-CGGGGCATGGCACTCTTGA-3′) containing the *GFP* gene sequence was carried out using specific primer pairs at an annealing temperature of 60 °C. DNA was purified using GeneJET™PCR Purification Kit columns (Thermo Scientific) according to the manufacturer's instructions. The quality of PCR products was assessed electrophoretically using the Gel Doc XR System (Bio-Rad, USA), the amount of DNA was determined using a NanoPhotometer NP80 spectrophotometer (IMPLEN, Germany).

Isolation of mitochondria. A crude mitochondrial extract was prepared from 3-week-old *A. thaliana* plants according to a previously described protocol (Sweetlove et al., 2007) by differential centrifugation. The purified mitochondrial fraction was obtained by separating the crude mitochondrial fraction in a stepwise Percoll density gradient (50–28–20 %) for 40 min at 40,000 g. A suspension of mitochondria was collected at the boundary of layers with 50 and 28 % Percoll concentrations.

Import of DNA substrates into Arabidopsis mitochondria *in organello*. DNA import was performed as described previously (Tarasenko et al., 2019). 200 μg of purified mitochondria were added to 200 μl of import buffer (0.4 M sucrose, 40 mM potassium phosphate, pH 7.0) containing 500 ng of DNA, then incubated at 25 °C for 30 min. Mitochondria were treated with DNase I (1 unit/μl) (Thermo Scientific) in 100 μl of import buffer in the presence of 10 mM $MgCl_2$ for 20 min at 25 °C. Samples were then washed in wash medium containing additional 10 mM EDTA and 10 mM EGTA, and mtDNA was extracted for further analysis of DNA import efficiency. As a control for the efficiency of DNase treatment, a sample without the addition of mitochondria was used. The level of the background signal obtained from such a sample was always at least two orders of magnitude lower than the level of the signal from import samples.

Preparation of protoplasts, transfection with DNA molecules and isolation of mitochondria. Protoplasts were obtained from *A. thaliana* leaves according to a previously described protocol (Wu et al., 2009) with modifications (Tarasenko et al., 2019). A DNA substrate (5 μg) was added to the suspension of isolated protoplasts, after which 300 μl of a solution containing 20 % PEG-2000, 0.2 M mannitol, 100 mM $CaCl_2$ was added to the samples. The protoplasts were incubated for 5 min, then the protoplast suspension was subjected to three cycles of centrifugation in 1.5 ml of washing medium for 1 min at 100 g and 20 °C. Further incubation of the protoplasts was carried out in W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 2 mM MES, pH 5.7) at 22 °C and low light for 20 hours. The protoplast suspension was centrifuged for 1 min at 100 g and 20 °C and mitochondria were isolated as described previously (Tarasenko et al., 2019).

DNA import assay. The amount of DNA imported into mitochondria was determined by quantitative PCR (qPCR) using the qPCRmix-HS SYBR kit (Evrogen, Russia) according to the manufacturer's instructions. Data were analyzed

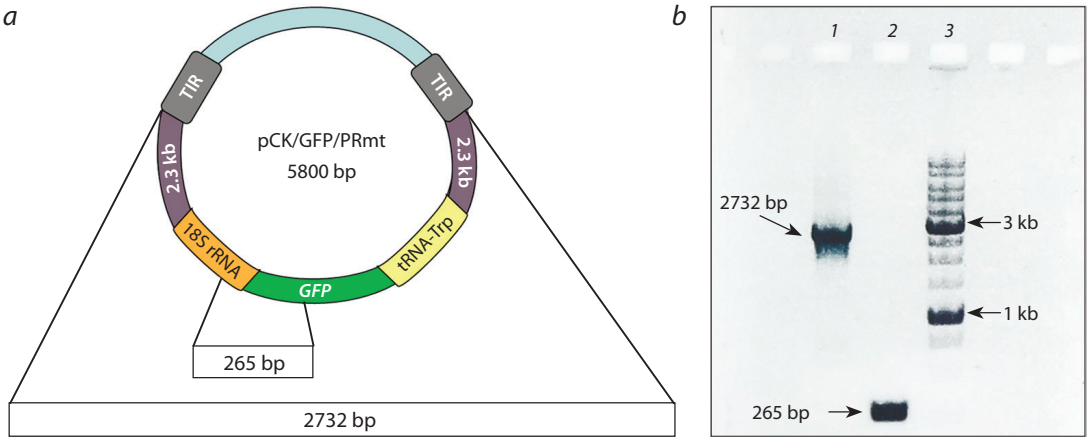

Fig. 1. DNA substrates used for import into *Arabidopsis* mitochondria.

a – scheme of the pCK/GFP/PRmt genetic construct (Koulintchenko et al., 2003), which served as a template for the synthesis of DNA import substrates. 2.3 kb – 2.3 kb plasmid sequence from the mitochondrial genome of *Zea mays*; TIR – terminal inverted repeats of the 11.6 kb plasmid from the mt-genome of *Brassica rapa*; *b* – electrophoretic analysis of DNA import substrates (200 ng each) prepared from pCK/GFP/PRmt. *1* – 2732 bp; *2* – 265 bp; *3* – DNA molecular weight marker.

using CFX Manager software (Bio-Rad). We used primer pairs specific for the *GFP* gene sequence (Forward: 5′-GAT GTGGAAAACAAGACAGGGGTTT-3′; Reverse: 5′-TGG TGAACCGGGCGTACTATTT-3′) and the *NAD4* gene sequence from the *Arabidopsis* mitochondrial genome (Forward: 5′-GCATTTCAGTGGGTTGGTCTGGT-3′; Reverse: 5′-AGGGATTGGCACGCTTTCGG-3′). The ratio of the content of imported DNA to mtDNA was calculated based on the ratio of the absolute values of the signal from the imported DNA and from the *NAD4* gene, taking into account the difference between the sizes of the imported fragments (265 bp and 2.7 kb) and the mt genome (367 kb) with the assumption that the mt genome is represented exclusively by the master chromosome.

Statistical analysis. Experiments were carried out in at least three biological replicates. The diagrams were constructed using the Microsoft Excel software package. The degree of significance of differences was assessed using Student's test.

Results

It is known that DNA import into mitochondria occurs through various transport pathways, the efficiency of which depends on the size of the imported molecules (Weber-Lotfi et al., 2015; Tarasenko et al., 2021). Based on this, the role of individual protein components of the mitochondrial membrane in the import of DNA molecules of small (265 bp) and medium (2732 bp) lengths was studied (Fig. 1).

DNA fragments 265 bp and 2.7 kb in size obtained by PCR (see Fig. 1, *b*) were imported into mitochondria isolated from *Arabidopsis* plants (*in organello*), wild-type (Col-0) and knockout mutants for Tim23-2 (*tim23-2*), Tim23-3 (*tim23-3*) and Tim17-1 (*tim17-1*), which are isoforms of key proteins of the Tim17:23 complex. For mitochondria of the *tim23-2* mutant, a significant decrease in the import level of a small-length fragment was shown in comparison with the wild type (Fig. 2, *a*), while the import level of a medium-sized fragment did not differ significantly. In mitochondria of the *tim23-3* mutant, no dependence of both DNA fragments import efficiency on the absence of functional Tim23-3 was found (see Fig. 2, *b*). Thus, it is obvious that the Tim23-2 protein isoform is part of the DNA import apparatus, performing a specific role in the transfer of DNA molecules, predominantly of short length.

When studying the import into *tim17-1* mutant mitochondria, we observed a decrease in the DNA transport efficiency, approximately similar for fragments of both short and medium length (see Fig. 2, *c*). These results suggest that the Tim17-1 protein may also be an important participant in the DNA translocation machinery into mitochondria.

In order to verify the data on the import of a DNA fragment of medium length (2.7 kb) into mitochondria isolated from the Tim17 and Tim23 knockout lines (see Fig. 2, *a*, *c*), we carried out experiments using *Arabidopsis* protoplasts obtained from these lines (*in vivo*) (Fig. 3, *a*). Protoplasts maintained their integrity for 20 hours (see Fig. 3, *b*). It was found that the import level of exogenous DNA from the cytoplasm of *tim17-1* knockout protoplasts into the mitochondria was indeed reduced, but to a lesser extent than that observed *in organello*. It can be concluded that the deficiency of mitochondrial Tim17-1 is apparently partially compensated *in vivo* by certain cellular factors. Compared to *tim17-1*, the level of mitochondrial import of *tim23-2* did not differ from the wild type, similar to what was shown in the isolated organelles (see Fig. 3, *a*). Taken together, the results obtained *in organello* using mutant lines lacking the Tim23-2 or Tim17-1 proteins reflect the patterns of DNA transfer *in vivo* into the mitochondria of protoplasts of these lines.

The next task was to investigate the possible relationship of the outer mitochondrial membrane protein VDAC with Tim17-1 and Tim23-2 in the import of short DNA molecules. The use of antibodies to VDAC1 potentially makes it possible to exclude this isoform from participation in the formation of a channel for DNA transfer. It has been shown (Koulintchenko et al., 2003) that the binding of antibodies specific to a certain protein of the outer mitochondrial membrane should inhibit

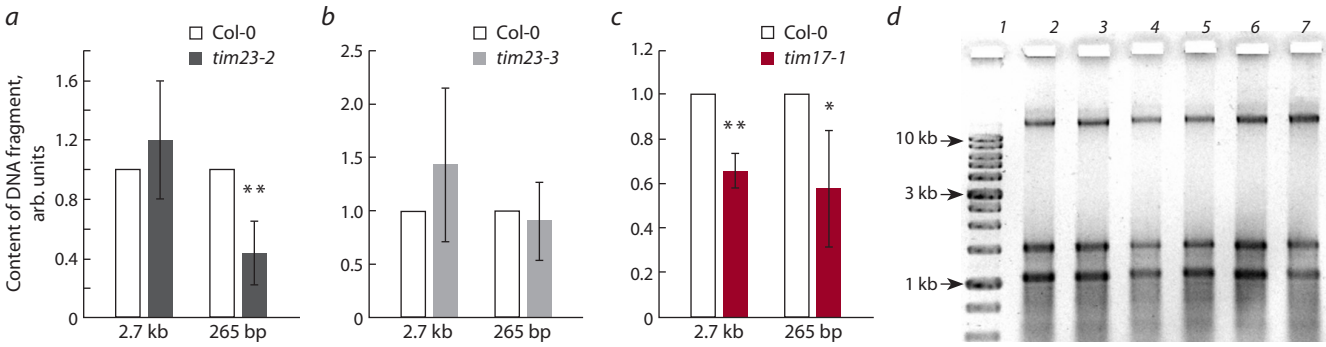

Fig. 2. Analysis of exogenous DNA import into isolated *Arabidopsis* mitochondria.

DNA fragments 265 bp and 2.7 kb in size were imported into mitochondria of wild-type and mutant *Arabidopsis* lines: (*a*) *tim23-2*, (*b*) *tim23-3*, and (*c*) *tim17-1*. Mitochondrial DNA extracted from mitochondria after import (*d*) was used for qPCR analysis. The amount of the *GFP* gene fragment (imported DNA) normalized to the content of the *NAD4* gene fragment (mtDNA) is shown. The import level in Col-0 is taken as an arbitrary unit. The mean values are shown with standard deviations. * and ** statistically significant differences at $p \le 0.05$ and $p \le 0.01$, respectively. *d* – mitochondrial nucleic acid preparation used for analysis: *1* – DNA molecular weight marker; *2*, *3* – mtDNA from *tim23-2*; *4*, *5* – mtDNA from *tim23-3*; *6*, *7* – mtDNA from *tim17-1*.

its transport activity. We applied this approach to analyze the efficiency of 265 bp DNA fragments import into isolated wild-type and *tim17-1* or *tim23-2* mutant mitochondria (Fig. 4).

At first, in order to exclude nonspecific inhibition, we assessed the effect of antibodies that specifically bind mitochondrial apocytochrome *b* (Cob). This inner membrane protein is the central catalytic subunit of ubiquinol-cytochrome c oxidoreductase (Islas-Osuna et al., 2006). The outer membrane of mitochondria is impermeable to antibodies, so inhibition of transport processes should not occur when using antibodies to Cob. Import level of the 265 bp fragment in wild-type mitochondria after their treatment with antibodies to Cob did not differ from the control sample (see Fig. 4, *a*). Based on this, experiments were carried out in which DNA fragments 265 bp in size were imported into isolated mitochondria of *tim17-1* or *tim23-2* mutant lines, pretreated with antibodies to the outer membrane protein VDAC1 (see Fig. 4, *b*).

According to the data obtained, the level of import of short-length DNA into wild-type mitochondria in the presence of antibodies to VDAC1 was significantly reduced (see Fig. 4, *b*). It is obvious that this porin isoform is directly involved in the transport of DNA of this length into *Arabidopsis* mitochondria. Analysis of import into mitochondria lacking functional Tim23-2 showed that in the presence of antibodies to VDAC1, the efficiency of this process did not differ from that in the wild type under the same conditions (see Fig. 4, *b*). Considering the role of Tim23-2 in the import of short-length DNA (see Fig. 2, *a*), these results indicate that VDAC1 presumably forms one transport channel with Tim23-2, since inhibition of either component results in an approximately equal decrease in import level. Moreover, the degree of import reduction does not change with simultaneous inactivation of these two transporter proteins located in different mitochondrial membranes (see Fig. 4, *b*). At the same time, in mitochondria of the *tim17-1* line treated with antibodies to VDAC1, we observed an additional decrease in the level of import of the 265 bp fragment (see Fig. 4, *b*), which indicates the participation of Tim17-1 in the formation of a DNA import channel independent of VDAC1.

It can be suggested that VDAC1 is a companion of Tim23-2, but not Tim17-1, in the process of translocation of short fragments across the double mitochondrial membrane. At the same time, the Tim17-1 protein, unlike Tim23-2, is involved in the process of import of fragments of both short and medium length, which serves as an additional argument in favor of the independence of Tim17-1 from the channel formed by Tim23-2 and VDAC1.

In order to estimate the actual efficiency of import of two DNA fragments into mitochondria of different lines, we calculated the content of imported DNA

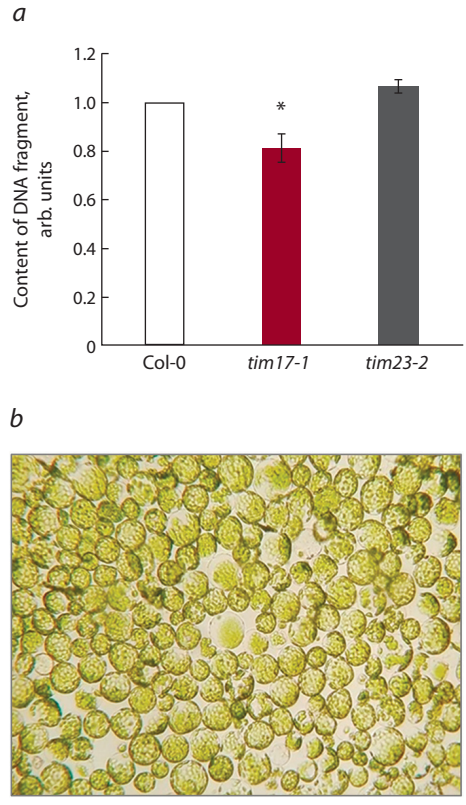

Fig. 3. Level of DNA import into mitochondria of protoplasts obtained from *Arabidopsis* leaves using qPCR.

a – 2.7 kb DNA fragment was imported into mitochondria of protoplasts of wild-type and *tim17-1* and *tim23-2* knockout lines. After transfection of protoplasts, mitochondria were isolated, followed by mtDNA extraction. The amount of the detected *GFP* gene fragment normalized to the content of the *NAD4* gene fragment is shown. The import level in Col-0 is taken as an arbitrary unit. The mean values are shown with standard deviations. * Statistically significant differences at $p \le 0.05$; *b* – light microscopy of protoplast integrity after transformation with a DNA fragment and incubation for 20 hours.

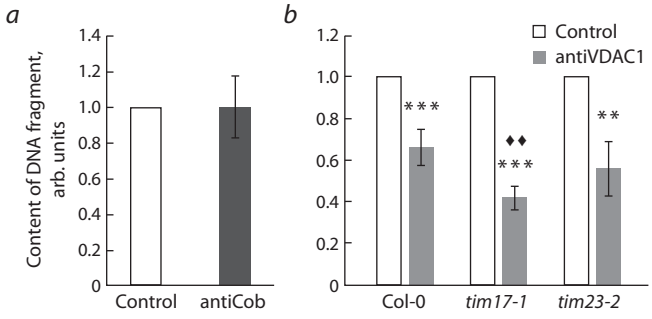

Fig. 4. Efficiency of short-length DNA import into isolated *Arabidopsis* mitochondria in the presence of antibodies to membrane proteins.

a – determination of possible nonspecific inhibition of transport channels involved in DNA import by antibodies to Cob; *b* – DNA import efficiency into mitochondria of Col-0 and *tim17-1* and *tim23-2* knockout lines in the presence of specific antibodies to VDAC1 (antiVDAC1). The amount of the *GFP* gene fragment normalized to the content of the *NAD4* gene fragment is shown. Import level of the 265 bp fragment in *Arabidopsis* mitochondria without pretreatment with antibodies (control) is taken as an arbitrary unit. The mean values are shown with standard deviations. ** (♦♦) and *** – statistically significant differences at $p \le 0.01$ and $p \le 0.001$, respectively; * – differences from control; ♦ – differences from the level of import into Col-0 mitochondria pre-treated with antiVDAC1 (*b*).

in mitochondria in relation to the content of mitochondrial DNA (see the Table). It was shown that the import efficiency of a short fragment is extremely high and amounts to up to 5 % of the amount of mtDNA. Import of the 2.7 kb fragment was much less effective; the amount of DNA penetrated into the organelles was 30 times less than the amount of the imported 265 bp fragment. The data obtained correlate well with known data on the efficiency of import (Koulintchenko et al., 2003), and also provide an additional argument in favor of the existence of separate import pathways for fragments of short and medium length, differing in the intensity of DNA transport through the membrane.

Discussion

The role of the Tim23 and Tim17 subunits in protein import into mitochondria has been extensively studied (Murcha et al., 2003, 2014; Lister et al., 2004). However, studies of the potential involvement of the TIM17:23 complex or its individual subunits in the DNA import into mitochondria have not been carried out until now. It was previously shown that different isoforms of the Tim17 and Tim23 proteins (Murcha et al., 2007) differ in their ability to complement knockout mutants of orthologous subunits in yeast, suggesting some functional specialization of different isoforms (Murcha et al., 2003). This study focused on the major isoform Tim23-2 and the minor isoform Tim17-1, the role of which in plant mitochondria remains poorly understood. To import foreign DNA fragments of different lengths, we used mitochondria isolated from *Arabidopsis* Tim23-2 and Tim17-1 knockout lines.

In the *in organello* system, we showed that both of these proteins are involved in DNA import, with Tim23-2 being more specific with respect to the size of the transferred molecule (see Fig. 2, *a*). These data were confirmed in the system of DNA import into mitochondria of protoplasts obtained from *Arabidopsis* knockout lines (Wu et al., 2009). Previously, we developed an effective method for studying the import of DNA fragments into mitochondria following transfection of *Arabidopsis* protoplasts with these molecules, i. e. under conditions of maintaining the native cellular environment of mitochondria (Tarasenko et al., 2019). This work established a number of regularities, the main one being that the results obtained in protoplasts are consistent with data from studies of DNA import into isolated mitochondria.

The results obtained using protoplasts allow us to conclude that the observed decrease in DNA import into mitochondria lacking Tim17-1 occurs *in vivo*. Likewise, using protoplasts, it was confirmed that Tim23-2 does not play a role in the import of medium-length DNA. This protein, however, according to *in organello* experiments, exhibits activity specific for the transfer of short DNA fragments. This property of Tim23-2 is another argument in favor of the existence of several pathways for DNA transfer through the inner membrane, specific to a certain extent with respect to the size of the imported molecules.

Another protein studied, the minor isoform Tim17-1, turned out to be involved in the import of DNA fragments of both short and medium length. The Tim17-1 isoform is characterized by a high level of expression during seed germination; therefore, it is assumed that Tim17-1 may be involved in mitochondrial biogenesis at this stage of development (Wang et al., 2014). The role of this isoform in protein import in the adult *Arabidopsis* plant is not obvious due to its low level of expression, given an increase in the expression of the other two isoforms throughout plant development. The gradual decrease in the expression level of the Tim17-1 subunit after germination (Wang et al., 2014) may indicate the potential specialization of this isoform in alternative and less important processes.

In addition to the use of knockout mutants, another approach to study the role of mitochondrial membrane proteins in mitochondrial transport processes is the use of specific antibodies to these proteins (Koulintchenko et al., 2003; Murcha et al., 2005). Studies of the VDAC role in DNA import into isolated potato and rat mitochondria were previously carried

Content of imported DNA fragments in mitochondria relative to mtDNA content

Arabidopsis line	265 bp, % of mtDNA content		2.7 kb, % of mtDNA content
	–antiVDAC1	+antiVDAC1	
Col-0	5.76 ± 1.68	3.53 ± 0.75	0.176 ± 0.011
tim17-1	2.84 ± 0.56	1.44 ± 0.47	0.119 ± 0.044
tim23-2	2.31 ± 1.02	1.47 ± 0.64	0.173 ± 0.021

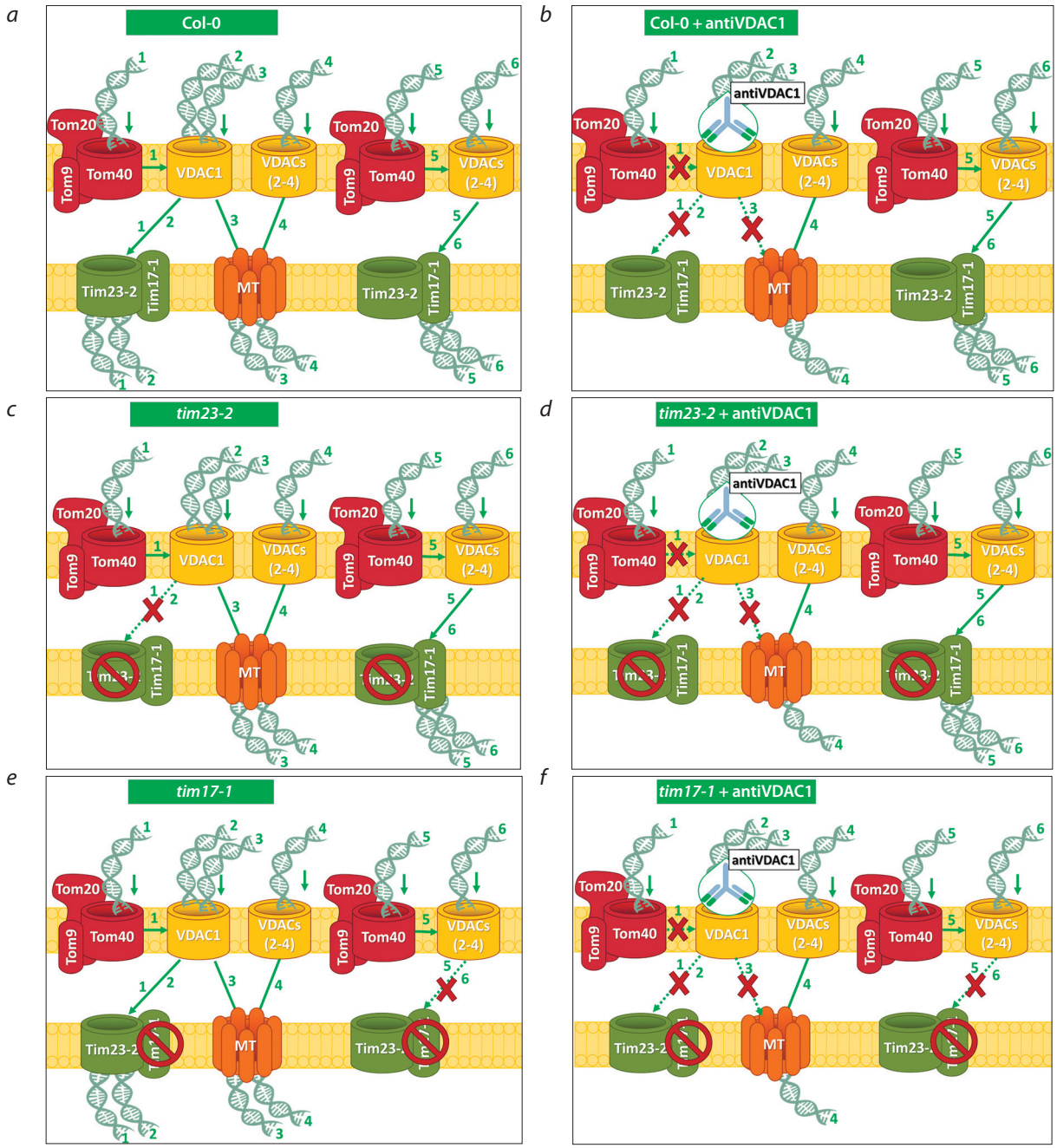

Fig. 5. Putative effect of inactivation of mitochondrial membrane proteins on the level of DNA import into mitochondria.

Inactivation of the transport process was ensured by (*a*, *c*, *e*) using *Arabidopsis* knockout mutants lacking functional Tim17-1 or Tim23-2 protein, and/or (*b*, *d*, *f*) inhibition of the VDAC1 protein with specific antibodies. In the case of simultaneous exclusion of VDAC1 and Tim23-2 from the transport process (*d*), there was a decrease in the level of import, comparable in intensity to that observed under conditions of inactivation of only one transporter (*b* or *c*). With the simultaneous exclusion of VDAC1 and Tim17-1 from the transport process (*f*), an additional decrease in efficiency was observed in comparison with the inactivation of one protein (*b* or *e*), indicating that these proteins belong to two different channels.
1–6 – pathways for transfer of DNA molecules into the matrix; arrows – direction of DNA transfer; dashed arrows crossed out with a red cross – blocking of the transport route; crossed out circle – absence of a functional protein in the mitochondrial membrane as a result of gene knockout. MT is a complex formed with the participation of mitochondrial transporters.

out using antibodies that recognize the conserved domain of VDAC proteins (Koulintchenko et al., 2003, 2006). However, for any group of organisms, there are no data so far on the role of a specific mitochondrial porin isoform in this process. Previously, we attempted to study the participation of one or another VDAC isoform in DNA import using knockout mutants; however, a decrease in the level of import was not detected in any of these lines, apparently due to compensation for the lack of the protein by other isoforms/transport mechanisms throughout plant development (Tarasenko et al., 2021).

It is known that the four isoforms of *Arabidopsis* VDAC, despite a high degree of homology (from 68 to 50 %), apparently have functional specialization (Tateda et al., 2011; Hemono et al., 2020). When assessing protein levels in *Ara-*

bidopsis mitochondria, the most abundant porin isoform was found to be VDAC1, which has approximately 44,400 copies per mitochondrion (Fuchs et al., 2020). In our work, we used isoform-specific antibodies to *Arabidopsis* VDAC1 (AT3G01280), which interact with the N-terminus of this protein. It was shown that inhibition of VDAC1 activity by antibodies leads to a decrease in DNA import intensity. This fact suggests that the use of specific antibodies to suppress porin activity has proven to be a more productive approach to studying the role of VDAC isoforms in DNA import than the use of knockout mutants. Thus, we have demonstrated for the first time the involvement of a specific porin isoform, VDAC1, in DNA transfer into mitochondria (Fig. 5, *a*, *b*). While a specific function of the VDAC4 isoform has been suggested in the mechanism of tRNA import into *Arabidopsis* mitochondria (Hemono et al., 2020), this work demonstrates the involvement of the VDAC1 isoform in DNA import. Thus, functional specialization of different VDAC isoforms with respect to the type of nucleic acids (DNA/RNA) and their size cannot be excluded.

We also investigated the possibility of VDAC1 interacting with Tim17-1 or Tim23-2 during DNA translocation. Due to the fact that both Tim17-1 and Tim23-2 are involved in the import of the short 265 bp fragment, we used a DNA substrate of this size. As a result, it was established for the first time that VDAC1 apparently forms a common transport channel with Tim23-2. We observed an equal decrease in the level of short-length DNA import upon inhibition of one of these proteins and the absence of an additional import decrease upon their simultaneous inactivation (see Fig. 5, *c*, *d*).

An additional DNA import decrease observed in the *tim17-1* knockout mitochondria upon inhibition of the VDAC1 protein with antibodies allows us to conclude that the Tim17-1 and VDAC1 proteins apparently belong to two independent transport channels (see Fig. 5, *e*, *f*). We hypothesize that the outer membrane companion protein for Tim17-1 might be one of the other porin isoforms (see Fig. 5, *a*, *e*). The absence of complete inhibition of DNA import into *Arabidopsis* mitochondria upon inactivation of any of the studied membrane proteins is consistent with the hypothesis that these organelles have multiple DNA transfer pathways (Weber-Lotfi et al., 2015; Tarasenko et al., 2021).

The extent to which other VDAC isoforms and Tim17:23 components participate in DNA import, whether there is specificity for them in terms of the size of the transferred molecule, and the nature of the relationship between the VDAC isoforms and the Tim17-2, Tim17-3, Tim23-1, Tim23-3 proteins remains to be determined in further studies.

Conclusion

The study of the role of the TIM17:23 complex proteins of the inner mitochondrial membrane in the DNA import mechanism made it possible to establish their participation in this process, as well as to reveal the possibility of their joint functioning with VDAC1. Given that the specificity of DNA import with respect to the size of the transferred molecule is likely determined at the level of the inner membrane, our data have deepened our understanding of the import mechanism and expanded the possibilities for developing a system for transforming the mitochondrial genome.

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ORCID

T.A. Tarasenko orcid.org/0000-0002-2830-4175
V.I. Tarasenko orcid.org/0000-0001-8208-6941
M.V. Koulintchenko orcid.org/0000-0003-0931-2863
Yu.M. Konstantinov orcid.org/0000-0002-0601-2788

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Photochemical activity in developing pea (*Pisum sativum* L.) cotyledons depends on the light transmittance of covering tissues and the spectral composition of light

G.N. Smolikova[1, 2] ✉, N.V. Stepanova[1, 2], A.M. Kamionskaya[2], S.S. Medvedev[1]

[1] Saint Petersburg State University, St. Petersburg, Russia
[2] Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences, Moscow, Russia
✉ g.smolikova@spbu.ru

Abstract. Many crops require not only leaf photosynthesis for their seed development but also the photochemical reactions that occur in the seeds. The purpose of this work was a comparative analysis of light transmittance and photochemical activity in the leaves of *Pisum sativum* L. and its pericarp, seed coat, and cotyledons at the early, middle, and late maturation stages. The spectral composition of light was measured using a spectroradiometer in the range of 390–760 nm. We assessed the light transmittance of plant tissues by placing the plant tissue between the light source and the spectroradiometer's sensor. PAM fluorometry was used to quantify the photochemical activity in plant tissues: this technique is handy for evaluating the efficiency of converting light energy into chemical energy through the analysis of the kinetics of chlorophyll fluorescence excitation and quenching. On average, a photochemically active green leaf of pea transmitted 15 % of solar radiation in the 390–760 nm, blue light was delayed entirely, and the transmitted red light never exceeded 5 %. Photochemically active radiation passing through the pericarp and coat and reaching the cotyledons at the early and middle seed maturation stages manifested a high proportion of green and far-red light; there was no blue light, and the percentage of red light was about 2 %. However, the cotyledons were photochemically active regardless of low irradiance and spectral ranges untypical of leaf photosynthesis. At the early and middle maturation stages, the maximum quantum yield of photosystem II (*Fv*/*Fm*) averaged 0.5 at the periphery of cotyledons and 0.3 at their center. Since the intensity of embryonic photochemical reactions significantly affects the efficiency of reserve nutrient accumulation, this parameter is a promising marker in pea breeding for seeds with improved nutritional qualities.
Key words: *Pisum sativum* L.; seed maturation; light transmittance of tissues; illumination intensity; photochemically active radiation; photochemical activity; PAM fluorometry.

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Фотохимическая активность формирующихся семядолей гороха (*Pisum sativum* L.) зависит от светопропускания покровных тканей и спектрального состава света

Г.Н. Смоликова[1, 2] ✉, Н.В. Степанова[1, 2], А.М. Камионская[2], С.С. Медведев[1]

[1] Санкт-Петербургский государственный университет, Санкт-Петербург, Россия
[2] Институт биоинженерии им. К.Г. Скрябина, Федеральный исследовательский центр «Фундаментальные основы биотехнологии» Российской академии наук, Москва, Россия
✉ g.smolikova@spbu.ru

Аннотация. У многих сельскохозяйственных растений для формирования семян необходимы не только фотосинтез листьев, но также фотохимические реакции, происходящие в семенах. Цель нашей работы заключалась в сравнительном анализе светопропускания и фотохимической активности листьев, перикарпия, кожуры и семядолей *Pisum sativum* L. на ранней, средней и поздней стадиях созревания семян. Спектральный состав света измеряли при помощи спектрорадиометра в области 390–760 нм. Светопропускание растительных тканей оценивали, разместив растительную ткань между источником освещения и датчиком спектрорадиометра. Фотохимическую активность растительных тканей определяли методом PAM-флуориметрии, позволяющим оценить эффективность преобразования световой энергии в химическую путем анализа кинетики возбуждения и гашения флуоресценции хлорофиллов. Фотохимически активный зеленый лист гороха пропускал в среднем 15 % солнечной радиации в диапазоне 390–760 нм; при этом синий свет задерживался полностью, а количество проходящего красного света составляло не более 5 %. Фотохимически активная радиация, проходящая сквозь перикарпий и кожуру и

достигающая семядолей на ранней и средней стадиях созревания семян, характеризовалась высокой долей зеленого и дальнего красного света, при этом синий свет отсутствовал, а количество красного света составляло около 2 %. Однако, несмотря на низкую энергетическую освещенность и спектральные диапазоны, не характерные для фотосинтеза листа, семядоли были фотохимически активными. На ранней и средней стадиях созревания максимальный квантовый выход фотосистемы II (*Fv/Fm*) в среднем составлял 0.5 на периферии семядолей и 0.3 в центре семядолей. Поскольку интенсивность эмбриональных фотохимических реакций в значительной степени влияет на эффективность накопления запасных питательных веществ, этот параметр является перспективным маркером для селекции семян гороха с улучшенными пищевыми качествами.
Ключевые слова: *Pisum sativum* L.; созревание семян; светопропускание тканей; интенсивность освещения; фотохимически активная радиация; фотохимическая активность; PAM-флуориметрия.

Introduction

Seed-based products represent nearly three-quarters of human food, making high-quality seed production the foundation of food security (Mattana et al., 2022). An essential factor in plant seed productivity is photosynthesis, which occurs in leaves and provides developing seeds with the necessary assimilates (Simkin et al., 2019a, 2010; Walter, Kromdijk, 2021). Therefore, most studies aimed at developing approaches that could increase crop productivity have focused on analyzing leaf photosynthetic processes. Meanwhile, other plant organs (petioles, stems, inner bark, and fruits) can also synthesize chlorophylls and develop actively functioning chloroplasts, where the so-called non-foliar photosynthesis occurs (Aschan, Pfanz, 2003; Tikhonov et al., 2017; Hu L. et al., 2019; Henry et al., 2020; Simkin et al., 2020; Yanykin et al., 2020).

The presence of green pigments in the embryos, as well as in the pericarp and seed coat of angiosperms, has been known since the middle of the 19th century (Hofmeister, 1859; Flahault, 1879; Monteverde, Lyubimenko, 1909). According to the analysis of the pigments in the maturing seeds of rape, they contained chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b*, and, in minor amounts, pheophorbide *a*, methyl pheophorbide *a*, and pyropheophorbide (Ward et al., 1994). At the same time, the total chlorophyll content per unit of wet weight and the ratio of chlorophylls *a*/*b* were lower in green embryos than in leaves (Bulda et al., 2008; Smolikova et al., 2011, 2018, 2020). Comparison of the chlorophylls and carotenoids content in the leaves of shade-adapted plants and in the green embryos of developing oilseeds revealed it to be approximately equal (Ruuska et al., 2004).

Non-foliar green tissues of C_3 plants can reassimilate CO_2 released during respiration, providing up to 15–48 % of the total carbon dioxide assimilated during photosynthesis (Hu L. et al., 2019). However, the contribution of these tissues to the total amount of assimilates synthesized in the light is often ignored. Non-foliar photosynthesis can also occur in the developing seeds of many plant species (Borisjuk et al., 2003; Allorent et al., 2015; Smolikova, Medvedev, 2016; Smolikova et al., 2017, 2018, 2020; Brazel, Ó'Maoiléidigh, 2019; Hu L. et al., 2019; Grulichova et al., 2022; Shackira et al., 2022).

Embryologists from the Komarov Botanical Institute of the Russian Academy of Sciences (St. Petersburg) were the first in the world to study the genesis and structure of plastids in the embryos of more than 1,000 plant species (Yakovlev, Zhukova, 1973, 1980). They identified 428 plant species, the embryos of which contained chlorophylls and plastids with well-developed thylakoid membranes. These species became known as chloroembryophytes. Later, it has been shown that the function of the photosynthetic apparatus in the developing seeds is directed to the synthesis of storage compounds (mainly fatty acids) rather than the monosaccharides as in leaves (Neuhaus, Emes, 2000; Ruuska et al., 2004; Weber et al., 2005; Allen et al., 2009; Hu Y. et al., 2018).

Expression of nuclear genes responsible for the process of photosynthesis was observed in *Arabidopsis* and rapeseed embryos starting from the globular stage of embryogenesis (Spencer et al., 2007; Le et al., 2010; Belmonte et al., 2013; Kremnev, Strand, 2014). The priority function of seed chloroplasts is the rapid synthesis of NADPH and ATP, which are used to convert sucrose supplied from the mother plant into acetyl-CoA and fatty acids and further into triglycerides (Ruuska et al., 2004; Allen et al., 2009; Puthur et al., 2013; Wu et al., 2014; Allorent et al., 2015; Shackira et al., 2022). It means that reserve nutrient accumulation in seeds depends on the efficiency of embryo photochemical reactions. For example, rape (*Brassica napus* L.) pods shielded from light during their development had significantly decreased seed weight and proteins and fatty acids content (Wang et al., 2023).

Seed embryos are typically covered with seed and pod coats, hindering the exchange of carbon dioxide and oxygen and shielding from sunlight. A crucial aspect of photo-dependent synthetic reactions in seed embryos involves using sucrose supplied from the mother plant and CO_2 released through respiration, rather than atmospheric CO_2, as a carbon source (Ruuska et al., 2004). At the same time, the O_2 released during photooxidation of water prevents hypoxia and supports mitochondrial respiration in developing seeds (Borisjuk et al., 2003; Weber et al., 2005; Borisjuk, Rolletschek, 2009; Tschiersch et al., 2011; Shackira et al., 2022). Recently, it has been shown in soybean (*Glycine max*) plants that non-foliar photosynthesis occurring in the pericarps and coats contributes up to 9 % of the total daily carbon assimilation and can compensate for up to 81 % of the carbon loss by respiration of these tissues (Cho et al., 2023). Nevertheless, in-depth studies are needed to investigate the mechanisms of photo-dependent synthetic reactions related to the accumulation of reserve nutrients.

Therefore, it remains unclear how developing seeds receive sufficient light to generate the energy for photochemical reactions. No detailed research has been conducted to determine the spectral characteristics of the light inside the seed embryos.

This study aimed to conduct a comparative analysis of light transmittance and photochemical activity between the leaves and tissues of developing pea seeds (pericarps, coats, cotyledons).

Materials and methods

Common pea (*Pisum sativum* L.) plants of the vegetable cv. Prima were used as the **material** in this study. This cultivar was approved for cultivation in the Central and North Caucasus and was added to the State Register (National List) in 2015. Seeds are wrinkled, large-sized, with green cotyledons (Besedin, 2015). Plants were grown in outdoor plots at St. Petersburg State University during the summer of 2022 under natural lighting conditions. We examined seeds at the early, middle, and late maturation stages, as shown in Fig. 1. The early maturation stage is marked by the end of embryo development and the start of reserve nutrient accumulation in the cotyledons (Smolikova et al., 2018, 2020). At the middle maturation stage, reserve nutrients are synthesized actively, causing the cotyledons to expand and fill the inner space of the seed. Finally, the seeds lose their moisture at late maturation, develop desiccation tolerance, and enter dormancy.

The spectral composition of light was measured using the spectroradiometer TKA "Spectr" (St. Petersburg, Russia). The device detects light spectral characteristics in the spectrum's visible range from 390 to 760 nm. The recorded irradiance spectral density was expressed in energy units per m^2 (mW/m^2).

We **evaluated the light transmittance of plant tissues by placing** the plant tissue between the light source and the sensor of the spectroradiometer. Natural solar radiation served as the source of light.

The photochemical activity of plant tissues was quantified by pulse amplitude modulation (PAM)-based fluorometry using a Walz MINI-PAM-II/B (Heinz Walz GmbH, Germany) according to the manufacturer's protocol (MINI-PAM-II: Manual for Standalone Use, 2018). The device is equipped with measuring and actinic light sources with an emission maximum of 470 nm and fluorescence detection at wavelengths > 630 nm. The measuring and active light intensities were 0.05 and 190 μM photons/($m^2 \cdot s$), respectively. The saturating pulse intensity was 5000 μM photons/($m^2 \cdot s$) with a duration of 0.6 s. Plant tissues (leaves, pericarps, coats, and cotyledons) were isolated from the mother plant, placed on moist filter paper (to prevent drying), and kept in light-proof boxes for 20 min for dark adaptation. Leaf clip 2030-B was used to hold the tissues. The following fluorescence ratio parameters were evaluated:

Fv/*Fm*, i. e., the maximum photochemical quantum yield of photosystem II (PSII) when all electron carriers in the electron transport chain (ETC) of chloroplasts are oxidized. It is detected immediately after dark adaptation of the tissue. *Fv*/*Fm* is calculated as the ratio of the light quantum used for the charge separation to the total amount absorbed by light-harvesting complexes (LHC):

$$Fv/Fm = (Fm–Fo)/Fm,$$

where *Fo* is the minimum level of fluorescence under measuring light that does not excite the transfer of electrons from donors to acceptors; *Fm* is the maximum level of fluorescence elicited by a saturation pulse that saturates all reaction centers (RC) of PS with electrons; *Fv* is the variable fluorescence, calculated by subtracting *Fo* from *Fm*.

Y(*II*), i. e., the effective quantum yield of photochemical quenching, measured in the light-adapted samples:

$$Y(II) = (Fm'–Fo)/Fm'.$$

NPQ, i. e., the non-photochemical quenching of fluorescence. It is calculated using the Stern–Volmer equation, according to which fluorescence quenching is proportional to the number of quenching centers in the LHC:

$$NPQ = Fm/Fm' – 1.$$

Statistical data processing and software. Three biological replicates were performed for each measurement. Quantitative chlorophyll fluorescence parameters and corresponding design ratios were obtained using the WinControl-3 program (Heinz Walz GmbH, Germany). Statistical processing was done in the Microsoft Excel 2023 software using a standard data analysis package. The graphs and tables present arithmetic means and standard deviations. All data were expressed as an arithmetic mean ± standard deviation and processed using Excel for Microsoft 365 with embedded statistical data analysis tools. A two-way analysis of variance (ANOVA) with replications was performed. Differences were considered statistically significant at a confidence level of $p \leq 0.05$.

Results

We studied the dynamics of light transmission in the pericarp, seed coat, and cotyledon tissues during the seed development of pea plants. The images of pods, seeds, and embryos are shown in Fig. 1.

Light transmittance was assessed by placing the plant tissue between the sunlight and the spectroradiometer's sensor. Solar radiation served as a control reference, taken as 100 %. We compared photosynthetically active green leaves, senescent yellow leaves, the pericarps, coats, and the summed combination of the pericarps and coats. The spectrum of solar radiation reaching the pod tissue is shown in Supplementary Material (*a*)[1]. Photosynthetically active green leaves of pea plants completely blocked blue and red light in the ranges corresponding to the chlorophyll and carotenoid absorption maxima, transmitted part of green and yellow light, and fully transmitted far-red light (see Supplementary Material, *b*). With leaf senescence, chlorophylls degraded, and the amount of transmitted blue and red light increased (see Supplementary Material, *c*).

Green tissues of the pericarps and coats at the middle maturation stage transmitted blue and red light (see Supplementary Material, *d*, *e*). However, together, they delayed it; as a result, the cotyledons received mainly light in the range of 500–650 and 700–770 nm and a small amount of light in the range of 600–700 nm (see Supplementary Material, *e*). The high light transmittance ("transparency") of the pericarp is illustrated in Fig. 1, *a*.

We then assessed the dynamics of light transmittance (Fig. 2) and the associated photochemical activity (Fig. 3) during pea seed maturation at the early and middle stages and the beginning of the late stage. The spectral radiance

[1] Supplementary Material is available at:
https://vavilov.elpub.ru/jour/manager/files/Suppl_Smolikova_Engl_27_8

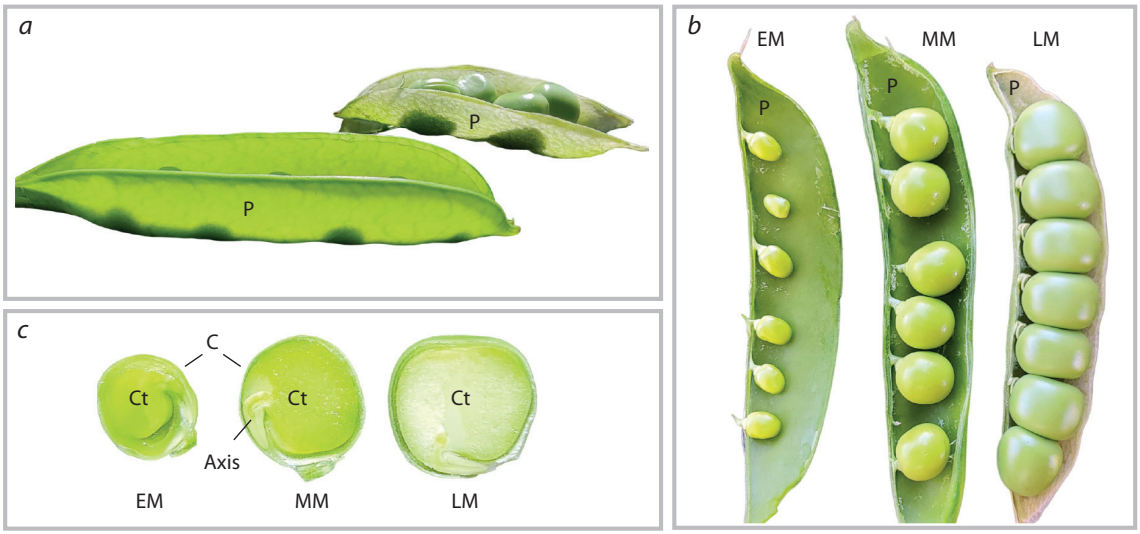

Fig. 1. The images of pea pods and seeds at the early, middle, and late maturation stages (EM, MM, and LM, respectively).

a – the photo demonstrates high light transmittance of the pericarp; *b* – pods with seeds; *c* – seeds in longitudinal section; P – pericarp; C – coat; Ct – cotyledon; Axis – embryonic axis including the root, hypocotyl, epicotyl, and plumule.

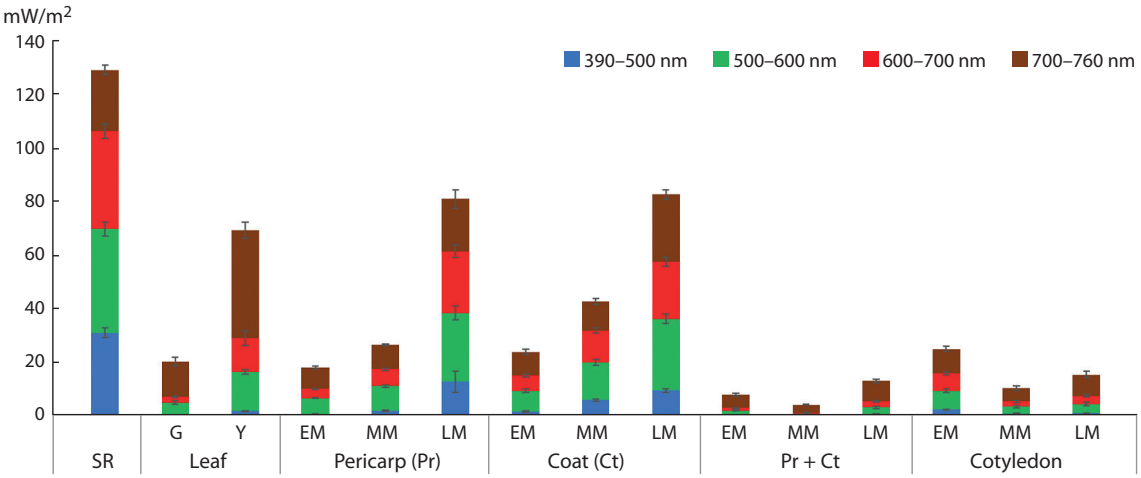

Fig. 2. Light transmittance in the tissues of *P. sativum* at the early (EM), middle (MM), and late (LM) maturation stages.

Blue, green, red, and brown bars show the spectral radiance density (mW/m^2) in the ranges of 390–500, 500–600, 600–700, and 700–760 nm, respectively. SR – solar radiation. Data are presented as the means ± standard deviation obtained in three biological replicates.

density (SRD) of solar radiation reaching the surface of the leaves and the pericarp averaged 136 mW/m^2 (see Fig. 2). The proportions of blue and red light were 32 and 39 mW/m^2 (1:1), respectively. We took these values as 100 % and then calculated the percent of the "transmitted" light.

The photochemical activity in green leaves was high (*Fv*/*Fm* = 0.71 ± 0.01, and *Y*(*II*) = 0.65 ± 0.01) (see Fig. 3). A green leaf transmitted about 20 mW/m^2 (15 %) (see Fig. 2). No blue light was transmitted, and the transmitted red light intensity was about 2 mW/m^2 (5 %). As the leaf senesced, chlorophylls degraded, and photochemical activity decreased (*Fv*/*Fm* = 0.33 ± 0.03, and *Y*(*II*) = 0.18 ± 0.03). As a result, 69 mW/m^2 (51 %) passed through the senesced yellow leaf, with the proportion of blue and red light increasing to 1.7 mW/m^2 (5 %) and 12.7 mW/m^2 (32 %), respectively. Leaf senescence was accompanied by an increase in the *NPQ* (non-photochemical fluorescence quenching) from 0.07 ± 0.01 up to 0.12 ± 0.01.

Pericarp. At the early stage of seed maturation, the pericarp transmitted 18 mW/m^2 (13 %), which is close to the values manifested by the green photosynthetic leaf (see Fig. 2). However, the proportions of blue and red light transmitted by the pericarp were higher, amounting to 0.5 mW/m^2 (1.5 %) and 3.5 mW/m^2 (8.9 %), respectively. The photochemical activity was increased (equivalent to that of the green leaf). It amounted to 0.69 ± 0.02 for *Fv*/*Fm* and 0.68 ± 0.01 for *Y*(*II*) (see Fig. 3). At the middle stage of seed maturation, the amount of light transmitted by the pericarp increased to 26 mW/m^2 (19 %), with the blue and red light reaching 1.8 mW/m^2 (5.6 %) and 6.4 mW/m^2 (16.4 %), respectively. Meanwhile, the

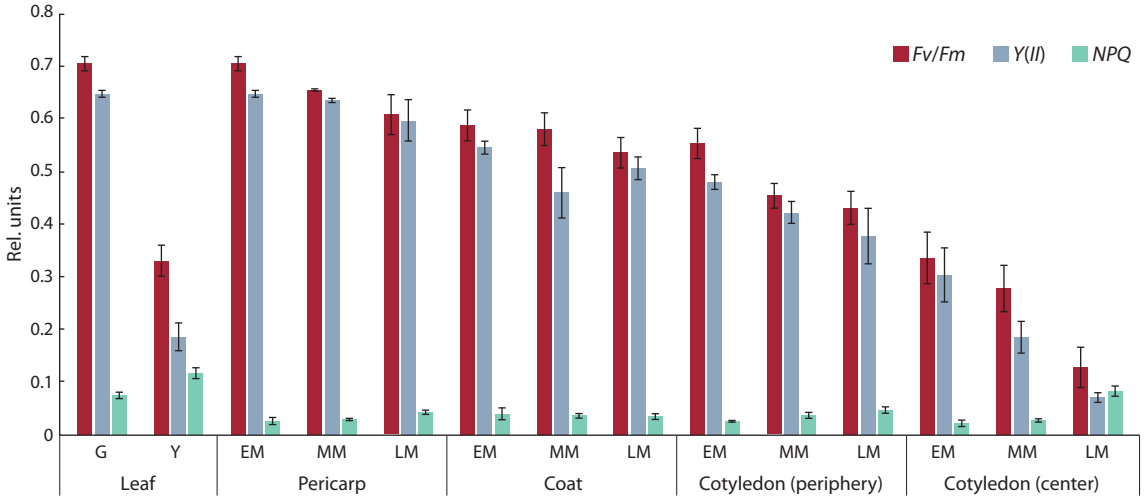

Fig. 3. Photochemical activity in the tissues of *P. sativum* at the early (EM), middle (MM), and late (LM) maturation stages.

Fv/*Fm* and *Y*(*II*) are the maximum and effective quantum yields of the PSII photochemical efficiency, respectively. *NPQ* is the non-photochemical quenching of chlorophyll fluorescence. Data are presented as the mean ± standard deviation obtained in three biological replicates. A two-way analysis of variance (ANOVA) with replications showed significant changes in the principal factor "photochemical activity" ($F(2) = 1282$, $p < 0.001$), the principal factor "plant tissues" ($F(13) = 63$, $p < 0.001$), and the interaction between the two factors ($F(26.84) = 19$, $p < 0.001$).

photochemical activity declined slightly (*Fv*/*Fm* = 0.65 ± 0.01; *Y*(*II*) = 0.64 ± 0.01). A more significant decrease in *Fv*/*Fm* occurred during the transition to late maturation (*Fv*/*Fm* = = 0.61 ± 0.04; *Y*(*II*) = 0.60 ± 0.04). At the same time, the pericarp tissue turned even more translucent: the transmitted light increased to 81 mW/m^2 (60 %), with 12.6 mW/m^2 (39 %) for blue light and 23.1 mW/m^2 (59 %) for red light.

In the **seed coat**, *Fv*/*Fm* and *Y*(*II*) did not change significantly from the early to late stage of maturation but were slightly lower than in the pericarp (see Fig. 3). The total transmitted light amount increased from 24 to 83 mW/m^2 (18 to 61 %), the amount of blue light, from 1.5 to 9.4 mW/m^2 (4.6 to 24.1 %), and red light, from 5.7 to 21.4 mW/m^2 (17.8 to 54.8 %) (see Fig. 2).

Cotyledons. "Pericarp + coat" (P + C) characterizes the amount and spectral composition of the light transmitted through the pericarp and coat and reaching the cotyledons (see Fig. 2). At the photochemically active early and middle stages of seed maturation, the amount of transmitted light never exceeded 8 mW/m^2 (6 %), with no blue light, and less than 1 mW/m^2 (less than 2 %) of red light (see Fig. 2). Surprisingly, photochemical processes took place in the cotyledons even at such low levels of light energy, albeit with low efficiency. The photochemical activity of the cotyledons was assessed externally (at the periphery) and internally (by longitudinal sectioning). At early maturation, *Fv*/*Fm* was 0.55 ± 0.03 at the periphery of the cotyledons and 0.33 ± 0.05 inside them (see Fig. 3). At late maturation, *Fv*/*Fm* decreased to 0.43 ± 0.03 at the periphery of the cotyledons and 0.13 ± 0.04 in their center. *Y*(*II*) showed similar dynamics but was lower than *Fv*/*Fm*. At this stage, we also observed an increase in the *NPQ* index, characterizing the non-photochemical fluorescence quenching (from 0.02 ± 0.01 to 0.08 ± 0.01).

It was interesting to note that the cotyledons were also transparent to sunlight. At early maturation, they transmitted 25 mW/m^2 (18 %), which was about the same as for the pericarp and coat (see Fig. 2). At the same time, they transmitted more blue light (2.2 mW/m^2, 6.9 %) and red light (6.4 mW/m^2, 16.4 %). Later, however, as reserve nutrients accumulated, the level of transmitted light decreased to 10–15 mW/m^2 (8–10 %).

Discussion

Seeds produce a wide variety of storage compounds that directly (as food) or indirectly (as animal feed) provide up to 70 % of the calories required by humans (Sreenivasulu, Wobus, 2013; Ingram et al., 2018; Mattana et al., 2022). The synthesis of storage compounds, limited by the low oxygen diffusion through seed tissues, is complex without significant energy and assimilates provided by photosynthesis (Walter, Kromdijk, 2021). Furthermore, seed development in many plant species (the so-called chloroembryophytes) requires not only the photosynthesis in the leaves of the mother plant but also photochemical processes of ATP and $NADPH^+$ synthesis in the embryos (Borisjuk et al., 2005; Weber et al., 2005; Puthur et al., 2013; Smolikova, Medvedev, 2016; Smolikova et al., 2018, 2020; Sela et al., 2020; Shackira et al., 2022; Cho et al., 2023).

We have previously shown that in the *P. sativum* embryos, the synthesis of chlorophylls and the appearance of chloroplasts with a well-developed granum structure occur at the earliest stages of embryogenesis (Smolikova et al., 2018, 2020). In other words, even though developing pea seeds have covering tissues (pericarps and coats) shielding them from sunlight, they receive sufficient light for synthesizing chlorophylls and transforming plastids into chloroplasts. However, the question remained about the spectral range of light that reaches green embryos and the intensity at which their photochemical activity occurs.

In this study, we examined pea seeds at the early, middle, and late stages of maturation (see Fig. 1). We carried out a comparative analysis of light transmission (see Fig. 2) and

photochemical activity (see Fig. 3) in leaves, pericarps, coats, and cotyledons of developing seeds using the spectroradiometer and the PAM fluorometer.

The 400–700 nm range is known to be the one in which green leaves absorb about 85 %, reflect about 10 %, and transmit about 5 % of light (Atwell et al., 1999). However, these values vary considerably depending on the plant species and growing conditions. In our experiments, photochemically active green pea leaves transmitted an average of 15 % of solar radiation (in the 390–760 nm) with no blue light and no more than 5 % red light.

The photochemically active pericarp tissue at the early and middle stages of pea seed maturation allowed 13 to 19 % of solar radiation to pass to the seed coat; in this case, the share of blue light ranged from 1.5 to 6 %, and that of red light, from 9 to 16 %. The periphery of developing cotyledons received light in 500–650 nm and 700–770 nm (6 % of solar radiation); blue light was utterly absent, and the amount of red light (620–700 nm) was about 2 %. With the senescence of covering tissues at the late stage of seed maturation, chlorophylls decomposed, and the transmitted red light amount that reached the cotyledons increased.

Interestingly, low light energy failed to stop photochemical processes from occurring even in the center of the cotyledons, although their efficiency was low. The photochemical activity of cotyledons was recorded in the almost complete absence of blue light, at a low level of red light, and a relatively high level of yellow and green light. At the early stage of seed maturation, the *Fv*/*Fm* index was 0.55 ± 0.03 at the periphery of the cotyledons and 0.33 ± 0.05 inside them (see Fig. 3).

How can we explain the photochemical activity in cotyledons at low light radiance densities and spectral ranges untypical for leaf photosynthesis? We hypothesize that green light may partially compensate for the lack of blue light in the cotyledons of developing seeds and thus increase the amount of light energy. Such compensation is likely to occur in the range of 500–550 nm, and the carotenoids present in embryos can absorb this light energy (Smolikova, Medvedev, 2015).

Light in the range of 500–600 nm was believed to be of minor importance in plant biology for a long time. Indeed, plant leaves do not absorb photons uniformly across the entire range of photosynthetically active radiation (PAR), and the spectral absorption of green light by chloroplast photosystems is much lower than in the case of blue and red light (Kume, 2017). However, evidence has emerged in recent years that green light is not only absorbed by plant tissues but is involved in the regulation of many physiological reactions (Golovatskaya, Karnachuk, 2015; Smith et al., 2017). Some authors assumed that the blue and red spectra are predominantly absorbed by the surface cells of the leaf's columnar mesophyll, while green light can penetrate deeper layers of the leaf tissue, promoting the excitation of photosystems in spongy mesophyll cells (Nishio, 2000; Terashima et al., 2009; Brodersen, Vogelmann, 2010).

J. Liu and M.W. van Iersel (Liu, van Iersel, 2021) assessed the quantum yield of assimilated CO_2 (*QY*) in lettuce leaves grown under different spectral ranges of illumination (blue, green, and red) and at different photosynthetic photon radiance densities (PPFD) (30–1300 mmol photons/m^2/s). It turned out that the *QY* was higher at a high PPFD under green light illumination than under blue or red-light illumination. The authors speculated that it was because, under intense illumination, green light is more evenly distributed within the leaf. Experiments with sunflower leaves also showed that adding green light under moderate to intense white-light illumination is more effective in stimulating photosynthesis than red light addition (Terashima et al., 2009).

A recently published study (Lv et al., 2022) on *Zingiber officinale* Roscoe plants demonstrated that the addition of green light to the white spectrum not only induced an increase in the *Fv*/*Fm* and *Y*(*II*) photochemical parameters but also led to an increase in the number of starch grains and leaf thickness. Intense green light, on the one hand, led to an increased rate of electron flow along the electron transport chain of PS II and, on the other hand, failed to trigger the accumulation of reactive oxygen species (ROS), usually occurring under light stress caused by red light. The authors attributed this phenomenon to more efficient thermal dissipation of excess green light energy.

Our experiments established that PAR reaches the periphery of developing cotyledons, which includes the green part of the spectrum and a small amount of red light. It is possible that green light penetrating through covering tissues to the embryo can affect carbon assimilation efficiency and serve as a good argument in favor of using green wavelengths in crop cultivation. However, this hypothesis requires additional research.

Conclusion

The data obtained make it possible to better understand the mechanisms of photochemical processes in seed embryos under low light intensity. We believe that the intensity of embryonic photochemical reactions significantly affects the efficiency of reserve nutrient accumulation and, therefore, can be considered a marker for plant breeders seeking to produce seeds with improved nutritional qualities. Research efforts to optimize the production of high-quality seeds by enhancing photochemical activity in their embryos through varying light parameters are also promising.

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ORCID

G.N. Smolikova orcid.org/0000-0001-5238-1851
A.M. Kamionskaya orcid.org/0000-0001-9815-9578
S.S. Medvedev orcid.org/0000-0003-1127-1343

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Studying a collection of common-wheat varieties for leaf rust resistance, crop yield and grain quality in the environmental conditions of Novosibirsk region

L.P. Sochalova[1], V.A. Aparina[1], N.I. Boyko[1], E.V. Zuev[3], E.V. Morozova[1], K.K. Musinov[1], N.A. Vinichenko[2], I.N. Leonova[2, 4], V.V. Piskarev[1]

[1] Siberian Research Institute of Plant Production and Breeding – Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Krasnoobsk, Novosibirsk region, Russia
[2] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[3] Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
[4] Novosibirsk State Agrarian University, Novosibirsk, Russia
piskaryov_v@mail.ru

Abstract. The relationship between a variety's genotype, environmental conditions and phytopathogenic load are the key factors contributing to high yields that should be taken into account in selecting donors for resistance and high manifestation of valuable traits. The study of leaf rust resistance in 49 common wheat varieties was carried out in the field against the natural pathogen background and under laboratory conditions using single-pustule isolates with virulence to *Lr9* and *Lr24*. It has been shown that the varieties carrying alien genes *Lr6Agi2* (Tulaikovskaya 10) and *Lr6Agi1* (Voevoda) were resistant to leaf rust infection both in the field and in the laboratory. Varieties KWS Buran, KWS Akvilon, KW 240-3-13, and Etyud producing crop yields from 417 to 514 g/m^2 comparable to the best standard variety Sibirskaya 17 can be reasonably used as *Lr24* resistance gene donors under West Siberian conditions. Omskaya 44 variety showing crop yield of 440g/m^2 can be used as a donor for *Lr19* and partially effective *Lr26*. Varieties Tuleevskaya and Altayskaya 110 with *Lr9* in their genomes are recommended for the development of resistance gene-pyramided genotypes. The highest protein and gluten contents were observed in the CS2A/2M sample, while KWS Buran, Altayskaya 110, Volgouralskaya, and KWS Akvilon showed the lowest values. Varieties CS2A/2M, Tulaikovskaya 10, Pavon, and Tuleevskaya were ranked the highest in micro- (Cu, Mn, Zn, Fe) and macronutrient (Ca, Mg, K) contents among the common wheat samples from the collection, while the lowest values for most elements were observed in KWS Buran, Novosibirskaya 15, and Volgouralskaya. Winter varieties demonstrating leaf rust resistance against the infectious background typically carry adult plant resistance genes (*Lr34*, *Lr12*, and *Lr13*), particularly combined with the juvenile *Lr26* gene. The presence of *Lr41* in a winter type line (KS 93 U 62) allowed it to maintain resistance against a leaf rust pathogen clone kLr24, despite the presence of *Lr24* in the genotype. Varieties Doka and Cheshskaya 17 may act as donors of resistance genes *Lr26* + *Lr34* and *Lr9* + *Lr12* + *Lr13* + *Lr34*, as well as sources of dwarfing without losses in winter hardiness and yield under West Siberian conditions.

Key words: common wheat; leaf rust; population; isolate; virulence; resistance gene; yield; microelement; macroelement; protein; gluten.

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Изучение устойчивости к бурой ржавчине, урожайности и качества зерна у образцов коллекции мягкой пшеницы в экологических условиях Новосибирской области

Л.П. Сочалова[1], В.А. Апарина[1], Н.И. Бойко[1], Е.В. Зуев[3], Е.В. Морозова[1], К.К. Мусинов[1], Н.А. Виниченко[2], И.Н. Леонова[2, 4], В.В. Пискарев[1]

[1] Сибирский научно-исследовательский институт растениеводства и селекции – филиал Федерального исследовательского центра Институт цитологии и генетики Сибирского отделения Российской академии наук, р.п. Краснообск, Новосибирская область, Россия
[2] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[3] Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
[4] Новосибирский государственный аграрный университет, Новосибирск, Россия
piskaryov_v@mail.ru

Аннотация. К основным факторам, влияющим на формирование высокого урожая, относятся связь генотипа сорта с условиями произрастания и фитопатогенная нагрузка, что необходимо учитывать в селекции для поиска доноров устойчивости и высокой выраженности ценных признаков. Изучение устойчивости 49 образцов мягкой пшеницы к поражению бурой ржавчиной проведено в полевых условиях естественного инфекционного фона и в лабораторных условиях к монопустульным изолятам с вирулентностью к генам *Lr9* и *Lr24*. Показано, что сорта, несущие чужеродные гены *Lr6Agi2* (Тулайковская 10) и *Lr6Agi1* (Воевода), устойчивы к поражению бурой ржавчиной как в полевых условиях, так и при заражении в лаборатории. Сорта KWS Buran, KWS Akvilon, KW 240-3-13 и Этюд, которые формировали урожайность от 417 до 514 г/м² – на уровне лучшего стандарта Сибирской 17, целесообразно использовать в условиях Западной Сибири в качестве доноров гена устойчивости *Lr24*. Донором генов устойчивости *Lr19* и частично эффективного *Lr26* может служить сорт Омская 44, характеризующийся урожайностью 440 г/м². Сорта Тулеевская и Алтайская 110, в геноме которых содержится ген *Lr9*, рекомендуется использовать при создании генотипов с пирамидой генов устойчивости. Наиболее высокие показатели содержания белка и клейковины выявлены у образца CS2A/2M, наименьшие – у сортообразцов KWS Buran, Алтайская 110, Волгоуральская и KWS Akvilon. Сравнение коллекции образцов мягкой пшеницы по микро- (Cu, Mn, Zn, Fe) и макроэлементам (Ca, Mg, K) продемонстрировало наиболее высокие показатели у группы, состоящей из образцов CS2A/2M, Тулайковская 10, Pavon и Тулеевская. Наименьшие показатели большинства элементов определены у сортов KWS Buran, Новосибирская 15 и Волгоуральская. Озимые сорта, характеризующиеся устойчивостью к поражению бурой ржавчиной в условиях инфекционного фона, как правило, несут возрастные гены устойчивости (*Lr34*, *Lr12* и *Lr13*), в том числе в сочетании с ювенильным геном *Lr26*. У линии с озимым типом развития (KS 93 U 62) выявлен ген *Lr41*, благодаря чему линия сохраняла устойчивость к поражению клоном патогена бурой ржавчины кLr24, несмотря на наличие в ее генотипе гена *Lr24*. Сорта Дока и Чешская 17 могут быть донорами генов устойчивости *Lr26*+*Lr34* и *Lr9*+*Lr12*+*Lr13*+*Lr34* и источниками короткостебельности без снижения зимостойкости и урожайности в условиях Западной Сибири.
Ключевые слова: пшеница мягкая; бурая ржавчина; популяция; изолят; вирулентность; ген устойчивости; урожайность; микроэлемент; макроэлемент; белок; клейковина.

Introduction

Common wheat (*Triticum aestivum* L.) is recognized as the primary food crop around the world. It is characterized by balanced composition of protein, starch, fiber, fat, and mineral elements, while also including vitamins C, B, A, E, D, K, beta carotene etc. (Roshan et al., 2016) and demonstrating high adaptability to growing conditions (Pryanishnikov, 2018). According to the data for 2022 (Rosstat), the area under crops for spring wheat varieties in the Novosibirsk region was 222,808 ha with crop yield of 21 centner/ha. The area for winter varieties was about 34,000 ha with crop yield of 28 centner/ha. The key factors contributing to high crop yields are the relationship between a variety's genotype, its growing conditions (Malchikov, Myasnikova, 2012) and phytopathogenic background. Developing a high-yielding variety requires taking these factors into account while selecting donors for resistance and high intensity of valuable agronomic traits (Volkova et al., 2016).

Wheat leaf rust is among the most common diseases found in bread wheat in West Siberia, as it affects both winter and spring varieties and reduces crop yields by 15–40 % in epiphytotic years (Kolmer et al., 2015). There is a set of requirements applying to developing and handling resistance gene donors, since the use of identical genes in spring and winter varieties may lead to an epiphytotic outbreak, if the pathogen overcomes the defenses ensured by the gene (Volkova et al., 2016; Pozherukova et al., 2019). Thus, winter and spring varieties require different effective resistance genes and their combinations for protection against the infection, which implies continuous research efforts to find new resistance genes.

Over 80 *Lr* genes have been identified around the world, with about 50 % classified as alien ones[1]. The list of genes used in commercial common wheat varieties includes *Lr9*, *Lr19*, *Lr21*, *Lr23*, *Lr24*, *Lr26*, *Lr28*, *Lr37*, *Lr39* (Aktar-Uz-Zaman et al., 2017; Leonova, 2018), *Lr6Agi1*, *Lr6Agi2* (Sibikeev et al., 2017), and *LrSp2* (Adonina et al., 2018). In Russia, breeding value is assigned to the samples carrying partially effective genes *Lr9*, *Lr19*, *Lr24*, *Lr25*, *Lr26*, *Lr6Agi1*, *Lr6Agi2* and highly effective protective genes *Lr28*, *Lr29*, *Lr39*, *Lr42*, *Lr45*, *Lr47*, *Lr50*, *Lr51*, *Lr66*, *LrSp2* (Gultyaeva, Shaydayuk, 2021; Sochalova et al., 2022).

The use of wheat varieties carrying resistance genes from relative species (*Aegilops*, *Agropyron*, *Secale cereale*, etc.) for hybridization makes it possible to extend the diversity of resistance genes, although the latter are often linked to the factors reducing crop yields or quality (Markelova, 2007; Krupin et al., 2019). It was found that the presence of a fragment carrying *Lr9* (transferred from *Aegilops umbellulata*) reduced crop yield in the United States (Friebe et al., 1996), while commercial varieties carrying this gene are available in Russia (Gultyaeva, Shaydayuk, 2021). The presence of alien material (gene *Y* determining an increase in yellow pigment synthesis in endosperm) linked to gene *Lr19* (transferred from *Agropyron elongatum*) reduced the

[1] Komugi – wheat genetic resources database. Available: https://shigen.nig.ac.jp/wheat/komugi/genes/symbolClassListAction.do?geneClassificationId=89 (accessed on March 9, 2023).

value of the first donors carrying this gene (Knott, 1968). Later, the locus carrying *Lr19/Sr25* was successfully separated from gene Y using *ph1b* deletion lines (Marais, 1992; Zhang et al., 2005). A chromosome segment carrying *Lr19* was shown to have a positive effect on crop yield (Singh et al., 1998), and a number of varieties with this gene are currently in production in Russia (Gultyaeva, Shaydayuk, 2021). The presence of a fragment carrying *Lr38* (transferred from *Thinopyrum intermedium*) in the wheat genome causes a significant reduction in crop yield (Mebrate et al., 2008) similarly to the presence of a chromosome segment including *Lr47* (transferred from *Aegilops speltoides*), which on top of that has a negative effect on quality (Brevis et al., 2008). Introduction of a wheat-rye translocation 1B.1R carrying genes *Lr26*, *Pm8*, *Sr31* leads to deteriorating quality of flour and bread (Kumlay et al., 2003). The use of currently available common wheat lines and varieties with alien translocations makes breeding efforts significantly easier, as it does not require obtaining new breeding material with the primary transfer from relative species (Timonova et al., 2012). Direct hybridization are not always successful, and translocations may be partially lost in the offspring upon further reproduction (Davoyan et al., 2015).

Among other things, selection of pairs for crosses is guided by environmental and geographic differences, which is explained by the high diversity of the genotypes obtained as a result of transgressions in segregating generations in crosses between varieties intended for and adapted to different conditions (Vyushkov, 2004). However, the adaptability of alien samples to local conditions is to be taken into account (Davydova, Kazachenko, 2013), because the use of environmentally distant samples with low adaptability produces a significant number of low-yielding genotypes in the offspring, which complicates the development of commercial varieties (Souza, Sorrells, 1991). The use of landraces as donors is complicated by the lack of research and their heterogeneity, since they were created as populations and have multiline nature. Thus, modern varieties of Russian and foreign breeds appear to be the best source for breeding, but a comprehensive investigation of their behavior under local conditions is required beforehand.

We suppose that selection of wheat leaf rust resistance donors is relevant in a close connection with target soil and climatic conditions, as well as with type of development. Therefore, the goal of the present paper was to perform a comprehensive investigation of the collection of common wheat varieties in the Novosibirsk region to identify donors of effective resistance genes for *Puccinia triticina* Erikss.

Materials and methods

In the present paper, we studied a collection common-wheat samples including 24 spring varieties and 25 winter varieties, among which 41 samples were from the VIR global collection and eight new spring varieties had been recently tested in Novosibirsk branch of the State commission of the Russian Federation for selection achievements test and protection (FSBI "GOSSORTCOMMISSION").

The field resistance to local population of leaf-rust pathogens was studied against the natural spread of the infection according to the VIR methodology (Merezhko et al., 1999) and against the artificially increased infectious background (sowing of susceptible winter wheat varieties, spraying the seedlings early in the morning with water and urediniospore mixture upon the emergence of the disease). Crop yield and its components (1000 grain weight, grain weight per spike, grain number per spike) was evaluated in the samples for 2–4 years (within the 2015–2020 evaluation of samples in collection nurseries) according to the VIR methodology developed for new acquisitions (Merezhko et al., 1999). The leaf rust resistance at juvenile stage was studied under laboratory conditions at the Siberian Research Institute of Plant Production and Breeding (SibNIIRS, Krasnoobsk, Novosibirsk region) in leaf fragments (Mikhailova, Kvitko, 1979). The samples were inoculated with water suspension of urediniospores prepared from the local population of *P. triticina* collected in 2020 from wheat varieties cultivated under natural conditions in the SibNIIRS fields (virulence for varieties and lines with genes *Lr1*, *Lr2a*, *Lr2c*, *Lr3a*, *Lr9*, *Lr16*, *Lr3ka*, *Lr11*, *Lr17*, *Lr30*, *Lr2b*, *Lr3bg*, *Lr14a*, *Lr14b*, *Lr15*, *Lr18*, *Lr20*; avirulence to *Lr24*, *Lr19*, *Lr41*, *Lr45*, *Lr47*, *Lr28*, *Lr6Agi1*, *Lr6Agi2*, *LrSp2*, and *Lr26*) and two testing clones: к*Lr24* (virulence to *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, *Lr3ka*, *Lr11*, ***Lr24***, *Lr17*, *Lr30*, *Lr2b*, *Lr3bg*, *Lr14a*, *Lr14b*, *Lr15*, *Lr18*, *Lr20*; avirulence to *Lr9*, *Lr16*, *Lr26*, *Lr19*) and *kLr9* (virulence to *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, ***Lr9***, *Lr16*, *Lr3ka*, *Lr11*, *Lr17*, *Lr30*, *Lr2b*, *Lr3bg*, *Lr14a*, *Lr14b*, *Lr15*, *Lr18*, *Lr20*; avirulence to *Lr24*, *Lr26*, *Lr19*). A clone with virulence to *p24* was isolated from variety Novosibirskaya 15 during the study of race composition of the population from the Kuibyshev District of the Novosibirsk region. A clone with virulence to *p9* was isolated from variety Chelyaba 2 (*Lr9*) cultivated in the collection nursery in the settlement of Krasnoobsk. Agent (with *Lr24*) and Udacha (with *Lr9*) were used as control varieties. Infection response type (IT) was determined on the 8–10th day after inoculation using the scale proposed by E.B. Mains and H.S. Jackson (1926), with 0, 1, 2 representing resistant response; 3, 4 susceptible response, and X heterogeneous response (Mains, Jackson, 1926). Virulence of the population and clones was determined in isogenic Thatcher lines and varieties carrying the known resistance genes. The severity of the damage done to the varieties in presence of artificial infectious background was estimated according to the quantitative scale proposed by R.F. Peterson et al. (1948). Novosibirskaya 15 variety was used as a susceptible control in the field and in the laboratory.

Total DNA was isolated from 5–7-day seedlings using the method proposed by J. Plaschke et al. (1995). Genotyping

of wheat varieties was performed using the DNA markers developed for wheat leaf rust resistance genes (Supplementary Material 1[2]). Protein and gluten contents were measured using an OmegAnalyzer G near-infrared spectrometer (Bruins Instruments, Germany). Macro- and micronutrient contents were measured using a ContrAA 800 D atomic absorption spectroscope (Analytik Jena, Germany).

Statistical processing of the results was performed using Statistica 10.0 and MS Excel.

Results

Evaluation of leaf rust resistance of the tested wheat varieties against the 2020 pathogen background has enabled us to identify 20 spring and 21 winter varieties with disease severity rates of 10 % and below (Table 1). The severity rate in Zauralochka, Udacha, Altayskaya 110, and Tuleevskaya varieties carrying *Lr9* gene reached up to 100 % of the susceptibility standard level (Novosibirskaya 15 variety) under field conditions. At the same time, almost all varieties were ranked moderately resistant (score 5) against the natural spread of the infection in the years with maximum pathogen background (Table 2). Juvenile resistance to *P. triticina* was maintained in 20 spring wheat varieties and only 10 winter varieties (Amigo, KS 93 U 50, KS 90 WGRC 10, KS 93 U 40, KS 93 U 62, Poema, Aivina, Kollega, Pervitsa, Vostorg), which implies the presence of adult plant resistance genes in the remaining 11 winter varieties (Knyaginya Olga, Doka, Lebed, Kuma, Batko, Grom, Lidiya, CO 07 W 245, Ritter, Cheshskaya 16, and Cheshskaya 17 (see Table 1).

The results of molecular testing using markers developed for resistance genes *Lr1*, *Lr9*, *Lr10*, *Lr12*, *Lr13*, *Lr16*, *Lr19*, *Lr24*, *Lr26*, *Lr28*, *Lr34*, *Lr41*, and *Lr47* confirmed the presence of postulated *Lr* genes in most varieties being studied. In addition, we found that KWS Buran and KW 240-3-13 varieties studied in collection nurseries carried *Lr24* gene similarly to the other modern varieties from the EU (KWS Akvilon and KWS Torridon). It is worth noting that KW 240-3-13 was infected by the к*Lr9* clone, but resisted the k*Lr24* clone (IT 2, i. e. moderately resistant), while KWS Buran showed heterogeneous response to the k*Lr24* clone and resisted the k*Lr9* clone.

Saratov breed varieties Tulaikovskaya 10 and Voevoda carrying alien genes *Lr6Agi2* and *Lr6Agi1* maintained resistance to pathogen in the field (in particular, against the artificial pathogen background) and to the clone with virulence to *Lr24*. We were unable to find any publicly available information on wheat leaf rust resistance genes carried by the H 15-3 variety, which demonstrated leaf rust immunity against pathogen in field and in the laboratory testing. Based on the genotyping results obtained using molecular DNA markers, resistance genes *Lr12* + *Lr16* + *Lr26* + *Lr34* were found.

The presence of *Lr41* gene detected in the genomes of the winter lines developed at the University of Kansas (USA) (KS 90 WGRC 10 and KS 93 U 62) allowed the line KS 93 U 62 maintain resistance to the к*Lr24* clone, despite the presence of the *Lr24* gene. The KS 93 U 40 line characterized by the presence of two *Lr* genes (*Lr19* + *Lr24*) also maintained resistance to the k*Lr24* clone as the spring varieties carrying *Lr19* (Yuliya, Volgouralskaya, Dobrynya). At the same time, according to the literature, the KS 93 U 50 line carrying the *Lr26* and *Lr24* genes was susceptible to the k*Lr24* clone, but maintained resistance to both the native population and the k*Lr9* clone in the context of increased infectious background. The *Lr19* and *Lr26* genes in the genotype of the Omskaya 44 spring variety effectively protected the plants from both the native population of wheat leaf rust pathogen and clones.

Noteworthy results were obtained for winter varieties characterized with different combinations of resistance genes, e. g., adult plant resistance gene *Lr34* combined with juvenile resistance gene *Lr26* in the Kollega, Poema, Aivina, and Doka varieties allowed them to maintain resistance both against natural pathogen background and pathogen clones (see Table 1). At the same time, the Lebed variety carrying *Lr13* (adult plant resistance gene) in addition to *Lr26* and *Lr24* genes was affected both by the native population and the clones in the juvenile phase and overcame infection in the field against the increased infectious background. A similar response was observed in Lidiya, Cheshskaya 16, and CO 07 W 245 varieties with two adult plant resistance genes (*Lr13* + *Lr34*) identified in the genome.

Effective use of resistance donors implies their fitness to the local conditions, which is why we analyzed the crop yields and manifestation of quantitative traits in a number of varieties tested in various experiments in different years. The selected varieties and lines had been under study for at least two years. Based on field evaluation of valuable agronomic traits, the following high-yielding spring wheat varieties stood out: Voevoda (509.8 g/m^2), KW 240-3-13 (514.1 g/m^2), and Altayskaya 110 (580.0 g/m^2) (see Table 2). On top of that, Voevoda and Altayskaya 110 produced high-yielding spikes (1.69 and 2.00 g) with high number of grains per spike (41.4 and 48.5). The KW 240-3-13 variety produced large grains with high 1000-grain weight (45.1 g). Other results of note included the Volgouralskaya (with high ear grain content of 39.1) and Chelyaba 75 (with 1000 grain weight of 45.3 g) varieties. In context of intensive crop farming practices, special attention is paid to dwarf varieties. Etyud, KWS Akvilon, KWS Torridon, and Tulaikovskaya 10 were not only resistant to the pathogen, but also showed crop yields comparable to the best standard variety Sibirskaya 17 (517.1 g/m^2) while being short-stemmed (62.1–83.8 cm) and so can be recommended as a source for developing leaf rust resistant varieties for intensive crop farming. Etyud, KWS Akvilon,

[2] Supplementary Materials 1 and 2 are available at: https://vavilov.elpub.ru/jour/manager/files/Suppl_Sochalova_Engl_27_8.pdf

Table 1. Evaluation of disease infection rate in common wheat varieties with established *Lr* resistance genes

Variety	Gene: literature data / established by PCR	Field severity, %	Seedling test, infection type			Reference
			kLr24	kLr9	Field population	
		Spring varieties				
Agent	***Lr24*** / ***Lr24***	0	3	0	1–2	GRIS, 2022
KWS Akvilon	*Lr24* / *Lr24*	0	3	1	1–2	Gultyaeva, 2018
Kvintus	*Lr24*, *Lr1* / *Lr24*	0	3	2	1	
KWS Torridon	*Lr24* / *Lr24*	0	1–2	0	1	
KWS Buran	*LrU* / *Lr24*	0	X	1	1–2	
KW 240-3-13	*LrU* / *Lr24*	0	2	3	1	
Cunningham	*Lr24* / *Lr24*	0	3	0	0	GRIS, 2022
Etyud	*Lr24* + *Lr26* / *Lr24*	0	3	0	1–2	Grib, 2019
Udacha	***Lr9*** / ***Lr9***	80–100	0	3	3	Gultyaeva, 2016
Zauralochka	*Lr9* / *Lr9*	80–100	0	3	3	
Altaiskaya 110	*Lr9* + *Lr10* + *Lr1* / *Lr9* + *Lr10* + *Lr1*	70–100	0	3	3	
Tuleevskaya	*Lr9* / *Lr9*	70–100	0	3	3	GRIS, 2022
Julia	*Lr19* / *Lr19*	0	1	1	0	
Volgouralskaya	*Lr19* / *Lr19*	0	1	1	0	
Dobrynya	*Lr19* / *Lr19*	0	0	1	0	
Tulaikovskaya 10	*Lr6Agi2* / –	0	0	0	0	
Voevoda	*Lr6Agi1* / –	0	0	0	0	
Chelyaba 75	*LrSp2* / –	0	0	0	0	Adonina et al., 2018
Odintsovskaya	*LrU* / –*	0	0	0	0	
Omskaya 44	*Lr19* + *Lr26* / *Lr1* + *Lr19* + *Lr26*	0	0	0	0	Meshkova et al., 2021
H 15-3	*LrU* / *Lr12* + *Lr16* + *Lr26* + *Lr34*	0	0	0	0	
CS2A/2M	*Lr28* / *Lr28*	0	0	0	0	Gultyaeva, 2012
Pavon	*Lr47* / *Lr47*	0	0	0	0	Gultyaeva, 2016
Novosibirskaya 15	***Lr10*** + ***Lr1*** / ***Lr10***	100	3	3	3	
		Озимые сорта				
Amigo	*Lr24* + *Lr26* / *Lr24*	0	3	1	1	GRIS, 2022
KS 93 U 50	*Lr42 или Lr24* + *Lr26* / *Lr24*	0	3	0	0	Germplasm releases…, 2022
KS 90 WGRC 10	*Lr41* + *Lr26* / *Lr41*	0	0	0	0	
KS 93 U 40	*LrU* / *Lr19* + *Lr24*	0	0	0	0	
KS 93 U 62	*Lr41* / *Lr24* + *Lr41*	0	0	0	0	Germplasm releases…, 2022
Poema	*LrU* / *Lr26* + *Lr34*	0	0	0	0	
Ivina	*Lr10*, *Lr26*, *Lr34* / *Lr10*, *Lr26*, *Lr34*	0	1	1	1–2	GRIS, 2022
Kollega	*Lr10*, *Lr26* / *Lr10*, *Lr26*, *Lr34*	0	1	0	1	
Knyaginya Olga	*Lr24* + *Lr1* + *Lr34* / *Lr1* + *Lr34*, no *Lr24*	1	3	1	3	

Table 1 (end)

Variety	Gene: literature data / established by PCR	Field severity, %	Seedling test, infection type			Reference
			kLr24	kLr9	Field population	
Pervitsa	*Lr26* / *Lr26* + *Lr1*	0	3	1	1–2	GRIS, 2022
Doka	*Lr26* + *Lr34* / *Lr26* + *Lr34*	5	2	2	X	
Vostorg	*Lr26* + *Lr34* / *Lr26* + *Lr34*	5	1–2	X	1	
Lebed	*LrU* / *Lr1* + *Lr13* + *Lr26* + *Lr34*	0	3	3	3	
Kuma	*Lr34* / *Lr34*	10	3	3	3	GRIS, 2022
Batko	*Lr10* + *LrU* / *Lr10* + *Lr1*	0	3	X	3	
Grom	*Lr10* + *Lr1* + *LrU* / *Lr1* + *Lr34*	0	3	X	3	
Lidiya	*Lr34* + *Lr3* + *LrU* / *Lr34* + *Lr13*	0	3	3	3	Shishkin et al., 2018
CO 07 W 245 (Antero)	*LrU* / *Lr13* + *Lr34*	0	3	3	3	
Ritter	*LrU* / нет *Lr24*	5	3	0	3	
Cheschskaya 16	*LrU* / *Lr13* + *Lr34*	5	3	X	3	
Cheschskaya 17	*LrU* / *Lr9* + *Lr12* + *Lr13* + *Lr34*	10	0	X	3	

Note. Resistance genes presented according to literature data are highlighted in red.
* According to the pedigree, the presence of the *LrSp2* gene is assumed, a dash (–) means that the identification of the *Lr* gene using the PCR method was not performed.

KW 240-3-13, Omskaya 44, and Tulaikovskaya 10 were characterized by high resistance (scores 7–99) to powdery mildew and septoria leaf spot during the years with high pathogen activity, high resistance to septoria leaf spot alone was observed in H 15-3 (score 9), Cunningham, and Pavon (7) varieties, while Voevoda, Tuleevskaya, and KWS Torridon were resistant to powdery mildew (7), which is also a significant trait for selecting pairs for crosses.

Among the leaf rust resistant winter varieties, high crop yields were demonstrated by Doka (589.2 g/m^2) and Cheshskaya 17 (547.7 g/m^2) also characterized by short stems (66.5 and 80.0 cm respectively) and winter hardiness comparable to standard variety Novosibirskaya 40 (score 4.1) (Table 3). In addition, Doka variety produced high number of grains per spike (100.6).

Protein and gluten content varied from 13.4 to 22.95 % and from 25.94 to 46.33 % respectively, with CS2A/2M demonstrating significantly higher values compared to other varieties ($p < 0.001$) (the Figure, Supplementary Material 2). KWS Buran, Altayskaya 110, Volgouralskaya, and KWS Akvilon varieties were characterized by the lowest protein contents below 14 %. The lowest gluten content values were observed in Volgouralskaya, KWS Akvilon, and KWS Buran varieties.

Comparison of micro- (Cu, Mn, Zn, Fe) and macronutrient (Ca, Mg, K) contents in the studied varieties showed that the highest values were observed in the CS2A/2M, Tulaikovskaya 10, Pavon, and Tuleevskaya varieties. The lowest values for most elements were observed in the KWS Buran, Novosibirskaya 15, and Volgouralskaya varieties.

Discussion

Despite the significant advances in biotechnology, hybridization of initial parental forms with further selection of morphotypes of interest (Gultyaeva et al., 2020; Marchenko et al., 2020), in particular, using marker-assisted selection (Stasyuk et al., 2017; Gultyaeva et al., 2018), still remains the prevalent method of developing new wheat varieties. Leaf rust is among the most dangerous diseases of wheat in West Siberia, as it affects both winter and spring varieties. To prevent epiphytotic outbreaks accompanied by dramatic reductions in crop yields of spring varieties, plant breeders have to use different effective resistance genes and their combinations for winter and spring varieties (Krupin et al., 2019). Another significant factor in selecting resistance donors is their fitness to target conditions, because resistance gene donors are often represented by foreign breeds (Gryaznov, Pigorev, 2019; Konkova et al., 2022) or the isogenic lines developed based on foreign cultivars (Koishybaev, 2019), and these genotypes can show reduced crop yields under local conditions due to low adaptability to adverse abiotic environmental stresses. In the present study, we have performed a comprehensive evaluation of wheat leaf rust resistant varieties. So, among the spring varieties carrying *Lr24* gene, German breed varieties (KWS Buran, KWS Akvilon), English KWS Torridon variety, or Ukrainian Etyud variety can be reasonably used as resistance donors for West Siberian conditions, unlike the Australian variety Cunningham producing much lower crop yield (242.4 g/m^2) compared to the minimum crop yield of a standard variety of 408.7 g/m^2 (Novosibirskaya 15). The latter drop in crop

Table 2. Field study results for spring common wheat varieties

Variety	Originator	Average values over the years of study						Resistance score in natural conditions (minimum value over the years of study)		
		Vegetation period, day	Yield, g/m²	Plant height, sm	Grain weight per spike, g	Grain number per spike, pc.	1000 grain weight, g	Powdery mildew	Leaf rust	Septoria
Novosibirskaya 15	Russia	70.0	408.7	86.8	0.91	25.9	34.8	3	1	3
Novosibirskaya 31	Russia	73.4	444.8	94.2	1.02	29.1	35.0	3	3	3
Sibirskaya 17	Russia	80.2	**517.1**	103.4	1.22	32.4	37.7	5	7	5
Etyud	Ukraine	75.0	428.0	60.0	0.95	25.8	36.8	7	99	5
CS2A/2M	Australia	79.0	125.0	70.0	0.29	15.6	19.0	9	99	–
KWS Akvilon	Germany	77.5	417.2	62.1	1.16	32.1	35.2	99	99	7
KW 240-3-13	Germany	83.1	**514.1**	92.0	1.48	32.5	**45.1**	7	99	7
KWS Buran	Germany	78.8	473.3	86.0	1.33	32.3	41.5	5	99	3
KWS Torridon	Great Britain	82.3	366.5	83.8	1.33	34.0	38.9	7	9	5
Cunningham	Australia	85.0	242.4	69.0	1.23	33.1	37.2	5	99	7
Zauralochka	Russia	76.8	356.9	93.4	1.02	29.9	33.9	1	5	3
Udacha	Russia	83.0	377.0	67.3	0.75	25.5	29.2	5	5	–
Tuleevskaya	Russia	83.0	424.8	66.7	0.83	28.6	28.6	7	5	–
Altaiskaya 110	Russia	84.0	**580.0**	100.0	**2.00**	**48.5**	41.2	1	3	–
Julia	Russia	86.5	391.6	86.0	1.14	28.2	39.2	5	9	3
Volgouralskaya	Russia	83.0	380.0	98.0	1.51	**39.1**	38.6	5	9	3
Voevoda	Russia	87.0	**509.8**	103.0	**1.69**	**41.4**	40.8	99	99	5
Chelyaba 75	Russia	81.0	404.4	103.0	1.34	29.6	**45.3**	3	99	3
Omskaya 44	Russia	81.3	440.8	87.5	1.41	37.9	37.6	7	99	7
H 15-3	Germany	92.0	251.0	65.0	1.10	35.7	30.8	5	99	9
Tulaikovskaya	Russia	92.5	405.3	79.9	1.14	31.9	34.6	9	99	7
Pavon	Mexico	96.0	130.0	60.0	0.59	15.9	30.0	3	99	7
Average		82.3	390.4	82.6	1.16	31.1	36.0	–	–	–
Standard deviation		6.2	115.4	15.1	0.37	7.4	6.0	–	–	–

* Septoria leaf spot resistance was not evaluated in the years when the sample was studied.

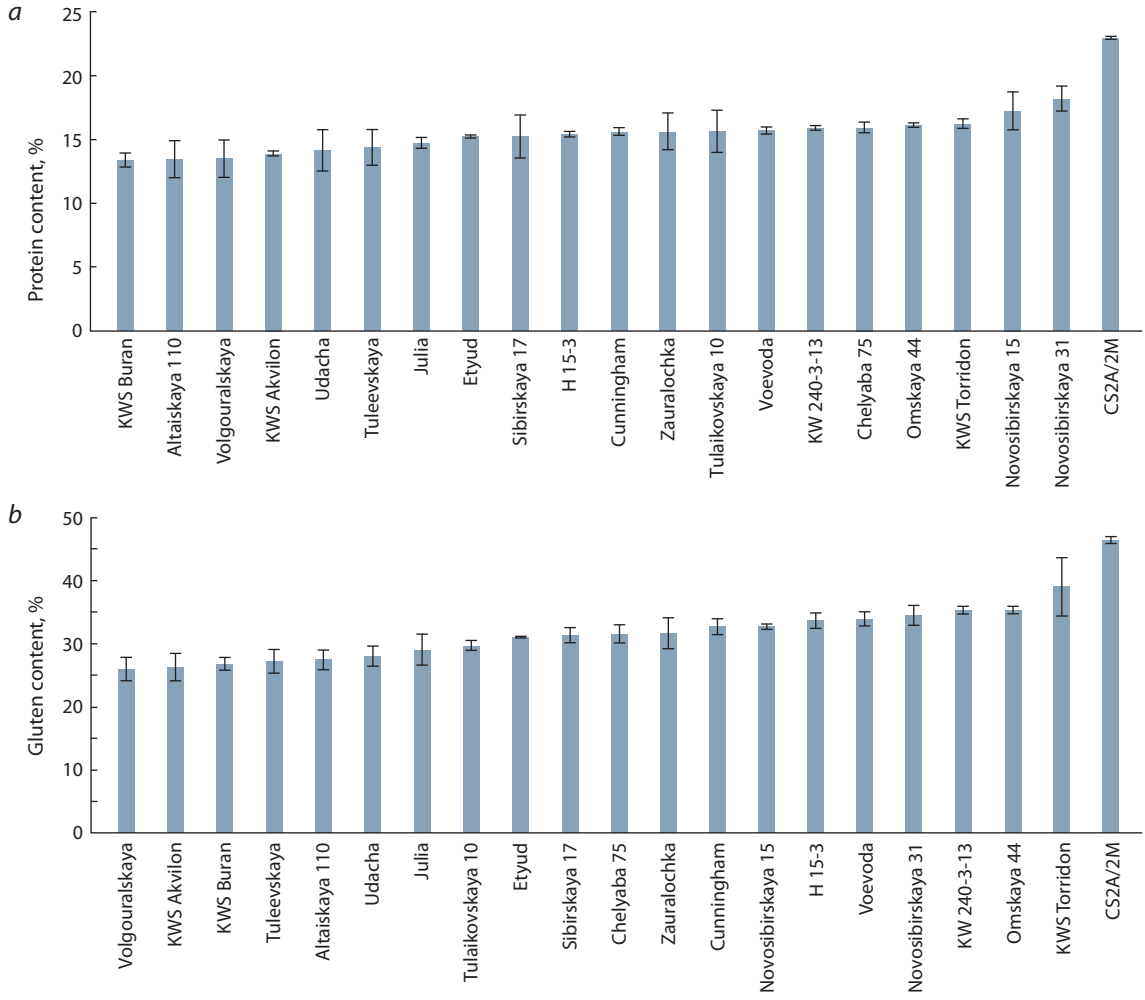
yield has nothing to do with the alien translocation from *Thinopyrum elongatum* (*Lr24*/*Sr24*), but is rather due to low adaptability of the genotype as a whole, which may have a detrimental effect on selection of high-yielding forms, if Cunningham variety is used as a donor for *Lr24* gene.

The *Lr19* gene commonly used in Russian breed varieties (Gultyaeva, Shaydayuk, 2021) still remains rather effective in protecting wheat varieties from leaf rust infection in West and East Siberia (Gultyaeva et al., 2018; Meshkova et al., 2019), despite its defense being compromised in the European part of Russia (Gultyaeva et al., 2020). The Omskaya 44 variety (440.8) can act as a donor for this resistance gene, since it is comparable to the best standard variety Sibirskaya 17 in crop yield (517.1 g/m²). Apart from that, this variety can also act as a donor for the *Lr26* gene, which is partially effective in protecting wheats from leaf rust in West (Gultyaeva et al., 2018) and East Siberia (Meshkova et al., 2019).

Despite the failure to resist infection in the context of infectious background, varieties carrying the *Lr9* gene still have breeding value, since it protects plants from severe infection in context of natural infection spread. Varieties

Table 3. Field study results for winter common wheat varieties

Variety	Originator	Average values over the years of study						
		Vegetation period, day	Winter hardiness, score	Yield, g/m^2	Plant height, sm	Grain weight per spike, g	Grain number per spike, pc.	1000 grain weight, g
Novosibirskaya 40, standart	Russia	319.5	4.1	396.5	107.0	1.66	86.5	27.3
Kollega	Russia	320.5	3.3	274.1	67.5	2.12	85.9	26.5
Doka	Russia	316.8	4.5	589.2	66.5	1.58	100.6	31.9
Cheschskaya 17	Czech Republic	321.5	3.8	547.7	80.0	1.61	72.4	24.8
Cheschskaya 16	Czech Republic	321.5	4.5	348.3	71.8	1.46	75.8	24.9
Standard deviation		1.7	0.7	80.5	13.7	0.21	10.2	4.7



Protein (*a*) and gluten (*b*) contents in grains of spring common wheat varieties.

carrying this gene (except for Altayskaya 110) were ranked as moderately resistant to the pathogen during the years of its maximum activity. The *Lr9* gene donors may be used for developing resistance gene-pyramided varieties, which may prolong the lifespan of the gene.

Breeding value of the donors of the *Lr28* (CS2A/2M) and *Lr47* (Pavon) resistance genes transferred from *Aegilops speltoides* seems questionable under West Siberian conditions, since their low fitness to the local conditions drastically affects the crop yields (125.0 and 130.0 g/m^2

respectively). On top of that, the evaluation of breeding material collected from hybrid populations F_3 and BC_1F_3 obtained earlier on the basis of two commercial varieties (Sibirskaya 17 and Novosibirskaya 31) crossed with lines Thatcher *Lr28* and Thatcher *Lr47* (Piskarev et al., 2021) showed a significant increase in vegetation period (+ 6.3 days) compared to the recipient Sibirskaya 17 (44.2 days) and in plant height (+ 11.4 cm) in recombinants with the *Lr28* gene. Adverse effects on crop yield, number of grains per spike, and stem length were observed in recombinants with Novosibirskaya 31 variety carrying the *Lr47* gene.

Despite the relatively high crop yield of the Chelyaba 75 variety (404.4 g/m^2) carrying the *LrSp2* gene from *Aegilops speltoides* Tausch linked to the gametocidal gene (Adonina et al., 2018), which is surely a valuable trait under West Siberian conditions, we were unable to obtain a variety outperforming the current standards while carrying this gene, despite the availability of vast source material (over 4000 lines from crosses between four varieties, namely Novosibirskaya 15, Novosibirskaya 31, Udacha, and Sibirskaya 17) as early as 2015.

Wheat leaf rust resistance of the Odintsovskaya variety (selection from population Chelyaba 75 x АНК-17B) may be controlled by the *LrSp2* gene transferred from Chelyaba 75 and linked to the gametocidal gene (Adonina et al., 2018), since the variety resisted infection, but no amplification products of markers linked to other resistance genes were detected. *Lr1* gene was detected as a result of genotyping in the Omskaya 44 variety in addition to *Lr19* and *Lr26* identified earlier by L.V. Meshkova et al. (Meshkova et al., 2021).

Voevoda and Tulaikovskaya 10 are of interest as a source material for developing varieties with all around resistance to leaf pathogen infections under West Siberian conditions. These varieties demonstrate crop yields (509.8 for Voevoda and 405.3 g/m^2 for Tulaikovskaya 10) on par with the best standard varieties. On top of that, Tulaikovskaya 10 stands out in stem length (79.9 cm), and Voevoda in high weight and number of grains per spike. Tulaikovskaya 10 was earlier used to develop the Novosibirskaya 61 spring common wheat variety, which was submitted to the FSBI "GOSSORTCOMMISSION" in 2017, but then withdrawn from testing due to lack of advantages compared to standard varieties in West Siberia branches of the FSBI "GOSSORTCOMMISSION". In addition, including Tulaikovskaya 10 into hybridization resulted in shorter vegetation period in the lines selected from combinations with middle-late variety Sibirskaya 17 (Leonova et al., 2019). The Voevoda variety has not been involved in hybridization yet.

The analysis of the genotyping results shows that the winter varieties characterized by wheat leaf rust resistance in context of infectious background typically carry adult plant resistance genes (*Lr34*, *Lr12*, and *Lr13*), in particular combined with the juvenile resistance gene *Lr26*, whereas the spring varieties are primarily represented by donors of juvenile resistance genes, which agrees with the findings of E.I. Gultyaeva and E.L. Shaidayuk (2021). We believe that these protective mechanisms are best suited for varieties with different type of development, because there is no evidence of leaf rust infection of winter wheat varieties before the ear emergence stage in West Siberia, and therefore the transition of the pathogen from winter varieties to the spring ones appears complicated.

The results of the present study with regard to intensity of quantitative traits and crop yields of winter varieties are rather modest, because the collection samples are often characterized by low winter hardiness under local conditions, which only allows us to evaluate resistance in the context of infectious background. However, the *Lr41* gene allowing the KS 93 U 62 line to resist the kLr24-clone infection despite the presence of *Lr24* in the genotype was only detected in winter lines (KS 90 WGRC 10, KS 93 U 62). In addition, the Doka (with plant height of 66.5 cm and crop yield of 589.2 g/m^2) and Cheshskaya 17 (80.0 cm and 547.7 g/m^2) varieties may be used not only as donors for effective resistance genes (*Lr26* + *Lr34* and *Lr9* + *Lr12* + *Lr13* + *Lr34*), but also as sources of dwarf genes not causing losses in winter hardiness and crop yields under West Siberian conditions.

Conclusions

The varieties carrying alien genes *Lr6Agi2* (Tulaikovskaya 10) and *Lr6Agi1* (Voevoda) show wheat leaf rust resistance both in the field and in laboratory setting. Among all spring varieties carrying the *Lr24* gene analyzed in the paper, the KWS Buran, KWS Akvilon, KW 240-3-13, and Etyud varieties producing crop yields (417.2–514.1 g/m^2) comparable to the best standard variety Sibirskaya 17 (517.1 g/m^2) can be reasonably used as donors under West Siberian conditions. Omskaya 44 (440.8) characterized by crop yield on par with the best standard variety can act as a donor for resistance gene *Lr19*, while also carrying the *Lr26* gene (which is partially effective in West and East Siberia). *Lr9* gene donors (Tuleevskaya and Altayskaya 110) are recommended as a source material for resistance gene-pyramided varieties. Breeding value of the donors of the *Lr28* (CS2A/2M) and *Lr47* (Pavon) resistance genes transferred from *Aegilops speltoides* seems low under West Siberian conditions due to low fitness of the samples to local conditions. The winter varieties characterized by white leaf rust resistance in the context of increased infectious background typically carry adult plant resistance genes (*Lr34*, *Lr12*, and *Lr13*), in particular, combined with juvenile resistance gene *Lr26*. *Lr41* identified in the winter type line (KS 93 U 62) allowed it to maintain resistance against the кLr24 clone, despite the presence of *Lr24* in the genotype. The Doka (with plant height of 66.5 cm and crop yield of 589.2 g/m^2) and Cheshskaya 17 (80.0 cm and 547.7 g/m^2) varieties may be used as donors for effective

resistance genes (*Lr26* + *Lr34* and *Lr9* + *Lr12* + *Lr13* + *Lr34*) and sources of dwarf genes not causing losses in cold hardiness and crop yields under West Siberian conditions.

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ORCID

L.P. Sochalova orcid.org/0000-0002-4674-6639
V.A. Aparina orcid.org/0000-0003-2714-7216
N.I. Boyko orcid.org/0000-0002-5026-4907
E.V. Zuev orcid.org/0000-0001-9259-4384
E.V. Morozova orcid.org/0000-0001-9439-9785
K.K. Musinov orcid.org/0000-0002-4500-836X
N.A. Vinichenko orcid.org/
I.N. Leonova orcid.org/ 0000-0002-6516-0545
V.V. Piskarev orcid.org/0000-0001-9225-5227

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The effect of salicylic and jasmonic acids on the activity of *SnAGO* genes in the fungus *Stagonospora nodorum* Berk. in *in vitro* culture and during infection of wheat plants

M.Yu. Shein, G.F. Burkhanova, I.V. Maksimov ✉

Institute of Biochemistry and Genetics – Subdivision of the Ufa Federal Research Centre of the Russian Academy of Sciences, Ufa, Russia
✉ igor.mak2011@yandex.ru

Abstract. RNA interference is a gene silencing mechanism that plays an important role in genetic regulation in a number of eukaryotes. Argonaute (AGO) proteins are central to the complex RNA interference system. However, their role in this mechanism, both in the host plant organism and in the pathogen, has not yet been fully elucidated. In this work, we identified and phylogenetically analyzed the *SnAGO1*, *SnAGO2*, *SnAGO3*, and *SnAGO18* genes of the pathogenic fungus *Stagonospora nodorum* Berk., and analyzed their expression under conditions of infection of plants with varying degrees of resistance to the pathogen. The expression level against the background of plant immunization with the resistance inducers salicylic and jasmonic acids was assessed. In addition, the activity of these genes in the culture of the fungus *in vitro* was studied under the direct influence of resistance inducers on the mycelium of the fungus. Earlier activation of the *SnAGO* genes in *in vitro* culture under the influence of salicylic and jasmonic acids suggests their sensitivity to it. In an *in vivo* system, plant immunization to induce the accumulation of pathogen *SnAGO* transcripts was found. At the same time, the *SnAGO* genes of the fungus *S. nodorum*, when interacting with plant cells, reacted depending on the degree of host resistance: the highest level of transcripts in the resistant variety was observed. Thus, our data prove that the *SnAGO* genes of the fungus *S. nodorum* effectively interact with the host defense system in direct proportion to the degree of resistance of the latter to the pathogen. It was proposed to use the ratio of the transcriptional activity of the fungal reference gene *SnTub* to the host *TaRLI* gene as a marker of disease development in the initial period of the infectious process.
Key words: RNA interference; *SnAGO* genes; fungus *Stagonospora nodorum*; common wheat; pathogenesis, salicylic acid; jasmonic acid.

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Влияние салициловой и жасмоновой кислот на активность генов *SnAGO* гриба *Stagonospora nodorum* Berk. в культуре и при инфицировании растений пшеницы

М.Ю. Шеин, Г.Ф. Бурханова, И.В. Максимов ✉

Институт биохимии и генетики – обособленное структурное подразделение Уфимского федерального исследовательского центра Российской академии наук, Уфа, Россия
✉ igor.mak2011@yandex.ru

Аннотация. РНК-интерференция представляет собой механизм подавления генов, играющий важную роль в генетической регуляции у эукариот. Белки Argonaute (AGO) занимают центральное место в сложной системе явления РНК-интерференции. Однако их роль в этом механизме, как в организме растения-хозяина, так и у патогена, до сих пор полностью не исследована. Мы провели идентификацию и филогенетический анализ генов *SnAGO1*, *SnAGO2*, *SnAGO3* и *SnAGO18* патогенного гриба *Stagonospora nodorum* Berk., возбудителя септориоза пшеницы, и проанализировали их экспрессию в условиях инфицирования растений с различной степенью устойчивости к патогену. Уровень экспрессии оценивали на фоне иммунизации растений индукторами устойчивости: салициловой и жасмоновой кислотами. Также изучена активность указанных генов в культуре гриба при непосредственном воздействии индукторов устойчивости на мицелий гриба. Выявленная более ранняя активация генов *SnAGO* в культуре под влиянием салициловой и жасмоновой кислот указывает на их чувствительность к ним. В системе *in vivo* обнаружено, что иммунизация растений индуцирует накопление транскриптов *SnAGO* патогена. При этом гены *SnAGO* гриба *S. nodorum* при взаимодействии с растительными

клетками реагировали в зависимости от степени устойчивости хозяина: наиболее высокий уровень транскриптов наблюдался в устойчивом сорте. Таким образом, полученные данные доказывают, что гены *SnAGO* гриба *S. nodorum* эффективно взаимодействуют с системой защиты хозяина в прямой зависимости от степени устойчивости последнего к патогену. Предложено использовать отношение транскрипционной активности грибного референсного гена *SnTub* к хозяйскому гену *TaRLI* в качестве маркера развития болезни в начальный период инфекционного процесса.
Ключевые слова: РНК-интерференция; гены *SnAGO*; гриб *Stagonospora nodorum*; мягкая пшеница; патогенез; салициловая кислота; жасмоновая кислота.

Introduction

Phytopathogenic fungi are a powerful risk to food security, which limits the biological potential of agricultural plants and reduces the quality of the resulting products. At the present stage, methods of plant protection are being developed based on natural systemic and cellular phytoimmunity, where a special place is occupied by a unique mechanism that disables gene expression, described by the term "RNA interference" (RNAi) – an evolutionarily conservative and at the same time highly specific immune component for almost all eukaryotes.

Methodologically, when creating modern approaches to plant protection, it is necessary to take into account that during the interaction of plants with pathogens, especially fungal ones, the RNAi components of not only the host, but also the pathogen are activated. Induction of the activity of a number of genes responsible for the functioning of RNAi in pathogenic fungi suggests the possibility of their participation in the suppression of genes of the host defense system (Weiberg et al., 2013). It should be noted that the role of the RNAi mechanism in the evolution and life of fungi is important and can vary quite significantly depending on the survival strategy, the method of infection and spread, as well as the pathogens that infect the fungus itself (Neupane et al., 2019). For example, it is known that artificial inactivation of one or two genes encoding RNAi proteins in phytopathogenic fungi disrupts virulence (Raman et al., 2017; Wang et al., 2018).

Argonaute proteins (AGOs) are considered key components in the complex RNAi phenomenon and bind short microRNAs (Feng et al., 2017; Neupane et al., 2019). The most important function of AGO proteins, actively discussed in the scientific literature, is participation in phytoimmunity. For example, we previously observed that pre-treatment of seeds with salicylic acid (SA) formed the resistance of wheat to Septoria nodorum blotch (SNB), and at the same time, active accumulation of *TaAGO1* gene transcripts was observed in plant tissues infected with the causative agent of this disease (Shein et al., 2021). So, in tobacco plants *Nicotiana attenuata* Torr. ex S.Watson, the accumulation of the *NaAGO4* protein turned out to be critical in the formation of resistance to the fungus *Fusarium brachygibbosum* Padwick (1945) through the jasmonate signaling pathway (Pradhan et al., 2020). Disruption of this process turned off the synthesis of jasmonic acid (JA) in plants and led to their infection, but resistance to the fungus was restored after treating the plants with JA. It can be assumed that the functioning of the plant defense system, regulated by JA, mediates the operation of the mechanism of the RNAi phenomenon. The importance of plant proteins AGO18 in the formation of antiviral defense in rice was discovered (Yang et al., 2020).

It is also known that AGO proteins are actively involved in physiological processes occurring in the mycelium of various types of fungi. Thus, genes encoding AGO proteins were identified in the genome of the fungi *Fusarium graminearum* (*FgAGO1*) (Chen et al., 2015) and *Metarhizium robertsii* (*MrAGO1*) (Meng et al., 2017). The participation of fungal proteins of the AGO family in the formation of compatibility between the host and the pathogen was demonstrated in the fungi *Verticillium dahlia* and *V. longisporum* (Shen et al., 2014). A similar effect was observed in the fungus *Scletotinia sclerotiorum*: mutants of the *AGO2* gene of this fungus had slow growth and reduced virulence (Neupane et al., 2019). Suppression of *AGO2* (*QDE-2*) gene expression also reduced virulence in the fungi *Valsa mali* (Feng et al., 2017) and *Fusarium oxysporum* f. sp. lycopersici (Jo et al., 2018).

Thus, proteins of the AGO family are key components in the functioning of not only plant but also fungal defense systems mediated by RNAi mechanisms. At the same time, it should be noted that so far little work has been devoted to the analysis of the expression of genes encoding proteins of the RNAi mechanism in various pathogens under conditions of plant infection and preliminary immunization with phytohormones SA and JA. In this work, such an analysis was carried out using a model of the phytopathogenic fungus *Stagonospora nodorum* Berk. (*Septoria*, *Parastagonospora*, *Phaeosphaeria*), which causes SNB – one of the most harmful wheat diseases. The main objective was to evaluate changes in the transcriptional activity of the *SNAGO* genes encoding AGO proteins in an *in vitro* culture of the fungus and under conditions of infection of plants with this fungus, contrasting in resistance to SNB against the background of SA and JA treatment.

Materials and methods

Object. In the experiment, we used a highly virulent strain of the phytopathogenic fungus *S. nodorum* SnB against common wheat *Triticum aestivum* L. (Zhnitsa cultivar) from the collection of the Institute of Biochemistry and Genetics – Subdivision of the Ufa Federal Research Centre of the Russian Academy of Sciences. As plant material, we used two cultivars of spring bread wheat *T. aestivum* (BAD, $2n = 42$) contrasting in their resistance to SNB: Zhnitsa (susceptible) and Omskaya 35 (resistant).

Cultivation of the fungus *in vitro*. The fungus was cultivated on a liquid potato-glucose nutrient medium in Petri dishes. To do this, a suspension of fungal spores was added to the nutrient medium at a rate of 10^5 spores/ml and cultivated in a KBW E6 climate chamber (Binder GmbH, Germany) at a temperature of 18 °C for 14 days with periodic 16-hour lighting. Solutions of SA with concentrations of 10^{-4}, 10^{-5} and

10^{-6} M, as well as JA solutions with concentrations of 10^{-5}, 10^{-6} and 10^{-7} M were previously added to the experimental versions of the nutrient media. These concentrations were chosen by us as optimal due to the data previously obtained in our laboratory on the influence of SA and JA on the degree of development of SNB in various wheat cultivars in the pathosystem (Yarullina et al., 2011).

The design of the experiment. Wheat seeds were presoaked for 24 hours in solutions containing 10^{-5} M SA and 10^{-7} M JA, or their composition. Control samples were kept in distilled water for the same period of time. Then, the seedlings in isolated vessels on Hoagland–Arnon nutrient medium were placed in a KBW E6 plant growth chamber (Binder GmbH, Germany), with a 16-hour photoperiod at a temperature of 20/24 ºC (night/day). The noted concentrations of SA and JA were selected as a result of preliminary experiments as the most effective in inducing resistance of wheat plants against *S. nodorum* (Yarullina et al., 2011). Then, sections of leaves of 7-day-old control and experimental wheat seedlings were placed in Petri dishes on damp cotton wool with the addition of benzimidazole (40 mg/L). Some of the leaves were infected with fungal spores by applying 4 μL of the suspension (105 spores/mL), according to the method (Veselova et al., 2021). Leaves inoculated with fungal spores in Petri dishes were placed in a thermostat for 24 hours and then transferred to a KBW E6 plant growth chamber (Binder GmbH, Germany).

Visual assessment of the degree of fungal development on wheat leaves. The development of the fungus *S. nodorum* on leaves was monitored daily. The area under the disease development curve in the variants was determined according to the method proposed by A.A. Marchenkova et al. (1991). Leaf lesion area was measured using ImageJ (https://imagej.nih.gov/ij/download.html).

RNA extraction and study of the relative gene expressions of the *SnAGO* genes. Isolation of total RNA from wheat leaves fixed in liquid nitrogen, as well as mycelium of the fungus *S. nodorum* SnB grown *in vitro*, was carried out using the Lyra reagent according to the protocol of Biolabmix (Russia, https://biolabmix.ru biolabmix.ru). The concentration of nucleic acids was measured using a Thermo Scientific™ NanoDrop™ 1000 spectrophotometer (ND1000WOC) at A260/A280. To synthesize cDNA, a reverse transcription reaction was performed using M-MuLV reverse transcriptase (Syntol, Russia). The nucleotide sequences of the studied *SnAGO* genes of the fungus *S. nodorum* were selected from the FunRNA database (http://funrna.riceblast.snu.ac.kr/, 04.12.2023). Primers for these genes were designed using the Internet programs "Primer-Blast" (https://www.ncbi.nlm.nih.gov/tools/primer-blast, 04.12.2023) and "PrimerQuest Tool" (https:/eu.idtdna.com/Primerquest, 04.12.2023) (the Table). To assess the level of gene transcription, we used the quantitative real-time PCR method on the CFX Connect Real-Time System device (Bio-Rad, USA). SYBR Blue reagent (Syntol, Russia) was used as an intercalating dye. The transcriptional activity of the fungal pathogen was assessed relative to the reference gene *SnTUB*, encoding the fungal tubulin protein (Fraaije et al., 2002). To assess the development of the fungus at the RNA level, we used an analysis of the ratio of transcripts of reference genes: the pathogen *SnTub* (Fraaije et al., 2002) and the host *TaRLI*, encoding a wheat RNase L inhibitor-like protein (Giménez et al., 2011) in experimental plants. According to the works of these authors, the expression of the noted genes of the fungus *S. nodorum* and wheat is not affected by environmental factors.

Primers of *SnAGO* gene loci of the fungus *S. nodorum*

Gene designation	Nucleotide sequence
SnAGO1 homologue of *QDE-2*	F GCAAGTTCGCCATGAACAATAA
	R CAAACCTTCTGGACCATCTCTC
SnAGO2	F GGAGACTCACAGTTCGAAGAAG
	R TAGGAGAGGCAGAGGTTGTAA
SnAGO3	F CGTTTCCTGGGTTGACATAGAT
	R GCCAGACGTTCACTCTGATATT
SnAGO18	F GTCAGTCGATCAAGGTGGATTTA
	R CGTATAGTGCTGACGTCTCTTG
SnTub(β)	F TGGTATGGGTACGCTTTTGATCTC
	R GTAGCGACCGTTGCGGAAGTCAGA
TaRLI(a)	F TTGAGCAACTCATGGACCAG
	R GCTTTCCAAGGCACAAACAT

Bioinformatic and statistical analyzes. The experiments were carried out in triplicate. Mean values with standard errors (± SE) are shown in the figures. Statistical analysis of the obtained data was carried out in the Bio-Rad CFX Maestro 1.1 Version: 4.1.2433.1219 program (Bio-Rad, USA). Differences in the studied parameters between individual treatments were analyzed using analysis of variance. Alignment of nucleotide sequences and construction of a phylogenetic tree were carried out using the MEGA11: Molecular Evolutionary Genetics Analysis version 11.0.13 program (Tamura et al., 2021). The MUSCLE algorithm was used for alignment; phylogenetic trees were constructed using the Maximum Likelihood method (Tamura et al., 2021).

Results

Based on previous studies, we selected bread wheat cultivars that contrast in their resistance to the fungus *S. nodorum* (Veselova et al., 2021). Previously, the effect of treating wheat seeds with SA and JA on the subsequent formation of resistance to the fungus *S. nodorum* in seedlings was also assessed (Yarullina et al., 2011). These works showed that SA and JA could significantly reduce the severity of the development of SNB in wheat.

In this work, at the first stage, we analyzed the degree of fungal development in plant leaf tissues under normal conditions and under conditions of inducing the plant defense system using SA and JA pretreatment. The intensity of the formation of an infectious spot and the rapid development of necrosis in the leaves of the Zhnitsa cultivar showed a high degree of susceptibility of this cultivar to the pathogen strain we used (Fig. 1, *a*). The disease symptoms began to appear in the form of brown spots already on the 4th day after

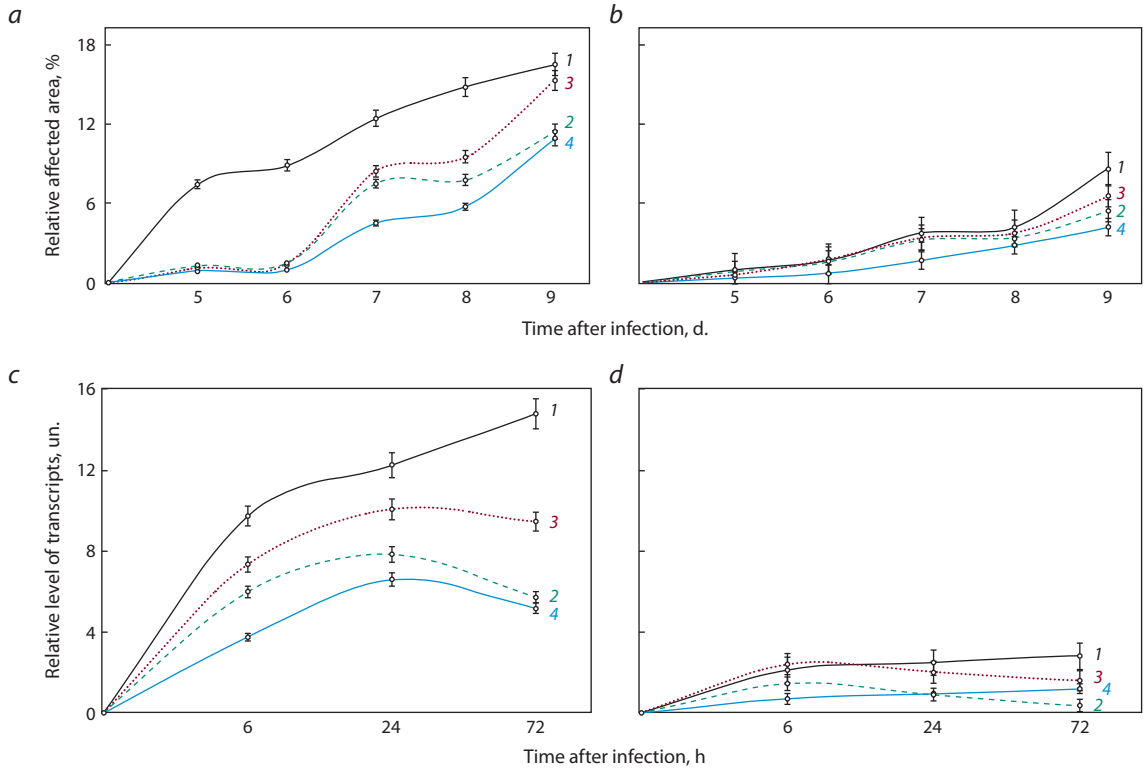

Fig. 1. Comparative changes in leaf damage area (*a*, *b*) and the level of transcripts of the fungal reference *SnTub* gene in relation to the wheat *TaRLI* gene (*c*, *d*) in the leaves of susceptible (*a*, *c*) and resistant (*b*, *d*) cultivars of common wheat in the control (*1*) and after pre-treatment with salicylic (*2*), jasmonic (*3*) acids and their composition (*4*).

inoculation of the leaves, and on the 7th day after infection, the leaves were significantly affected. Under the same conditions, on the resistant cultivar Omskaya 35, SNB developed less intensively (see Fig. 1, *b*, *d*). Accordingly, during the experiment, we confirmed the degree of susceptibility of the studied cultivars in terms of resistance to the fungus *S. nodorum*. In the variant of pre-treatment of seeds with SA and JA, as previously found (Yarullina et al., 2011), inhibition of the development of SNB on leaves infected with the fungus was observed, which suggests a systemic immunizing effect of these compounds. We found a particularly good protective effect in the variant of pre-sowing treatment of wheat seeds with a composition of SA and JA.

It is obvious that the successful development of the fungus *S. nodorum* in plant tissues is accompanied by the accumulation of its biomass and, accordingly, a change in the ratio of proteins and nucleic acids between the host and the pathogen. Based on this, we analyzed the ratio of transcript levels of reference genes: the pathogen *SnTub* and the host *TaRLI* in experimental plants. As can be seen from the data obtained (see Fig. 1, *c*, *d*), the ratio of cDNA of the *SnTub* gene to the *TaRLI* gene in the susceptible Zhnitsa cultivar during the observed period was much higher than in the resistant one. This ratio noticeably decreased in plants pre-treated with SA and JA, as well as their composition (most noticeable after treatment of seeds with SA and JA).

In the resistant cultivars, the most pronounced decrease in the *SnTub*/*TaRLI* ratio was observed after treatment with SA. These data indicate the important contribution of the salicylate-induced pathway in the formation of resistance of wheat plants against the *S. nodorum* pathogen. Accordingly, we can say that the *SnTub*/*TaRLI* indicator is a convenient marker for early rapid diagnosis of resistance of wheat plants to the pathogenic fungus *S. nodorum*, as well as for assessing changes in this resistance at different stages of the formation of the relationship between the host plant and the specified pathogen.

To identify *SnAGO* genes encoding AGO family proteins in the *S. nodorum* genome, an analysis of the FunRNA database (Choi et al., 2014) was carried out using the annotated *S. nodorum* genome sequence (Hane et al., 2007). This approach allowed us to identify the SNOG_12157 locus. The phylogenetic tree of *AGO* genes is presented in Figure 2. As can be seen, the structure of the same *AGO* genes in different organisms has greater homology than the structure of different genes of a given family in representatives of the same genus/taxon. Thus, the *AGO1* gene we selected for analysis in the pathogen *S. nodorum* turned out to be on the same branch as the *AGO1* genes of fungi from other genera or even divisions, and not with the *AGO2*, *AGO3* and *AGO18* genes of the same species. It is also noteworthy that, according to the obtained result, the *Qde2* genes, being homologues of *AGO1* (Jo et al., 2018), are located on a completely different phylogenetic branch of fungal genes. This classification of *AGO1* into a separate group from *AGO2* and *AGO3* correlates with similar results obtained in other studies (Zhang et al., 2015; Ahmed et al., 2021).

Subsequent BlastP sequence analysis identified multiple genes putatively encoding fungal *SnAGOs* based on matches

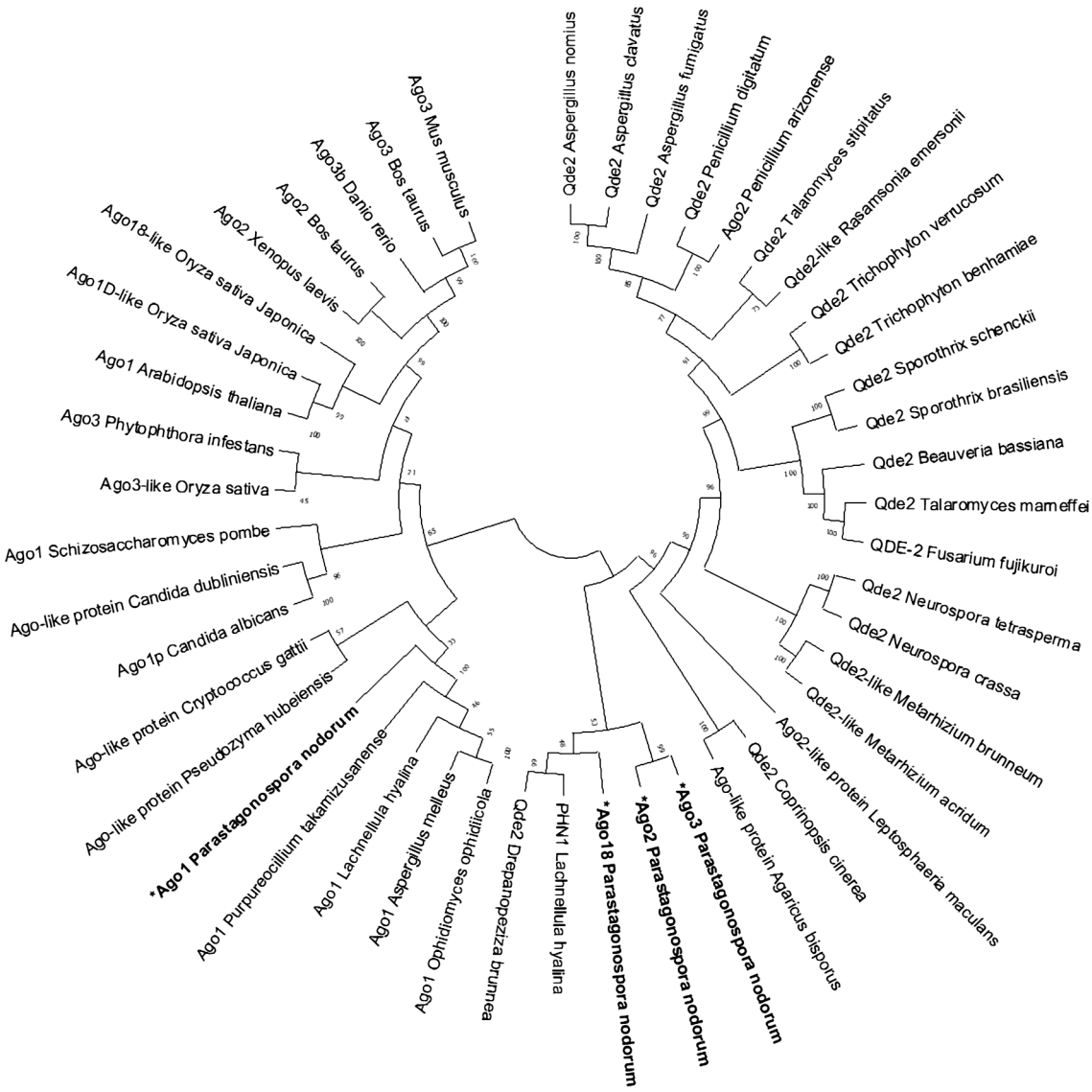

Fig. 2. Phylogenetic tree of the *AGO* and *QDE* genes in various organisms. Sequences of fungi studied in this work are highlighted in bold.

to known motifs characteristic of *AGO* genes. On this basis, the *SnAGO* genes were named *SnAGO1*, *SnAGO2*, *SnAGO3*, and *SnAGO18*, respectively, and were selected for further analysis of transcription activity. Primers for assessing the expression of the *SnAGO1*, *SnAGO2*, *SnAGO3* and *SnAGO18* genes are presented in the Table.

Previously, it was not known how the transcriptional activity of fungal genes responsible for the formation of the RNAi phenomenon changes under conditions of infection of susceptible and resistant wheat cultivars. To do this, we analyzed the level of *SnAGO* gene transcripts identified in the fungus *S. nodorum* under infection conditions in bread wheat cultivars contrasting in resistance to this pathogen, pretreated with SA and JA, as well as with the SA + JA composite (Fig. 3). As can be seen, when wheat leaves are infected with fungal spores during the experiment, transcripts of the *SnAGO1*, *SnAGO2* and *SnAGO3* genes accumulate. Moreover, it is noteworthy that on the resistant cultivar Omskaya 35, the level of gene transcripts increases during the experiment.

Treatment of seeds with SA and JA, as well as their composition, was accompanied by locus-specific changes in the levels of transcripts of fungal *SnAGO* genes in a pathogenic system with both resistant and susceptible wheat cultivars (see Fig. 3). Most loci showed transcript accumulation; however, the extent of accumulation varied depending on the locus and the treatment. For example, the *SnAGO1*, *SnAGO2* and *SnAGO18* genes on the resistant cultivar Omskaya 35 as a result of seed treatment with JA were expressed to a greater extent than under the influence of SA. The composition of SA and JA reduced the activity of the *SnAGO2* and *SnAGO3* genes more pronouncedly in comparison with untreated and infected samples.

In fungus-infected leaf tissues of a resistant cultivar, pretreated with both SA, JA, and their composition, after 24 hours of the experiment, the level of transcription of all studied genes (mostly *SnAGO1*) was higher compared to untreated plants after the same time of infection, but after 72 hours, it was lower than the corresponding levels in untreated plants

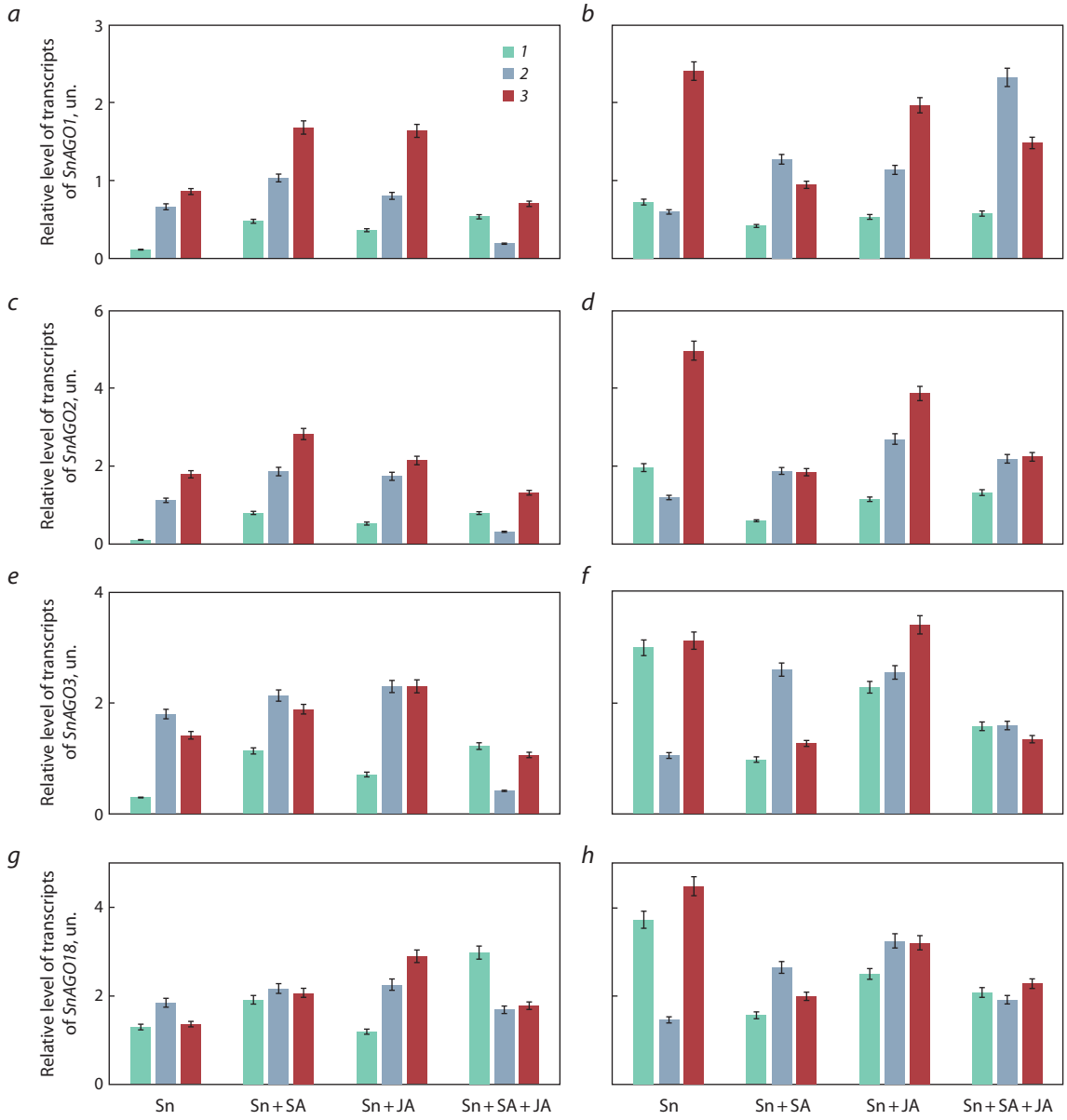

Fig. 3. Changes in the level of transcripts of genes of the *AGO* family in the fungus *S. nodorum* in the leaves of susceptible (Zhnitsa) (*a*, *c*, *e*, *g*) and resistant (Omskaya 35) (*b*, *d*, *f*, *h*) wheat grown from salicylic (SA) and jasmonic (JA) acids treated seeds in 6 (*1*), 24 (*2*) and 72 (*3*) hours after infection.

a, *b*, *SnAGO1* (Snog_12157); *c*, *d*, *SnAGO2* (Snog_10544); *e*, *f*, *SnAGO3* (Snog_10546); *g*, *h*, *SnAGO18* (Snog_12309).

(with the exception of *SnAGO3* after JA treatment). In susceptible plants, throughout the experiment, the expression of the *SnAGO1* and *SnAGO3* genes increased approximately equally under the influence of both SA and JA; separately, the expression of the following genes increased to the greatest extent: *SnAGO2* under the influence of SA and the *SnAGO18* gene under the influence of JA, compared with control infected plants. When wheat seeds were treated with a mixture of SA and JA, the expression level of all *SnAGO* genes in the susceptible cultivar increased after 6 hours of infection (3-fold in the case of *SnAGO18*).

Thus, the obtained results showed that the *SnAGO* genes, encoding one of the key enzymes of the RNAi mechanism, in fungal cells, when interacting with plant cells, respond to the degree of host resistance and increase their transcriptional activity in resistant cultivars. Accordingly, the higher level of *SnAGO* transcripts in pathogen-infected wheat leaves confirms previous suggestions about the important role of this group of proteins in the formation of compatible relationships between wheat and the fungus *S. nodorum* (Shen et al., 2014).

From our point of view, it is interesting to evaluate the direct response of the fungal genome to the effects of SA and JA on a nutrient medium. Thus, using mycelium of the fungus *S. nodorum* grown in liquid culture, we assessed the expression status of *SnAGO* genes when SA and JA solutions of various concentrations were added to the medium on days 5, 7, and 14 after planting on the medium (Fig. 4). It was found that when the fungus was cultivated on a nutrient medium, the activity

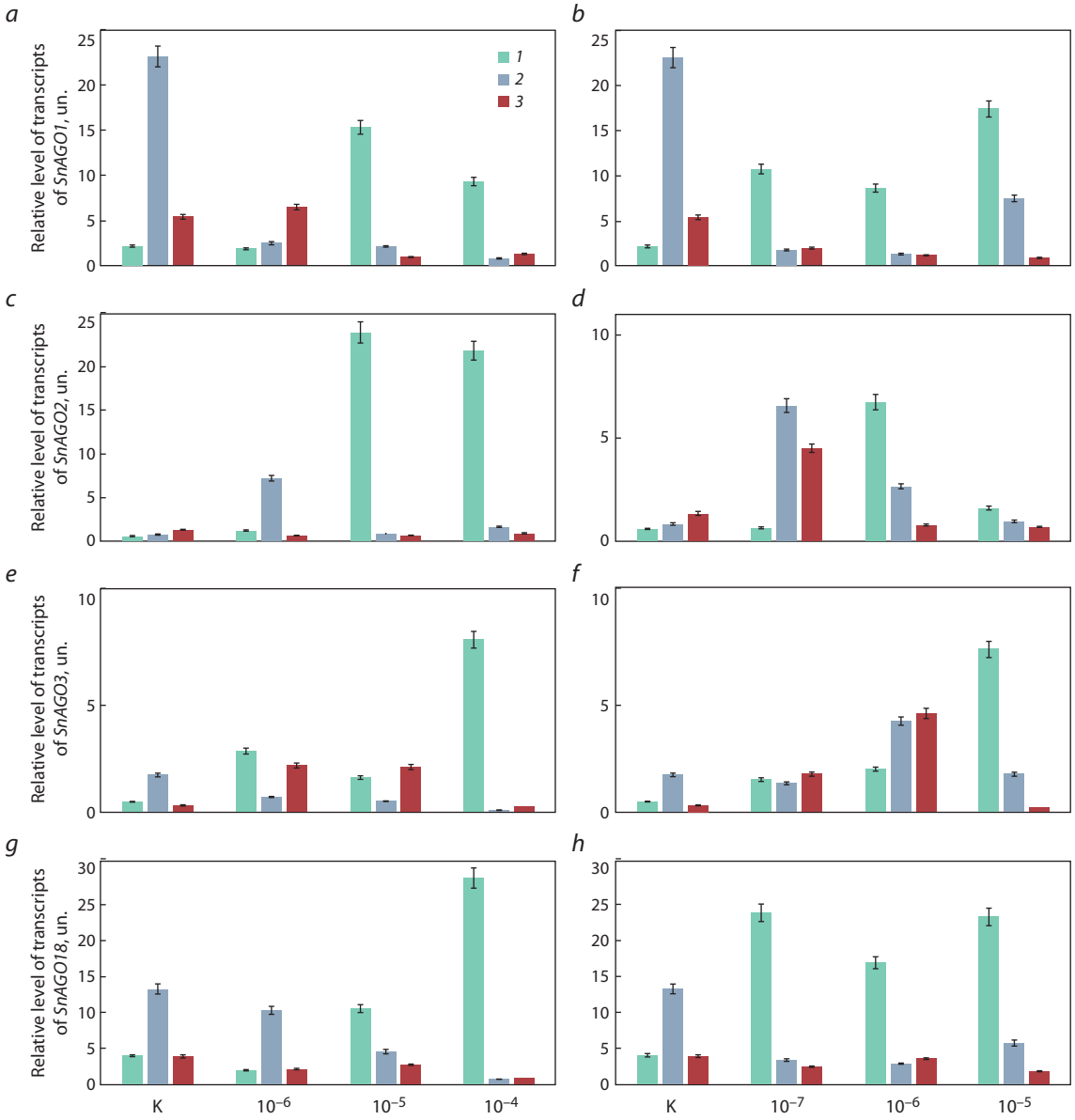

Fig. 4. Changes in the level of transcripts of the *SnAGO* genes of *S. nodorum* grown on a liquid nutrient medium with the addition of salicylic (SA) (*a*, *c*, *e*, *g*) and jasmonic (JA) (*b*, *d*, *f*, *h*) acids of various concentrations (M) in 5 (*1*), 7 (*2*) and 14 (*3*) days after planting.
a, *b*, *SnAGO1* (Snog_12157); *c*, *d*, *SnAGO2* (Snog_10544); *e*, *f*, *SnAGO3* (Snog_10546); *g*, *h*, *SnAGO18* (Snog_12309).

of the *SnAGO1* gene in control samples increased on the 7th day after the start of cultivation. The activity of the *SnAGO2* gene in control colonies of the fungus increased during the experiment, but the effect was less pronounced. When SA was added to the nutrient medium, a pronounced increase in the level of transcripts of all the studied *SnAGO* genes was observed already on the 5th day, and the degree of accumulation of transcripts of these genes was directly proportional to the concentration of the added substance. Similar data were obtained when JA was added to the medium. At the same time, it is noteworthy that the addition of signaling molecules at all JA concentrations and relatively high SA concentrations shifts the accumulation of transcripts to the earliest period – 5 days. Particular attention should be paid to the fact that the *SnAGO1* and *SnAGO18* genes turned out to be the most sensitive to the addition of JA to the nutrient medium.

Discussion

Assessing the mechanisms of formation of plant resistance, especially against pathogens, is an urgent task, the solution of which will make it possible to effectively regulate it and achieve higher productive properties. The diversity of feeding methods of phytopathogenic fungi contributed to the evolutionary formation of various ways of protecting plants from them (McCombe et al., 2022). For example, against biotrophic pathogens that feed on living plant tissues, defense mechanisms are launched, where the SA-dependent signaling pathway plays an active role, forming systemic acquired resistance. JA triggers another signaling pathway, called systemic induced resistance, which protects plants from necrotrophic pathogens and insects. Numerous studies have shown that the causative agent of SNB is characterized by a hemibiotrophic mode of nutrition on wheat plants, combining

a biotrophic phase of development at the very beginning of pathogenesis with a subsequent necrotrophic one; however, it is also capable of saprotrophic growth on nutrient media (Oliver et al., 2012). Accordingly, depending on the timing of pathogen development in plant tissues, host defense systems must clearly recognize these transition stages.

We have previously shown that the highly virulent strain of the pathogenic fungus *S. nodorum* SnB can overcome the defense of wheat associated with the pro-/antioxidant system, including the activation of catalases (Troshina et al., 2010) and chitin deacetylases (Maksimov et al., 2011), and also the accumulation of various effector molecules (Veselova et al., 2021). In this work, we analyzed a number of *S. nodorum* genes encoding *SnAGO* nuclease proteins involved in the RNAi mechanism, using it as a model object.

The RNAi phenomenon represents a unique and ancient mechanism for protecting the genome of eukaryotes, including fungi, from foreign genetic information, and also serves to regulate physiological processes. The most important key components of this complex immune mechanism are AGO RNA nucleases, which have been functionally characterized in model organisms (Choi et al., 2014). It has been shown that the RNAi mechanism in the eukaryotic immune system during the interaction of hosts with their parasites is a "double-edged instrument", which, on the one hand, protects the host from the pathogen, and on the other hand, helps the pathogen disable the accumulation of the most essential key protective proteins of the host, in order to use plant resources for its own functioning. However, to date, the mechanisms of RNAi operation in fungal systems, especially during the development of diseases, have not been sufficiently studied, which limits our understanding of this mechanism.

For example, the formation of compatibility between the host and the pathogen with the participation of plant proteins AGO1 of *Brassica napus* was shown under conditions of plant infection by the fungi *V. dahliae* and *V. longisporum* (Shen et al., 2014). In tomato and *Arabidopsis* plants, microRNAs secreted by the fungus *Botrytis cinerea* used the host AGO1 proteins to selectively suppress the translation of host mitogen-activated protein kinases (MAPKs), peroxiredoxin, and cell wall-associated kinase, and suppression of the accumulation of this protein in mutant *Arabidopsis* plants resulted in reduced susceptibility to the fungus (Weiberg et al., 2013). F. Dunker and co-workers (2020) showed on *Arabidopsis* plants that similar recruitment of the host protein complex AGO1 is also characteristic of the oomycete *Hyaloperonospora arabidopsidis*, a pathogen phylogenetically distant from fungi, suggesting a property common to pathogens.

An important role of the RNAi mechanism in the growth and development of fungi has been revealed, which is naturally reflected in their ability to infect plants. Thus, Δago1 mutants of the fungus *Colletotrichum higginsianum* showed severe defects in conidial morphology (Campo et al., 2016). Deletion of the *FgAGO2* gene of the fungus *Fusarium graminearum* did not affect the fungal phenotype during the asexual phase (Chen et al., 2015), but this gene was found to be important during the fungal sporulation and ascospore maturation stages (Zeng et al., 2018). In another species of fungus, *Fusarium oxysporum* f. sp. *lycopersici* strain 4287, mutants with suppressed expression of the *FoQDE-2* (*AGO1*) gene (Jo et al., 2018) exhibited reduced virulence against tomato plants.

At the moment, there are practically no works that would discuss the behavior of fungal pathogen genes encoding proteins responsible for RNAi under conditions of direct exposure to biostimulants and resistance inducers, for example, SA and JA, or indirect exposure through plant infection. There is conflicting information about the role of plant resistance inducers in the operation of the RNAi mechanism in plant tissues under the direct influence of SA and JA and in response to infection by pathogens. For example, some authors show that SA-induced pathogen resistance affects plant RNAi mechanisms only through the host RNA-dependent RNA polymerase (RDR1) and is coordinated by this protein (Lee et al., 2016). At the same time, there is information that in plants, for example, rice, the expression levels of the *OsAGO1a*, *OsAGO2* and *OsAGO18* genes turned out to be associated with JA, and in mutant rice plants coi1-13, unable to transmit JA signals, they were lower than in wild plants (Yang et al., 2020). This is especially interesting due to the fact that in the pathogenic wheat fungus *S. nodorum*, the role of RNAi in pathogenicity still remains unresolved. At the same time, the participation of RNAi in the development of pathogenicity of *Zymoseptoria tritici*, another fungus that causes septoria leaf blight on wheat, was recently analyzed after knockout of the *AGO1* and *AGO2* genes in its genome (Kettles et al., 2019; Ma et al., 2020). The analysis did not reveal qualitative phenotypic changes in the development of SNB symptoms on the susceptible wheat cultivar Bobwhite. Fungal strains mutant for the *AGO* genes did not lose their virulence properties against wheat plants. From the results obtained, the authors conclude that these proteins do not play a significant role in the development of SNB. At the same time, analysis of the expression status of genes responsible for the operation of the RNAi mechanism in the pathogenic fungus *V. nonalfalfae*, which causes verticillium wilt in hops (*Humulus lupulus* L.), showed that more virulent strains of the pathogen have a higher level of accumulation of *VnAGO* transcripts (Jeseničnik et al., 2019).

We showed that in the pathogen-resistant to *S. nodorum* wheat cultivar Omskaya 35, the accumulation of transcripts of the studied *SnAGO* genes during fungal pathogenesis in leaf tissues was more active than in the susceptible cultivar, which suggests the involvement of the studied genes in the process of overcoming the host's defense system and the possibility of regulating activity of these genes depending on the degree of host resistance. These assumptions are further confirmed in experimental variants where plants were immunized by pre-sowing seed treatment with SA and JA, as well as their composition. As can be seen, the level of transcripts of pathogenic *SnAGO* genes in these variants also turns out to be higher than in the control ones, although, judging by the ratio of housekeeping genes *SnTub*/*TaRLI*, the content of the *SnTub* gene in plants immunized with SA and JA is significantly lower.

We obtained interesting results when assessing the transcriptional activity of *SnAGO* genes after direct exposure of the fungal mycelium in culture to SA and JA. High concentrations of SA enhanced the accumulation of transcripts of the studied *SnAGO* genes at earlier times of cultivation, while low

concentrations did not produce this effect. JA stimulated the accumulation of *SnAGO1*, *SnAGO3*, *SnAGO18* transcripts at early stages of cultivation and at lower concentrations compared to SA. It can be assumed that fungal *SnAGO* genes are sensitive to the direct effects on the fungal mycelium of these signaling molecules, which are positioned as inducers of plant defense systems against pathogens. Thus, we have shown that the fungal RNAi system actively responds to the addition of SA and JA to the cultivation medium, and also participates in the process of plant infection. At the same time, artificial stimulation of the protective properties of the plant also triggers the accumulation of *SnAGO* transcripts of the pathogen. Our data suggest that the *AGO* genes involved in the RNAi system of the fungus *S. nodorum* strain SnB interact effectively with the infecting plant and, most likely, this interaction depends on the degree of host resistance.

The possibility of assessing the degree of development of pathogens in plant tissues using molecular biological methods that assess the level of accumulation of pathogen nucleic acids in tissues is very important, since visual assessment of disease symptoms is often subjective and influenced by various factors. This work uses the *SnTub*/*TaRLI* method for assessing the accumulation of pathogen genetic material in plant tissues by comparing the ratio of transcript levels of fungal and wheat housekeeping genes. Estimation of the *SnTub*/*TaRLI* transcript ratio correlated with the results of visual observation of disease development on leaves. The results also showed that the most effective in inducing the protective properties of wheat plants against SNB was pre-sowing treatment of seeds with JA, as well as with a composition of JA and SA (see Fig. 1). Using the level of the *SnTub*/*TaRLI* ratio, we confirmed the dynamics of accumulation of biological material of the pathogenic fungus, but at earlier periods of observation. In addition, we demonstrated the possibility of using the data obtained on the transcriptional activity of the *AGO* genes of the fungus *S. nodorum* strain SnB as an objective indicator of the activation of its RNAi system.

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ORCID

M.Yu. Shein orcid.org/0000-0002-3743-9928
G.F. Burkhanova orcid.org/0000-0003-2346-3502
I.V. Maksimov orcid.org/0000-0002-5707-3265

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Chitosan and its derivatives as promising plant protection tools

A.B. Shcherban

Kurchatov Genomic Center of ICG SB RAS, Novosibirsk, Russia
atos@bionet.nsc.ru

Abstract. In modern conditions, the increase in the yield of agricultural crops is provided not by expanding the areas of their cultivation, but mainly by introducing advanced technologies. The most effective strategy for this purpose is the development of genetically resistant and productive cultivars in combination with the use of a variety of plant protection products (PPPs). However, traditional, chemical PPPs, despite their effectiveness, have significant drawbacks, namely, pollution of environment, ecological damage, toxicity to humans. Recently, biological PPPs based on natural compounds have attracted more attention, since they do not have these disadvantages, but at the same time they can be no less effective. One of such agents is chitosan, a deacetylation product of chitin, one of the most common polysaccharides in nature. The high biological activity, biocompatibility, and safety of chitosan determine the breadth and effectiveness of its use in medicine, industry, and agrobiology. The review considers various mechanisms of action of chitosan as a biopesticide, including both a direct inhibitory effect on pathogens and the induction of plant internal defense systems as a result of chitosan binding to cell surface receptors. The effect of chitosan on the formation of resistance to the main classes of pathogens: fungi, bacteria, and viruses has been shown on a variety of plant objects. The review also discusses various ways of using chitosan: for the treatment of seeds, leaves, fruits, soil, as well as its specific biological effects corresponding to these ways. A separate chapter is devoted to protection products based on chitosan, obtained by its chemical modifications, or by means of combining of a certain molecular forms of chitosan with various substances that enhance its antipathogenic effect. The data presented in the review generally give an idea of chitosan and its derivatives as very effective and promising plant protection products and biostimulants.
Key words: plant protection products; pesticide; chitosan; novohizol; pathogen; resistance; yield.

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Хитозан и его производные как перспективные средства защиты растений

А.Б. Щербань

Курчатовский геномный центр ИЦиГ СО РАН, Новосибирск, Россия
atos@bionet.nsc.ru

Аннотация. В современных условиях прирост урожайности сельскохозяйственных культур обеспечивается не за счет расширения площадей их возделывания, а главным образом благодаря внедрению передовых технологий. Наиболее эффективная стратегия включает создание генетически устойчивых к неблагоприятным факторам и продуктивных сортов в сочетании с использованием разнообразных средств защиты растений. Однако традиционные, химические, средства защиты, несмотря на эффективность, имеют существенные недостатки: загрязнение окружающей среды, нарушение экологии, токсичность для человека. В последнее время все больше внимания привлекают биологические (на основе природных соединений) средства защиты растений – они лишены этих недостатков, при этом могут быть не менее эффективными. К таким средствам относится хитозан – продукт деацетилирования хитина, одного из наиболее распространенных в природе полисахаридов. Высокая биологическая активность, биосовместимость и безопасность хитозана определяют широту и эффективность его применения в медицине, промышленности и агробиологии. В обзоре рассмотрены механизмы действия хитозана в качестве биопестицида, включающие как прямое подавляющее воздействие на патогены, так и индукцию внутренних защитных систем растения в результате связывания хитозана поверхностными рецепторами клеток. На множестве растительных объектов показано влияние хитозана на формирование устойчивости к основным классам патогенов: грибам, бактериям и вирусам. Кроме того, в работе оценены способы применения хитозана, включающие обработку семян, листьев, плодов, почвы, а также соответствующие этим методам специфические биологические эффекты. Отдельный раздел посвящен средствам защиты на основе хитозана, полученным как путем его химической модификации, так и с помощью комбинирования тех или иных молекулярных форм с различными веществами, усиливающими его антипатогенное действие. Представленные в обзоре данные дают представление о хитозане и его производных как об эффективных и перспективных средствах защиты растений и биостимуляторах.
Ключевые слова: средства защиты растений; пестицид; хитозан; новохизоль; патоген; устойчивость; урожайность.

Introduction

The intensive growth of the world's population poses a global problem for agriculture to increase the yield of the main cultivated plant crops. However, yield losses due to numerous bio- and abiotic factors can be very significant. Particularly actual is the control of various pathogens: bacteria, viruses, fungi, which not only reduce yields, but also reduce the quality of plant products as a result of the accumulation of toxins and other metabolites during the infectious process. For a long time this control has been carried out through the use of chemical pesticides, which cover a wide range of pests, are easy to use and have a low cost. But, along with this, they greatly pollute the environment and negatively affect human health (Igbedioh, 1991). In addition, their accumulation in the environment and living organisms can lead to irreversible consequences in ecosystems and a decrease in biodiversity (Yasmin, D'Souza, 2010). The effect of chemical plant protection products can be significantly weakened due to the emergence of resistant forms of pathogens, which makes it necessary to increase the rate of use of these agents or to create new ones (Kumaraswamy et al., 2018).

Another direction is the creation of new plant varieties that are genetically resistant to stress factors and have increased yields in various environmental conditions. However, although this method is the most reliable and effective means of protection, it can also have a temporary effect due to the emergence of new aggressive forms of pathogens. A typical example is the emergence of a new Uganda 99 race of stem rust, a dangerous fungal pathogen of cereals (Singh et al., 2011). In addition, there is a risk of transfer from other areas of such forms of pests to which certain varieties are susceptible.

Apparently, the most effective strategy for plant protection is a combination of methods for the formation of genetic resistance with the use of biostimulants, or biopesticides, which, unlike chemical pesticides, do not cause environmental pollution, ecosystem changes and a negative impact on human health, but are no less effective (Tyuterev, 2014). Over the past decades, a number of biostimulants have been developed that are used to control the processes of plant growth and development, increase their productivity, and also reduce sensitivity to pathogens (Rouphael, Colla, 2020). Among them, a special place is occupied by chitosan, a product of the processing of chitin, the second most widespread natural biopolymer after cellulose.

The aim of this review is to analyze the accumulated scientific data on the effectiveness of the use of chitosan and its derivatives to control plant diseases and increase their productivity. The mechanisms of induction of plant resistance to stress factors under the influence of these plant protection agents are discussed.

Chitosan

The precursor of chitosan is chitin, a biopolymer of the group of nitrogen-containing polysaccharides, consisting of N-acetyl-D-glucosamine and D-glucosamine (Fig. 1). Chitin forms the external skeleton of most invertebrates and is also a component of the cell walls of fungi, yeasts, and algae, accounting for up to 16% of the body's dry weight as a structural polysaccharide (Muzzarelli, 2010).

The use of chitosan began in the 80s of the last century, and since then there have been many works devoted to its use in chemistry, medicine, and agrobiology (Rinaudo, 2006; Malerba, Cerana, 2016). These applications are due to the unique physicochemical properties of chitosan, such as: biocompatibility, non-toxicity and biodegradation. Some organisms, such as zygomycetes, are capable of synthesizing chitosan in significant amounts, which allows to use them to obtain this valuable chitin derivative in various fields of biotechnology (Karimi, Zamani, 2013).

In industry, chitosan is usually obtained from chitin by deacetylation during a chemical process using NaOH (Skryabin et al., 2002). The products of this process are very heterogeneous in terms of the degree of deacetylation, molecular weight, and other chemical parameters determining the differences in their physical properties (viscosity, solubility), which, in turn, determine the possibilities of using chitosan and its biological effects (Orzali et al., 2017). In medicine, it is successfully used for tissue regeneration due to its ability to form elastic biofilms on the wound surface; it has also found application in the creation of anticoagulant and antisclerotic drugs (Skryabin et al., 2002; Chen et al., 2021). Among other applications there are cosmetics, food processing, wastewater treatment, environmental protection (Morin-Crini et al., 2019). In many countries, chitosan and its derivatives have been used for a long time as biostimulants that increase plant productivity and their resistance to pathogens (Tyuterev, 2015). All these effects of chitosan, along with its availability and relatively low cost, make its use as a biological plant protection product economically viable and justified (Xing et al., 2015).

Chitosan as an inducer of plant immunity

The induction of the internal mechanism of plant protection against pathogens is an effective and safe alternative to chemical methods of protection. It is known that a number of

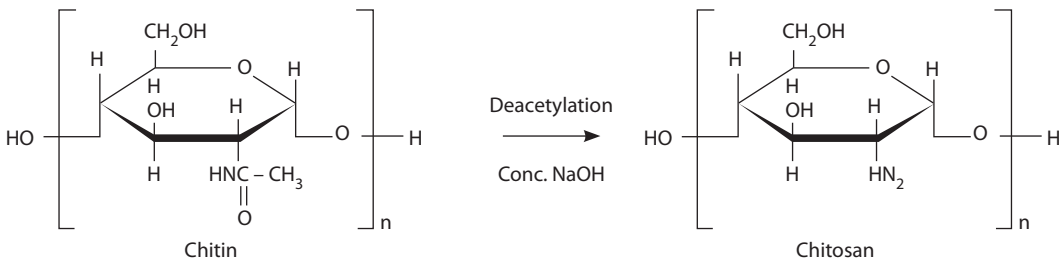

Fig. 1. The structure of chitin and its derivative – chitosan.

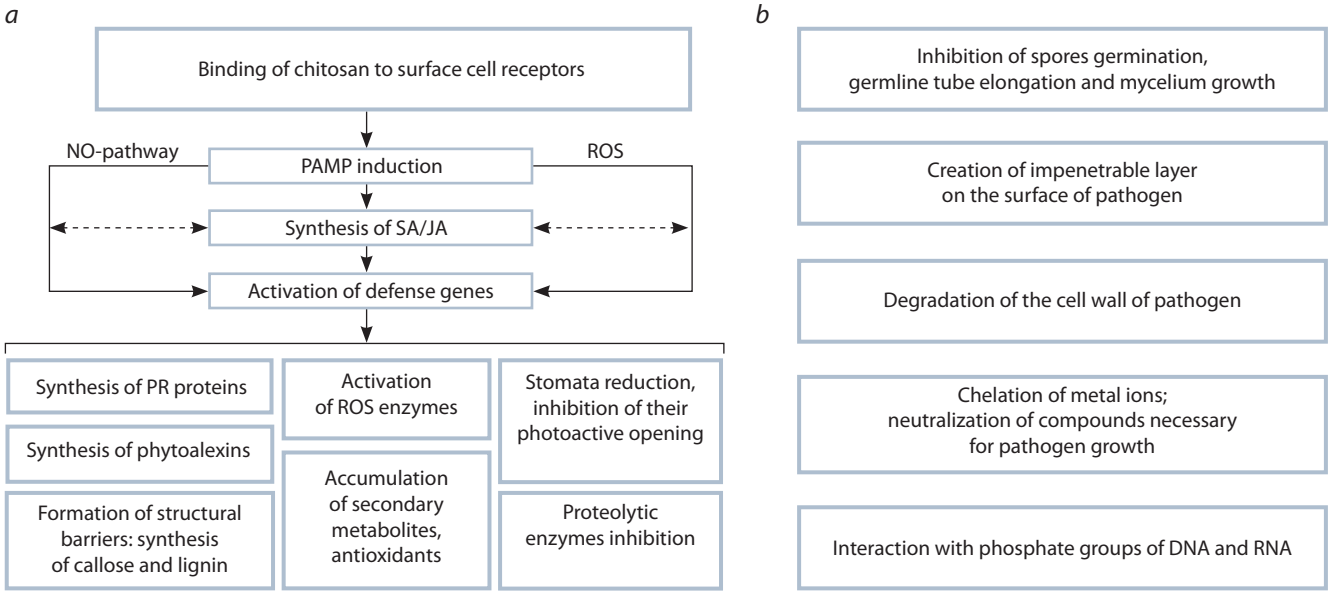

Fig. 2. The effect of chitosan on plant defense mechanisms (*a*) and its antipathogenic effects (*b*).

substances can enhance resistance to pathogens as elicitors (Gaffney et al., 1993; Malerba, Cerana, 2016). The polysaccharide chitosan is one of the most effective resistance stimulators (Falcón-Rodríguez et al., 2012). Its mechanism of action is not yet well understood. It is assumed that chitosan binds to transmembrane cell receptors, which are not currently identified. Also, no protein kinase cascades transmitting a signal from receptors to transcription factors or protection genes have been identified. Various models have been proposed to explain the role of chitosan in plant immunity (Orzali et al., 2017). The most common model suggests the induction of nonspecific PAMP (pathogen-associated molecular pattern) by chitosan, an immune system that includes a number of interrelated signaling cascades (Tyuterev, 2002; Tang et al., 2012). The central role in this system is played by hormonal pathways associated with the synthesis of salicylic and jasmonic acids (SA and JA). In particular, the octadecanoid pathway is activated, leading to the accumulation of JA in tissues (Ishiguro et al., 2001). This hormone, along with SA, activates defense genes encoding various PR (pathogenesis related) proteins (Reinbothe et al., 2009).

Another pathway is initiated by the accumulation of free oxygen radicals (ROS, reactive oxygen species), which are formed in tissues at the earliest stage of stress. Besides the direct toxic effects on pathogens, ROS are functioning as cell signaling molecules that trigger plant defense responses such as cell wall strengthening, hormone synthesis, and programmed cell death (Grant, Loake, 2000). The development of systemic resistance also involves the nitric oxide (NO) signaling pathway, which activates an early protective response, including a hypersensitivity reaction, the formation of a callose layer and the expression of a number of proteins: PR-1 and PR-5, chitinase (CHI), polyphenol oxidase (PPO), peroxidase (POX), superoxide dismutase (SOD), catalase (CAT), and phenylalanine ammonium lyase (PAL) (Manjunatha et al., 2008, 2009). Enzymes PPO, POX, SOD, and CAT are the main enzymes that neutralize excess oxygen radicals (Elsharkawy et al., 2022). PAL is involved in the biosynthesis of protective phenolic compounds such as flavonoids, phenylpropanoids, and lignin (Appert et al., 1994).

As a result of treatment with chitosan, phytoalexins, low molecular weight antibiotic substances, accumulate in plant tissues (Hadwiger, 2013). The synthesis of callose, a polysaccharide, is also induced, which is deposited in the cell wall and serves as a barrier to the penetration of pathogenic organisms (Köhle et al., 1985; Conrath et al., 1989). The process of lignification, which is enhanced under the influence of chitosan, serves the same purpose (Hirano et al., 1999). In particular, it was shown that the formation of structural barriers to the path of the pathogen is the main plant response to chitosan in the tomato *Solanum lycopersicum* L. (Benhamou et al., 2001). Under the influence of chitosan, the suppression of proteolytic enzymes released by pathogens for penetration into plant tissues is enhanced (Peña-Cortes et al., 1988). The effect of chitosan also manifests itself in the reduction of the size of stomata as a result of a decrease in their sensitivity to light (Lee et al., 1999). Possibly, this effect is related to the hormonal activity of JA similar to that of abscisic acid, which is a key regulator of the transpiration process (Sembdner, Parthier, 1993). Other authors have revealed the role of chitosan in the biosynthesis of curcumin, a powerful natural antioxidant deposited in the root tissue of turmeric *Curcuma longa* L. (Sathiyabama et al., 2016). Thus, a wide range of regulatory effects was established that enhance plant immunity under the treatment with chitosan (Fig. 2, *a*).

In addition to the eliciting effect on plant cells, chitosan is able to have a direct effect on pathogens.

Mechanisms of antipathogenic action of chitosan

Chitosan exhibits a variety of antipathogenic activity, which depends, on the one hand, on its chemical properties and method of preparation, and, on the other hand, on the charac-

teristics of the host plant and environmental conditions. In some studies, oligomeric forms of chitosan (penta- or heptamers) exhibited higher fungicidal activity compared to larger molecules (Rabea et al., 2003), while in other studies, on the contrary, an increase in the antipathogenic effect was observed with increasing molecular weight (Kulikov et al., 2006).

Unlike natural chitin, the molecules of which are not charged and have no antimicrobial activity, chitosan has a positive charge. According to one model, electrostatic interaction of chitosan molecules with negatively charged surfaces of pathogen cells results in an increase in the permeability of plasma membranes and destruction of the cell wall (Je, Kim, 2006). Another mechanism implies the formation of an impermeable chitosan polymer layer on the cell surface, which prevents the absorption of nutrients and, at the same time, the excretion of metabolites into the intercellular space (Xing et al., 2015). Chitosan is also able to chelate metal ions and some nutrients necessary for the development of bacteria or fungi, thereby inhibiting the reproduction of the latter and the production of toxins by them (El Hadrami et al., 2010; Xing et al., 2015). In a number of works, the inhibitory effect of chitosan on various stages of pathogen development was established (Rabea et al., 2005; Meng et al., 2010; Reglinski et al., 2010; Badawy, Rabea, 2011). The mechanisms of the antipathogenic action of chitosan are shown in Fig. 2, *b*.

The use of chitosan for protection against various pathogens

Due to climate change, over the past 10–15 years, there has been an increasingly intensive development of various infectious diseases of the main crops of plants, which has led to a significant drop in their productivity and a decrease in product quality. The most widespread are fungal diseases, which account for more than 80 % of all diseases of agricultural plants (Garibova, Sidorova, 1997). So, for example, common wheat *Triticum aestivum* L. ($2n = 42$) can be affected by 25 fungal diseases, including smut, rust, root rots, etc. Yield losses from these diseases in separate areas of distribution can reach 70% or more (Singh et al., 2011).

Under *in vitro* conditions, the fungicidal effect of chitosan was shown against a number of pathogenic fungi, representatives of the genera *Botrytis*, *Alternaria*, *Colletotrichum*, *Rhizoctonia*, etc. (Orzali et al., 2017). At the same time, the suppressive effect of chitosan on various stages of fungal development was demonstrated: mycelium growth, sporulation stage, viability of spores and the efficiency of their germination, and the ability of fungus to produce virulence factors (Badawy, Rabea, 2011). For example, chitosan completely inhibited spore germination and mycelial growth in *Alternaria kikuchiana* S. Tanaka and *Physalospora piricola* Nose (Meng et al., 2010). Also, in grape, it effectively suppressed the growth of mycelium of the fungus *Botrytis cinerea* Pers *in vitro*, as well as on leaves and fruit clusters (Reglinski et al., 2010). E.I. Rabea et al. (2005) reported increased fungicidal activity of 24 chemically modified chitosan derivatives compared to conventional chitosan in a radial growth model of hyphae of *B. cinerea* and *Pyricularia grisea* fungi. Other authors showed that chitosan is able to penetrate the plasma membrane of *Neurospora crassa* Shear and cause cell death as a result of energy imbalance (Palma-Guerrero et al., 2009). An increase in the resistance of tomato to Alternaria under the influence of chitosan was demonstrated (Bayrambekov et al., 2012). Its effectiveness against the anthracnose pathogen (*Colletotrichum* sp.) in cucumbers is comparable to that of chemical fungicides (Dodgson J.L.A., Dodgson W., 2017). Chitosan treatment of common wheat plants prior to infection with the fungal pathogen *Fusarium graminearum* Schwabe, the causative agent of Fusarium rot, has been shown to significantly reduce the number of affected ears (Kheiri et al., 2016). In the same culture, the effect of chitosan on resistance to another dangerous fungal disease, brown leaf rust caused by *Puccinia triticina* Erikss., was shown (Elsharkawy et al., 2022).

Chitosan and its derivatives inhibit the growth of various bacteria (Fei Liu et al., 2001; Wiśniewska-Wrona et al., 2007; Rabea, Steurbaut, 2010; Badawy et al., 2014). However, the latter are less sensitive to the action of chitosan than fungi (Kong et al., 2010). Its minimum inhibitory concentration varies from 0.05 to 0.1 % depending on the type of bacteria, the molecular weight of chitosan, and the pH of the solution (Katiyar et al., 2014). Some authors showed a stronger effect of chitosan on Gram-positive bacteria compared to Gram-negative ones (No et al., 2002; Tayel et al., 2010). This can be explained by the fact that the latter form an additional outer membrane, which is impermeable to high molecular weight chitosan (Xing et al., 2015). However, as shown in other studies, under certain conditions (pH, Mg^{2+} content), chitosan is able to overcome this barrier, making Gram-negative bacteria more sensitive to its action (Helander et al., 2001). Chitosan negatively affects the growth of a number of pathogenic bacteria, including *Xanthomonas* (Li et al., 2008), *Pseudomonas syringae* van Hall (Mansilla et al., 2013), *Agrobacterium tumefaciens* (Smith et Townsend) Conn. and *Erwinia carotovora* (Jones) Waldee (Badawy et al., 2014). The antimicrobial activity of chitosan derivatives against *Escherichia coli* Migula and *Staphylococcus aureu* Rosenbach was also shown (Su et al., 2009).

There are a lot of works devoted to the antiviral effects of chitosan (Su et al., 2009). In plants, chitosan induces resistance to viral diseases, preventing the spread of viruses and viroids so that most treated plants do not develop a systemic viral infection (Chirkov, 2002). It was found that chitosan enhances the expression of RNases associated with the development of resistance to potato virus X (PVX), suppressing its replication in cells (Iriti, Varoni, 2015). Chitosan-treated tomato plants not only show resistance to tomato mosaic virus, but also increased vegetative growth (Abd El-Gawad, Bondok, 2015). Chitosan also effectively inhibits the development of alfalfa mosaic virus (AIMV), tobacco mosaic virus (TMV), squash mosaic virus (SMV) (Nagorskaya et al., 2014). The level of suppression of viral infection varies depending on the molecular weight of chitosan. Low molecular weight chitosan suppresses the formation of local necrosis caused by TMV in tobacco by 50–90 % (Davydova et al., 2011).

Examples of the protective action of chitosan against various plant pathogens are given in the Table. The defense reaction induced by chitosan depends not only on the type of plant or pathogen, but also on the conditions and method of its application.

Examples of the protective action of chitosan in plants

Host	Pathogen	Effects	References
		Fungi	
Durum wheat	*F. graminearum*	Activation of defense genes (*PAL*, *POD*) and accumulation of phenolic compounds	Orzali et al., 2014
Common wheat	*F. graminearum*	Accumulation of lignin and phenols	Bhaskara Reddy et al., 1999
Millet	*A. kikuchiana*	ROS enzymes activation	Meng et al., 2010
Pearl millet	*S. graminicola*	Increasing nitric oxide (NO) level, activation of early protection genes (*PR-1*), callose synthesis	Manjunatha et al., 2008, 2009
Sunflower	*P. halstedii*	Activation of defense genes (*PR-1a*, *CHI*, *POX*, *GLU*, etc.)	Nandeeshkumar et al., 2008
Cherry tomato	*A. alternata*	Activation of defense genes (*PPO*, *POD*, *PAL*)	Chen et al., 2014
Sweet pepper	*B. cinerea*	Fungal germ tube inhibition, polygalacturonase suppression	Ghaouth et al., 1997
Carrot	*S. sclerotiorum*	Destruction of the plasma membrane of the pathogen, activation of defense genes (*POX*, *PPO*)	Qing et al., 2015
Chilli pepper	*Colletotrichum sp.*	Lignin accumulation	Photchanachai et al., 2006
Grape	*B. cinerea*	Activation of defense genes (*PAL*, etc.)	Reglinski et al., 2010
Cucumber	*P. aphanidermatum*	Formation of structural barriers and activation of defense genes	Ghauoth et al., 1994
Lychee	*P. litchii*	Accumulation of lignin, increased expression of *CHI* and *APX* genes	Jiang et al., 2018
Peach	*M. fructicola*	Increased expression of *POX* and *GLU* genes	Ma et al., 2013
Date palm	*F. oxysporum* f. sp. *albedinis*	Activation of defense genes (*POX*, *PPO*) and accumulation of phenolic compounds	Hassni et al., 2004
Potato	*V. dahlia*	Inhibition of fungal growth	Amini, 2015
	P. infestans	SA accumulation, activation of *PAL*, etc.	Zheng et al., 2021
	A. solani	Pathogen cell wall degradation (*CHI* activation)	Abd El-Kareem, Haggag, 2014
Tomato	*F. oxysporum* f. sp. *radicis-lycopersici*	Induction of hormones (SA, JA, abscisic acid), accumulation of phenolic compounds and other stress-induced metabolites	Suarez-Fernandez et al., 2020
	B. cinerea	Callose synthesis, JA accumulation, Avr9/cf-9 expression	De Vega et al., 2021
	P. expansum	Destruction of spore membranes, activation of defense genes	Liu et al., 2007
		Bacteria	
Apricot	*B. seminalis*	Pathogen membrane destruction, bacterial lysis	Lou et al., 2011
Tomato	*R. solanacearum*	Activation of *CHI* and *GLU* defense genes	Algam et al., 2010
Melon	*A. citrulli*	Pathogen membrane destruction, bacterial lysis	Li et al., 2013b
		Viruses	
Potato	PVX (virus X)	Increased expression of RNases	Iriti, Varoni, 2015
Tobacco	TMV (mosaic virus)	Increased activity of hydrolases, destruction of the virus	Nagorskaya et al., 2014
	TNV (necrosis virus)	Callose accumulation, microoxidative bursts, hypersensitivity reaction	Iriti et al., 2006
Tomato	ToMV (mosaic virus)	Stimulating the vegetative growth of plants, synthesis of antioxidants (ascorbic acid)	Abd El-Gawad, Bondok, 2015
		Nematodes	
Tomato	*M. incognita*	Direct effect on the parasite	Khalil, Badawy, 2012
	M. javanica	Stimulating the vegetative growth of plants; direct effect on the parasite	El-Sayed, Mahdy, 2015

Methods of chitosan application

Seed treatment

There are many examples of the effect of seed treatment on plant resistance to infections (Benhamou et al., 1994; Algam et al., 2010; Amini, 2015). In most cases, low molecular weight chitosan demonstrated the highest efficiency (Orzali et al., 2017). Mechanisms for increasing resistance in this case differ depending on the pathogen. For example, it was shown that the treatment of pearl millet seeds with a 4 % solution of chitosan increased resistance to downy mildew caused by the oomycete *Sclerospora graminicola* (Sacc.) J. Schröt (Sharathchandra et al., 2004) by 48 %. In addition, an increase in the expression of a number of proteins associated with the NO signaling pathway was found (see above). A similar effect of seed treatment was found in sunflower in relation to the causative agent of downy mildew *Plasmopara halstedii* (Farl.) Berl. et de Toni (Nandeeshkumar et al., 2008). Chitosan treatment of *T. aestivum* seeds increased resistance to obligate phytopathogens due to the accumulation of phenolic compounds and lignification of cell walls at subsequent stages of plant development after germination (Bhaskara Reddy et al., 1999). An intensification of the lignification process was found during the treatment of chili pepper seeds with chitosan, which increased the survival rate of seedlings infected with the anthracnose pathogen (Photchanachai et al., 2006). Seed treatment with chitosan induced resistance in tetraploid wheat *Triticum durum* Desf. to the causative agent of Fusarium *F. graminearum* (Orzali et al., 2014). At the same time, the analysis of plant tissues showed an increase in the activity of enzymes: guaiacol-dependent peroxidase (POD), ascorbate-dependent peroxidase (APX), as well as PPO and PAL.

Besides the antipathogenic effect, the effect of seed treatment with chitosan is based on the enhancement of metabolic processes in host plant. Thus, it was shown that soaking wheat seeds in a solution of chitosan (in the form of a poly- or oligomer) increased the length of the stem and roots in seedlings 6 days after treatment (Krivtsov et al., 1996). Later, these data were confirmed by Chinese authors, who found that treatment with low molecular weight chitosan increases the vigor of wheat seed germination, as well as plant viability, biomass, and yield, which is associated with accelerated carbon and nitrogen metabolism (Zhang et al., 2017).

Treatment of soil

It is assumed that the addition of chitosan improves soil structure, and also affects the ratio of soil microorganisms, shifting it towards beneficial ones. There is evidence of an increase in the population of actinomycetes and pseudomonads, as well as *Bacillus subtilis* in soils treated with chitosan (Mulawarman et al., 2001). The latter also favorably affects the growth of mycorrhizal fungi (Park, Chang, 2012). In addition, chitosan is able to chemically neutralize toxic substances, pesticides, and fertilizers (Xing et al., 2015). The positive effect of chitosan in the soil also includes the induction of plant defense mechanisms against soil pathogens. For example, in tomato, significant inhibition of the pathogenic fungus *Fusarium oxysporum* f. sp. radicis-lycopersici and nematode *Meloidogyne javanica* Treub was observed as a result of depolarization of root cell membranes that produce hormones, signal lipids, and various protective substances, including phenolic compounds (Suarez-Fernandez et al., 2020). However, in another work, it was shown that the treatment of roots with chitosan did not affect the development of fusariosis in sensitive celery varieties, but effectively reduced the manifestations of the disease in a tolerant variety (Bell et al., 1998).

Chitosan, applied as soil drainage, controlled the development of the bacterial pathogen *Ralstonia solanacearum* Smith in tomato, both as a result of direct action on the pathogen and through eliciting effects, such as the synthesis of CHI and B-2,3-glucanase (GLU), an enzyme that decomposes large polysaccharides (Algam et al., 2010). Soil treatment with chitosan effectively controlled the development of late blight in sweet pepper (Kim et al., 1997) and strawberries (Eikemo et al., 2003). In the date palm, chitosan activated such enzymes as POD and PPO in root cells, as well as the production of hydroxycinnamic acid, which promotes resistance to *F. oxysporum* f. sp albedinis (Hassni et al., 2004). There are a number of works showing the high efficiency of chitosan applied to the soil to control nematodes of various species, so that its action reduces the nematode population, egg weight, and the degree of root damage (Khalil, Badawy, 2012; El-Sayed, Mahdy, 2015).

Leaf treatment

Treatment of vegetative plants with chitosan has been used for many species for various purposes. For example, in the barley *Hordeum vulgare* L., it caused an oxidative burst and production of phenolic compounds in the leaves, which created an unfavorable environment for the spread of fungi (Faoro et al., 2008). Processes such as callose accumulation, microoxidative bursts, and hypersensitivity reaction also developed during tobacco leaf treatment, which ensured its resistance to tobacco necrosis virus (TNV) (Iriti et al., 2006). In another study, the effects of chitosan formulations on the suppression of powdery mildew in grapes were studied (Iriti, Varoni, 2015). In tomato, treatment of leaves with a solution of chitosan caused resistance to the pathogenic fungus *B. cinerea* (De Vega et al., 2021). This resistance correlated with callose deposition at sites of infection, JA accumulation, and expression of the Avr9/cf-9 elicitor protein.

In cucumber leaves, chitosan activated a number of defense reactions against the oomycete *Pythium aphanidermatum* (Edson) Fitzp., including the induction of protective barriers (see above), activation of CHI, chitosanase, and GLU (Ghauoth et al., 1994). The effect of chitosan preparations against the fungus *Phytophthora infestans* (Mont.) de Bary during leaf treatment of potatoes manifested in an increase in the content of polyphenols in plant tissues and suppression of the growth of the pathogen (Zheng et al., 2021). In the same species, a similar effect was also demonstrated against the causative agent of early late blight *Alternaria solani* Sorauer (Abd El-Kareem, Haggag, 2014). In rice, several mechanisms of inhibition of bacterial pathogens have been identified by treating plant leaves with chitosan. On the one hand, there is a direct effect causing lysis of cell membranes and destruction

of bacterial biofilms, and on the other hand, an increase in the production of plant defense proteins, including oxidative stress proteins (peroxidases and oxidases), PAL, etc. (Li et al., 2013a; Stanley-Raja et al., 2021). All these mechanisms provided rice resistance to such pathogenic bacteria as *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas oryzae* pv. *oryzicola*, pathogens of bacterial late blight and leaf streak, respectively. The positive effect of leaf treatment on resistance has also been shown in other plant species (Reglinski et al., 2010; Lou et al., 2011; Li et al., 2013b).

Fruit treatment

The treatment of fruits with biostimulants is of great interest in connection with the problem of tolerance of many pathogens that develop on fruits after harvest to conventional chemical pesticides, as well as in connection with the toxicity of the latter to humans. It has been shown that chitosan reduces the rate of respiration, the production of ethylene, the aging hormone, and moisture loss, thereby contributing to the long-term preservation of the quality of fruits and vegetables (Li, Yu, 2001). Thus, the production of macerating enzymes of cell walls that destroy pectins and cellulose in sweet pepper fruits under the action of chitosan is reduced (Ghaouth et al., 1997). In cherry tomato fruits, chitosan and its complex with methyl jasmonate enhance the activity of PPO, POD, and PAL in the presence of the fungus *Alternaria alternata* (Fr.) Keissl. (Chen et al., 2014). Papaya fruits treated only with chitosan or chitosan in combination with plant extracts remain resistant to the anthracnose pathogen (Bautista-Baños et al., 2003). Treatment of lychee fruits with kadosan (a new formulation of chitosan) effectively reduces their sensitivity to late blight by increasing the activity of CHI, GLU, APX, as well as the accumulation of lignin during storage (Jiang et al., 2018). Chitosan treatment suppresses *B. cinerea* and *Penicillium expansum* Link fungi (causative agents of gray and blue mold, respectively) during storage of tomato fruits, through a direct fungicidal mechanism, including destruction of the spore coat, and also due to the high activity of PPO and POD in fruit tissues (Liu et al., 2007).

Another study showed that the combination of chitosan with beeswax and lime essential oil had a fungicidal effect on *Rhizopus stolonifer* (Ehrenb.) Vuill. by inhibiting mycelium growth, spore germination and sporulation of this fungus in potato (Ramos-García et al., 2012). W. Qing et al. evaluated the effect of chitosan on the control of *Sclerotinia sclerotiorum* (Lib.) de Bary (sclerotinia rot) in carrot (2015). As a result, various antipathogenic effects have been established, including damage to plasma membranes, lipid peroxidation, protein loss, along with an increase in PPO and POD activity in fruits tissues. Other authors showed that soaking harvested sweet cherries or irrigating them with a chitosan solution before harvest effectively suppresses a range of fungal pathogens, namely: *B. cinerea*, *P. expansum*, *R. stolonifera*, *A. alternata*, and *Cladosporium* spp. (Romanazzi et al., 2003). The reduction in infection symptoms correlated with a protective response associated with PAL accumulation. Z. Ma et al. found that chitosan-induced induction of GLU, POD, CAT, CHI, and other enzymes controls brown rot (*Monillinia fructicola*) affecting peach fruits (2013). However, the effect of chitosan per se was not effective in all cases. For example, it did not provide complete protection of pear fruits against blue mold (*P. expansum*), although it was very effective in combination with *Cryptococcus laurentii* and calcium chloride (Meng et al., 2010).

Plant protection products based on chitosan

Despite the presence of a large number of positive effects of chitosan in terms of plant disease control, at present, its use in its pure form is rather limited due to insufficient efficiency. An increase in the biological efficiency of preparations based on chitosan is achieved by its chemical modification, which affects the physical properties, by selecting the optimal ratio of low- and high-molecular forms of chitosan for a particular pathogen-host system, and also by creating complexes with other biologically active substances. The latter, in particular, include organic acids: salicylic, arachidonic, succinic, glutamic, etc., which induce the mechanisms of local and systemic plant resistance to pathogens and thereby increase plant productivity under adverse conditions.

At the moment, a number of complex preparations have been developed in Russia, such as “Narcissus”, “Chitozar”, “Ecogel”, etc. Of particular interest is “Narcissus” (JSC Agroprom – MDT Group of Companies), which includes chitosan (50 %), succinic (30 %) and glutamic (20 %) acids. It increases the resistance of wheat to leaf rust and root rot, rice to blast, tomatoes to late blight and fusarium, cucumbers to powdery mildew, etc. (Badanova et al., 2016). In addition, the preparation destroys the chitinous membrane of root-knot nematodes (Dobrokhotov, 2000; Gol’din, 2014). “Ecogel” (Biochemical Technologies Ltd., Moscow) was obtained by magnetic enrichment of chitosan lactate with silver ions (http://ekogel.ru/poleznaya-informaciya/laktat-hitozana-dlya-rasteniy-svoystva-primenenie/). It improves plant growth and root formation, increases the resistance of a number of crops, such as sugar beet, sunflower, potato, etc., to fungal, bacterial and viral diseases when applied by seed treatment and spraying of plants (Tyuterev, 2015). The All-Russian Institute of Plant Protection (St. Petersburg, Pushkin) has developed a number of preparations under the general name “Chitozar” based on chitosan and other biologically active substances. In addition to chitosan, their composition includes: SA and potassium phosphate (“Chitozar M”), arachidonic acid (“Chitozar F”). These combined preparations were effective against such pests as powdery mildew and downy mildew fungi, California thrips (Kirillova, 2015; Badanova et al., 2016). In particular, the activity of preparations with arachidonic acid and SA against *Phytophthora infestans* (Mont.) de Bary and virus Y, respectively, was demonstrated on potato. In the case of phytophthora, the biological efficiency of the complex was 15 % higher compared to treatment with chitosan alone, and in the case of virus Y plants showed complete resistance after treatment with the complex (6.7 % infected in plants pretreated with chitosan only) (Tyuterev, 2015).

As known, according to the type of nutrition, pathogens are classified into biotrophs, necrotrophs, and hemibiotrophs having different sensitivity to ROS, the level of which is con-

trolled by the antioxidant system. The effect of immunomodulators based on chitosan, vanillin, and SA on the resistance of wheat to pathogens of leaf rust and dark brown spotting differing in the type of nutrition was studied. Combined preparations of chitosan with a certain ratio of vanillin and SA were developed, which provided a high antipathogenic effect against both pathogens due to the modulation of the activity of enzymes of the antioxidant system (Popova et al., 2018).

A perspective direction in plant protection is the use of a complex of chitosan with alginate – a polysaccharide that is part of the cell wall of brown algae. This complex provides encapsulation of beneficial microorganisms that can be used as probiotics and pathogen antagonists (Saberi Riseh et al., 2021).

As mentioned above, there are conflicting data on the antipathogenic activity of low and high molecular weight chitosan, which is largely due to the lack of a unified and reliable method for determining its molecular weight, as well as the fact that in most cases chitosan preparations are a mixture of molecules of different sizes. Along with the complexity and high cost of analyzing the composition of these preparations (the level of polymerization of molecules, the degree of their acetylation, etc.), some chemical features of chitosan also limit its use. For instance, the solubility and, consequently, the efficiency of chitosan in neutral or alkaline media (soil or aqueous solution) is significantly inferior to those in an acidic environment (Katiyar et al., 2014). The solubility of chitosan in a wide pH range can be increased by chemical modification of the polymer molecule, for example, by interaction with mannose (Yu et al., 2023), addition of methyl groups (Wang et al., 2015), and also by intramolecular crosslinking. Recently, a new chitosan derivative, novochizol, was obtained by the last method. Unlike the linear chitosan molecule, the novochizol molecule has a globular, close to spherical shape (https://www.novochizol.ch). Such a molecular design gives it a number of advantages over chitosan, namely: higher chemical stability, low degree of biodegradation, solubility in aqueous solutions with pH > 6, increased adhesion, and the ability to retain various active substances, such as fungicides, in globules and slowly release them. The latter feature provides a significant decrease in the effective concentrations of active substances and, accordingly, a decrease in their negative impact on ecosystems and humans.

The unique capabilities of novochizol allow to combine it with almost any substances (of low or high molecular weight, hydrophilic, hydrophobic, even insoluble), as well as bacteria, fungi and their spores, viruses. Various combination methods (by impregnation or emulsification) make it possible to control the dose of active component and its release rate, the degree of adhesion, and other parameters. It has recently been shown that treatment with novochizol stimulates the germination of common wheat seeds in the soil, and also increases both the root biomass and the total seedling biomass (by 1.5 and 1.8 times, respectively) (Teplyakova et al., 2022). Unlike chitosan, the effect of novochizol and its complexes on plant resistance to pathogens is still poorly studied. It is only assumed that such an action may have a much more pronounced effect due to the synergistic action of novochizol *per se*, and the action of other biologically active substances, for which it can serve as a carrier. There are already preliminary data confirming this assumption obtained on various plant objects (https://www.novochizol.ch/agrotechnology/).

Conclusion

Among the approaches aimed at increasing the resistance of plants to certain factors, biological protection products have great prospects, since, unlike most of the chemical pesticides used, they do not pollute the environment and are non-toxic to humans. These products include chitosan, a deacetylated derivative of chitin. According to numerous authors, treatment with chitosan leads to an increase in plant biomass and an increase in their resistance to abiotic and biotic environmental factors. The antipathogenic effects of chitosan are associated both with a direct effect on pathogens and with its elicitor action associated with the induction of PAMP. The specific biological effects of chitosan are determined by the types of pathogen and host plant, environmental conditions and method of application, depending on the plant organ being treated. Despite the facts of the successful use of chitosan in agrobiology, some of its physical and chemical properties: low solubility and adhesion, chemical instability, limit this application. Recently, a number of different preparations of chitosan have been developed in combination with biologically active substances that enhance its action, as well as an improved chemical derivative, novochizol, which has great potential for use as a biostimulant and an effective plant protection agent.

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ORCID
A.B. Shcherban 0000-0003-1000-8228

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Study of wheat (*Triticum aestivum* L.) breeding material potential for *in vitro* androgenesis

N.V. Petrash ✉, T.N. Kapko, V.V. Sovetov

Siberian Research Institute of Plant Production and Breeding – Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
✉ pnv11@bionet.nsc.ru

Abstract. Doubled haploid technology is a valuable biotechnological approach in plant breeding that enables one to quickly create new varieties through the single-stage production of homozygous lines. The aim of this study was to assess the indicators of *in vitro* androgenesis in the anther culture of the initial breeding material of varieties and combinations of F_1 and F_2 and to identify promising accessions with good responsiveness. For that purpose, the plant material that proved promising for the breeding programs of Siberian Research Institute of Plant Production and Breeding (SibRIPP&B) was used. Ten cultivars of common wheat and the F_1 and F_2 hybrids of nine combinations were evaluated for the main parameters of *in vitro* androgenesis such as the number of new formations, albino, green and all regenerated plants. Induction of androgenesis *in vitro* was carried out in anther culture in growth medium Chu (N6) containing 1 mg/l of growth regulator 2,4-D. The studied samples showed different responses to induction. The maximum level of new formations was found in F_2 hybrids Novosibirskaya 15 × Lutescens ShT-335. The largest number of green plants was found in F_1 Novosibirskaya 15 × Lutescens ShT-335. According to the results of variance analysis, a significant ($p < 0.01$) influence of genotype on the studied traits was established. Varieties with good responsiveness to anther culture (Novosibirskaya 15) and lack of responsiveness to *in vitro* androgenesis (Novosibirskaya 31) were identified. Novosibirskaya 16 was characterized by a low regeneration capacity of new formations. A significant heterotic effect was revealed considering the number of new formations per 100 anthers among the hybrids of such combinations as Novosibirskaya 15 × Lutescens ShT-335, Novosibirskaya 15 × Lutescens 111/09, and Zagora Novosibirskaya × Obskaya 2. Novosibirskaya 15 was recommended for inclusion in crossings as a parental form that provides high hybrid responsiveness during *in vitro* androgenesis. The use of doubled haploid technology made it possible to quickly create DH-lines based on the breeding material.
Key words: doubled haploids; *in vitro* androgenesis; anther culture; *Triticum aestivum* L.; heterosis.

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Изучение потенциала исходного селекционного материала пшеницы (*Triticum aestivum* L.) в андрогенезе *in vitro*

Н.В. Петраш ✉, Т.Н. Капко, В.В. Советов

Сибирский научно-исследовательский институт растениеводства и селекции – филиал Федерального исследовательского центра Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
✉ pnv11@bionet.nsc.ru

Аннотация. Создание удвоенных гаплоидов – ценный биотехнологический подход в селекции растений, позволяющий ускоренно создавать новые сорта за счет одноэтапного получения гомозиготных линий. Целью настоящего исследования было проведение оценки показателей андрогенеза *in vitro* в культуре пыльников исходного селекционного материала сортов и комбинаций F_1 и F_2 и выявление перспективных образцов с хорошей отзывчивостью. В работе использован растительный материал, перспективный для селекционных программ Сибирского научно-исследовательского института растениеводства и селекции – филиала ИЦиГ СО РАН. Десять сортов мягкой пшеницы и гибриды F_1 и F_2 девяти комбинаций скрещивания оценивали по основным параметрам андрогенеза *in vitro*: числу новообразований, числу альбиносов и зеленых растений-регенерантов и всех регенерировавших растений. Индукцию андрогенеза *in vitro* проводили в культуре пыльников на питательной среде Chu (N6), в качестве регулятора роста использовали 1 мг/л 2.4-Д. У изучаемых образцов обнаружен различный ответ на индукцию андрогенеза *in vitro*. Отмечен максимальный выход новообразований у гибридов F_2 Новосибирская 15 × Лютесценс ШТ-335. Наибольшее количество зеленых растений-регенерантов обнаружено у F_1 Новосибирская 15 × Лютесценс ШТ-335. По результатам дисперсионного анализа установлено достоверное ($p < 0.01$) влияние генотипа на изучаемые признаки. Выявлены сорта с хорошей отзывчивостью

в культуре пыльников (Новосибирская 15) и с отсутствием отзывчивости к андрогенезу *in vitro* (Новосибирская 31). Сорт Новосибирская 16 характеризовался низкой регенерационной способностью новообразований. Среди гибридов значительный гетерозисный эффект отмечен по признаку «число новообразований на 100 пыльников» в комбинациях Новосибирская 15 × Лютесценс ШТ-335, Новосибирская 15 × Лютесценс 111/09, Загора Новосибирская × Обская 2. Сорт Новосибирская 15 рекомендован к включению в скрещивания как сорт, обеспечивающий высокую отзывчивость в андрогенезе *in vitro* гибридов. Применение технологии удвоенных гаплоидов позволило быстро создать DH-линии на основе изучаемого материала.
Ключевые слова: удвоенные гаплоиды; андрогенез *in vitro*; культура пыльников; *Triticum aestivum* L.; гетерозисный эффект.

Introduction

Common wheat (*Triticum aestivum* L.) is a critical cereal crop and the main source of vegetable protein for humans. According to the Food and Agriculture Organization of the United Nations (FAO), over 760 million tons of wheat was annually produced around the world in 2019–2021, with Russia having harvested around 78.8 million tons[1]. As the world's population grows, increasing cereal production becomes a necessity. According to projections, the world cereal production is expected to reach 840 million tons by 2030 thanks to, among other things, higher wheat yields[2].

As for breeding efforts, their main goal of is to develop new varieties combining high productivity, environmental plasticity, and resistance to diseases and other environmental stresses. Reaching this goal requires the use of new breeding material and advanced biotechnological methods.

In addition to conventional wheat breeding methods including hybridization and multistage selection followed by a series of self-pollinations to achieve homogeneity and persistence, various optimization approaches have been widely used in recent years, such as production of DH (doubled haploids) lines. The latter are completely homozygous lines obtained by doubling the number of chromosomes in haploid plants. Their use accelerates the breeding process and makes it less laborious, while also providing unique genetic material for mapping populations, phenotyping, and genotyping (Hao et al., 2013; Hale et al., 2022).

DH make it possible to obtain homozygous lines from hybrid material in one generation, while conventional methods take five-six self-pollination generations. This allows plant breeders to produce a new variety in five-seven years and respond quickly to the needs of the grain market.

In recent years, researchers have focused on improving DH production protocols, which has allowed DH technology to become a fast and accurate tool for achieving homozygosity of the original breeding material (Maluszynski et al., 2003; Wędzony et al., 2009; Seguí-Simarro et al., 2021b). The research received a boost with the discovery of *Datura* anther culture's ability to form haploid embryos and seedlings (Guha, Maheshvari, 1964). At present, DH production protocols are available for almost 400 species (Seguí-Simarro et al., 2021a). According to some authors, over 300 varieties have been produced using DH technologies in 12 plant species around the world (Forster, Thomas, 2005).

Doubled haploids may be obtained *in vivo* and *in vitro*. The use of *in vivo* systems implies obtaining a haploid embryo by parthenogenesis, pseudogamy, distant hybridization with subsequent elimination of alien pollinator chromosomes or as a result of intraspecific crosses (pollination by pretreated pollen, crosses with haploid induction lines). Chromosome doubling is a required step in all these DH production techniques. *In vitro* methods are based on obtaining plants from gametophyte cells by gynogenesis (cultivation of ovaries and flowers on nutrient media) or androgenesis (cultivation of anthers and isolated microspores) (Forster, Thomas, 2005; Seguí-Simarro et al., 2021b).

The isolated microspore culture and anther culture are widely used for production of haploids and DH plants in wheat breeding programs (Dunwell, 2010; Lantos et al., 2013; Seguí-Simarro et al., 2021a). DH production by *in vitro* androgenesis in anther culture (AC) is a simple and efficient method of obtaining pure lines (Castillo et al., 2015; Urazaliyev, 2015; Lantos, Pauk, 2016; Kolesnikova et al., 2021). The process is based on changing microspore development program from gametophyte way (pollen grain formation) to sporophyte, and the obtained embryo-like structures (ELS) and calluses are then used to grow regenerated plants (Embryological Foundations..., 2005). These plants are of significant breeding value because they develop from cells following the meiotic division, and thus have unique gene combinations. Haploid cells on the nutrient medium may undergo genome doubling and produce spontaneous DH plants with 100 % homozygosity as a result. In homozygous organisms, the effect of recessive genes can be seen along with that of dominant genes, which significantly accelerates genotype selection (Kasha, Maluszynski, 2003).

The efficiency of androgenesis in AC is affected by many factors, including donor growth conditions, microspore development stage, pretreatment conditions, nutrient medium composition, but genotype is what affects it the most (Tuvesson et al., 2000; Lantos, Pauk, 2020; Seguí-Simarro et al., 2021b; Hale, 2022). The success in obtaining androgenic regenerant plants is limited due to albinism and significant genotypic dependency (Li et al., 2013; Zhao L. et al., 2015). Genotype-dependent variation in responsiveness can be seen both at intraspecific and interspecific levels. For example, hexaploid winter wheats show better *in vitro* androgenic responsiveness than the spring ones (Sharma et al., 2005; Lazaridou et al., 2016). A wheat-rye 1RS.1BL translocation has a positive effect on plant regeneration in *in vitro* androgenesis (Agache et al., 1989; Pershina et al., 2013; Timonova et al., 2022).

[1] Crops and livestock products. https://www.fao.org/faostat/en/#data/QCL
[2] OECD/FAO (2021), OECD-FAO Agricultural Outlook 2021–2030, OECD Publishing, Paris. https://doi.org/10.1787/19428846-en

Table 1. F_1–F_2 combinations and their parent varieties assessed with respect to *in vitro* androgenic responsiveness in the anther culture

No.	Varieties	No.	Combinations, generation F_1, F_2	No. combination
1	Novosibirskaya 15	11	Novosibirskaya 15 × Lutescens ShT-335	No. 3
2	Novosibirskaya 16	12	Novosibirskaya 15 × Lutescens 111/09	No. 2
3	Novosibirskaya 18	13	Novosibirskaya 16 × Lutescens 111/09	No. 7
4	Novosibirskaya 31	14	Novosibirskaya 18 × Lutescens 111/09	No. 9
5	Novosibirskaya 75	15	Novosibirskaya 18 × Sigma	No. 61
6	Zagora Novosibirskaya	16	Novosibirskaya 75 × Lutescens 111/09	No. 23
7	Obskaya 2	17	Novosibirskaya 31 × Lutescens 111/09	No. 14
8	Sigma	18	Zagora Novosibirskaya × Lutescens 111/09	No. 26
9	Lutescens 111/09	19	Zagora Novosibirskaya × Obskaya 2	No. 24
10	Lutescens ShT-335			

Additive, dominant, and epistatic relationships between genes responsible for inheritance of androgenic traits in AC were observed (Chaudhary et al., 2003; Dagüstü, 2008; Grauda et al., 2016). At the same time, some authors showed that androgenic responsiveness in AC followed a simple inheritance scheme and was controlled by dominant genes (El-Hennawy et al., 2011). B.E.S. Abd El-Fatah et al. (2020) demonstrated that additive effects prevailed over dominance effects in terms of genetic control of *in vitro* androgenic traits.

A viable strategy of overcoming genotypic dependency is to use breeding material with high *in vitro* androgenic responsiveness, i. e. one of the parents in the cross should induce the development of green regenerants in hybrids (Tuvesson et al., 2003; Kondic-Špika et al., 2011; Lantos, Pauk, 2020). Thus, it seems reasonable to assess the initial breeding samples and use the ones with good *in vitro* androgenic responsiveness in crosses.

The goal of the present study was to assess *in vitro* androgenic indicators in the anther culture of the initial breeding material from spring varieties of common wheat and combinations of F_1 and F_2, as well as identify promising accessions with good responsiveness.

Materials and methods

Spring common wheat samples showing promise under the breeding program of Siberian Research Institute of Plant Production and Breeding (SibRIPP&B) – Branch of ICG SB RAS were used as breeding material. Nine combinations of F_1 and F_2 and ten parent varieties were selected for the assessment of *in vitro* androgenic responsiveness (Table 1).

Anther donor plants were grown on the field of Siberian Research Institute of Plant Production and Breeding in 2022. The spikes were harvested from leading shoots while most of the microspores were at the mid to late uninucleate stage. In terms of visual evidence, it meant that the middle of the spike was at the same height as the second top leaf sheath. Microspore development stage was identified using a Leica CME microscope (Leica Microsistems, Russia) in acetocarmine-stained cytological squash preparations.

The harvested spikes were stored in a temperature controlled container with cooling agents, transported to the laboratory, placed in test tubes with distilled water, and kept in a refrigerated thermostat TVL-K at +4 °C for seven days. After the cold pretreatment, the spikes were sterilized with wipes soaked in 70 % and then 96 % alcohol and transported to a biosafety box. The anthers were obtained from lateral flowers from the middle of the spike, with the average of about 50 anthers per spike. The experiments were performed in triplicate with one Petri dish for each measurement and with at least 100 anthers obtained for each accession.

The anthers from two spikes with the same genotype were inoculated in 100 mm Ø Petri dish with 15–20 ml of Chu's N6 induction medium (Chu, 1978), 90 g/l sugars (sucrose: maltose in the ratio of 2:1); 100 mg/l myo-inositol; 1 mg/l 2,4-D, 0.5 mg/l kinetin, and 6 g/l plant agar. Petri dishes with anthers were incubated in the dark at 28 °C until the emergence of the first microspore-derived structures, and then at 25 °C for the further growth of the obtained structures. Following the incubation period of 30–40 days, the ELSs and calluses reaching 1.5–2 mm in diameter were placed in quantities of 3 to 5 in 28 mm Ø test tubes with Gamborg's B5 medium (Gamborg et al., 1968), 30 g/l sucrose, 5 g/l plant agar without growth regulators. Plantlets regenerated under LED lights with photosynthetic photon flux density (PPFD) of 751.6 μmol/m^2/s at 18–20 °C for 20–30 days with photoperiod of 16 hours.

Green plantlets with well-developed roots and leaves were taken out from the test tubes, with the remains of the nutrient medium thoroughly washed away from the roots, and planted into separate pots (0.8 l) with a mixture of coconut substrate, all-purpose soil, and vermiculite in the ratio of 3:1:1. The rooted plants were grown under the same LED lights at temperatures of 19–21 °C and humidity of about 50–60 %. The plants were grown to full maturity. Only the fertile plants (spontaneous DH) were selected for further study, while partially fertile or sterile plants were discarded.

The responsiveness of the AC was assessed using the following indicators: number of neoplasms (ELSs and calluses) per 100 isolated anthers (N/100A); number of albino plantlets

per 100 isolated anthers (AP/100A); number of green plantlets per 100 isolated anthers (GP/100A); total plantlets per 100 neoplasms (TP/100N).

Statistical processing of the data was performed using Microsoft Excel 2010. Analysis of variance was carried out using SNEDECOR software (Sorokin, 2004). True (H_{tr}, %) and hypothetical (H_{hyp}, %) heterosis were calculated using Eqs. (1) and (2) based on (Omarov, 1975):

$$H_{tr} = F_1 - P_{best} / P_{best} \times 100\ \%, \quad (1)$$

$$H_{hyp} = F_1 - P_{av} / P_{av} \times 100\ \%, \quad (2)$$

where F_1 is the value of interest in the hybrid; P_{best} is the same value in the best parent; P_{av} is the average value between parents (P1 + P2)/2.

The degree of phenotypic dominance (Hp) acting as an inheritance indicator in the controlled crosses was calculated using Eq. (3) based on (Griffing, 1956):

$$Hp = F_1 - MF/HF - MF, \quad (3)$$

where Hp is the dominance value; F_1 is the observed mean of F_1; MF is the average attribute value between parents; and HF is the attribute value in the best parent. The interpretation was as follows: Hp > 1 was recognized as positive heterosis, Hp = 0.5–1.0 as positive dominance, Hp from +0.5 to –0.5 as intermediate inheritance, Hp = –0.5 to –1.0 as negative dominance, and Hp < –1.0 as negative heterosis. Inbreeding depression (ID %) was calculated using Eq. (4) based on (Pederson, 1971):

$$ID = (F_2 - F_1/F_1) \times 100\ \%, \quad (4)$$

where ID is the inbreeding depression, F_1 is the average attribute value in the first-generation hybrid family, F_2 is the average attribute value in the second-generation hybrid family.

Results and discussion

The success of DH technology in breeding programs depends on the genotype's ability to regenerate green plants in *in vitro* androgenesis.

In the present paper, the assessment of *in vitro* androgenic responsiveness is presented for 10 varieties and 9 combinations, generations F_1 and F_2. A total of 16,598 anthers have been isolated and placed in induction medium, with at least 100 anthers analyzed in triplicate for each accession. The single-factor analysis of variance showed a significant effect of genotype on all *in vitro* androgenic indicators of interest (Table 2).

The studied samples showed a variety of *in vitro* androgenic responses (Table 3). The number of neoplasms per 100 isolated anthers (N/100A) indicates the quantity of structures (ELSs and calluses) developing from microspores. This attribute varied from 0 to 17.20 (Novosibirskaya 15 × Lutescens ShT-335, F_2), the average being 3.74. The average number of green regenerants per 100 anthers (GP/100A) was 1.45. Maximum values were observed in F_1 Novosibirskaya 15 × Lutescens ShT-335 and Novosibirskaya 15 × Lutescens 111/09, at 12.15 and 12.50, respectively. The average number of albino plantlets per 100 anthers (AP/100A) was 0.63. Maximum values were observed in Novosibirskaya 15 (2.67), F_2 Novosibirskaya 15 × Lutescens ShT-335 (2.40), and F_1 Zagora Novosibirskaya × Obskaya 2 (2.92). The average total number of regenerants per 100 anthers was 2.08. Maximum values with prevalence of green regenerants were observed in F_1 Novosibirskaya 15 × Lutescens ShT-335 and Novosibirskaya 15 × Lutescens 111/09. A total of 150 green plantlets were obtained in the experiment.

The analysis showed that high neoplasm production was not directly associated with a high number of regenerants. For instance, varieties Novosibirskaya 15 ($p < 0.10$) and Novosibirskaya 16 ($p < 0.05$) both showed above average neoplasm production, but Novosibirskaya 15 also showed higher regeneration ability (TP/100A = 4.33, $p < 0.05$). Novosibirskaya 16 produced 12.40 neoplasms per 100 anthers with 1.80 regenerated plantlets per 100 anthers (see Table 3). This observation confirms the literature data that *in vitro* androgenic indicators are polygenically controlled and independently inherited (Ekiz, Konzak, 1994; Nielsen et al., 2015; Abd El-Fatah et al., 2020). Novosibirskaya 31 and, notably, its combinations in the first and second generations did not produce any structures, allowing us to assume that a non-responsive genotype worthy of further research has been discovered.

The ability of calli and embryo structures to regenerate into plantlets is reflected in the number of green regenerants per 100 neoplasms and the number of albino plantlets per 100 neoplasms (see the Figure). The experiment showed that the average number of regenerated green plantlets per 100 neoplasms was higher than the number of albino plantlets, the respective values being 26.41 and 18.74. The highest regeneration ability, with more than half of neoplasms regenerating into plants, was observed in hybrids F_1 No. 3 (Novosibirskaya 15 × Lutescens ShT-335), No. 2 (Novosibirskaya 15 × Lutescens 111/09), No. 7 (Novosibirskaya 16 × Lutescens 111/09), No. 61 (Novosibirskaya 18 × Sigma), No. 26 (Zagora Novosibirskaya × Lutescens 111/09), and F_2 No. 26 (Zagora Novosibirskaya × Lutescens 111/09) (see the Figure). Notably, the number of green regenerants per 100 neoplasms was above 100 for hybrid F_1 No. 3 (Novosibirskaya 15 × Lutescens ShT-335), possibly

Table 2. Single-factor analysis of variance for *in vitro* androgenic responsiveness indicators in the anther culture of wheat varieties and F_1–F_2 hybrids

Source of variation	*df*	N/100A		GP/100A		AP/100A	
		Effect, %	F_{fact}	Effect, %	F_{fact}	Effect, %	F_{fact}
Genotype	27	73.44	9.30*	78.57	12.00*	51.82	4.23*
Random factors	56	26.56	–	21.43	–	48.18	–

*$p < 0.01$ ($F_{tab.\ 0.99} = 2.18$); *df* is the number of degrees of freedom; F_{fact} is the calculated Fisher test value; N/100A is the number of neoplasms per 100 anthers; GP/100A is the number of green plantlets per 100 anthers; AP/100A is the number of albino plantlets per 100 anthers.

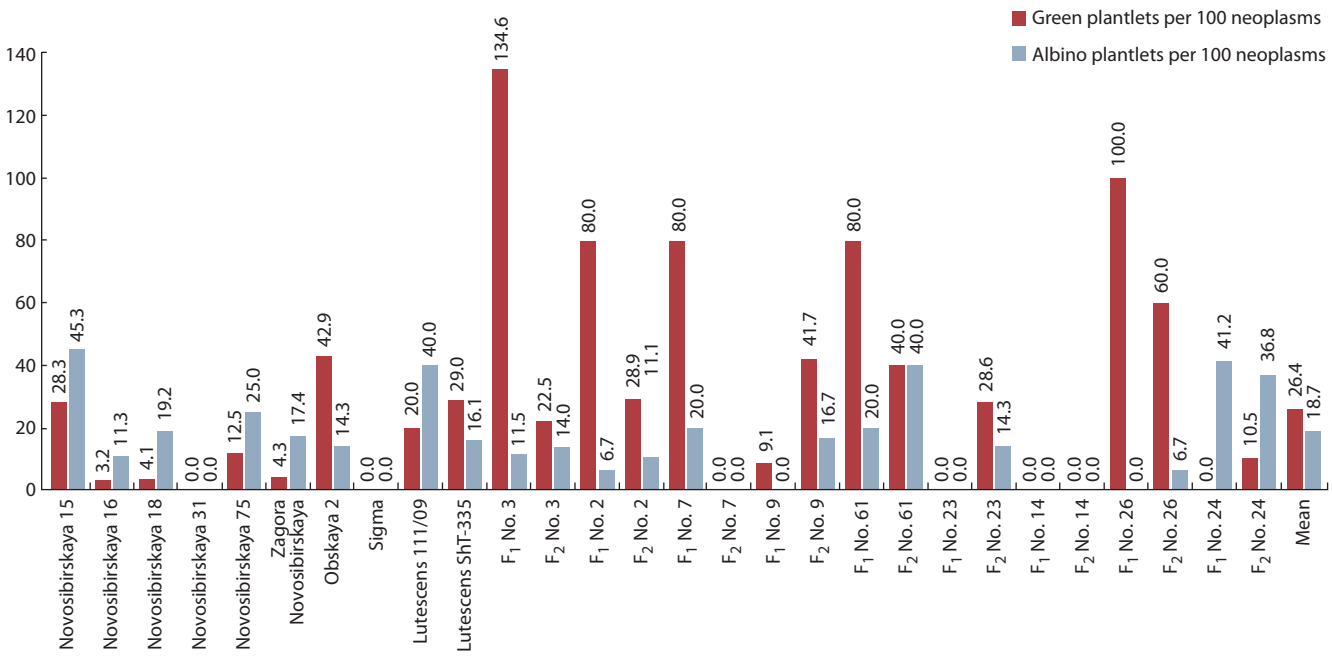
Table 3. *In vitro* androgenic responsiveness indicators in the anther culture of wheat varieties and F_1–F_2 hybrids

Genotype	N/100A	GP/100A	AP/100A	TP/100A
Novosibirskaya 15	5.89[2] ± 1.26	1.67 ± 0.33	2.67[1] ± 0.33	4.33[1] ± 0.58
Novosibirskaya 16	12.40[1] ± 2.67	0.40 ± 0.40	1.40[1] ± 0.36	1.80 ± 0.73
Novosibirskaya 18	5.21 ± 0.70	0.21 ± 0.22	1.00 ± 0.55	1.21 ± 0.70
Novosibirskaya 31	0.00	0.00	0.00	0.00
Novosibirskaya 75	2.13 ± 0.46	0.27 ± 0.46	0.53 ± 0.32	0.80 ± 0.78
Zagora Novosibirskaya	2.71 ± 0.66	0.12 ± 0.21	0.47 ± 0.41	0.59 ± 0.21
Obskaya 2	0.47 ± 0.31	0.20 ± 0.35	0.07 ± 0.12	0.27 ± 0.46
Sigma	0.40 ± 0.29	0.00	0.00	0.00
Lutescens 111/09	1.43 ± 0.73	0.29 ± 0.08	0.57 ± 0.31	0.86 ± 0.37
Lutescens ShT-335	2.58 ± 0.25	0.75 ± 0.25	0.42 ± 0.52	1.17 ± 0.52
F_1 (Novosib. 15 × Lut. ShT-335)	9.03[1] ± 1.35	12.15[1] ± 4.51	1.04[2] ± 0.00	13.19[1] ± 4.51
F_2 (Novosib. 15 × Lut. ShT-335)	17.20[1] ± 2.95	3.87[1] ± 0.61	2.40[1] ± 1.06	6.27[1] ± 1.40
F_1 (Novosib. 15 × Lut. 111/09)	15.63[1] ± 2.76	12.50[1] ± 2.08	1.04[2] ± 0.00	13.54[1] ± 2.08
F_2 (Novosib. 15 × Lut. 111/09)	4.50 ± 0.81	1.30 ± 0.63	0.50 ± 0.17	1.80 ± 0.61
F_1 (Novosib. 16 × Lut. 111/09)	1.74 ± 1.54	1.39 ± 0.68	0.35 ± 0.60	1.74 ± 1.28
F_2 (Novosib. 16 × Lut. 111/09)	0.40 ± 0.23	0.00	0.00	0.00
F_1 (Novosib. 18 × Lut. 111/09)	3.27 ± 1.68	0.30 ± 0.26	0.00	0.30 ± 0.26
F_2 (Novosib. 18 × Lut. 111/09)	1.33 ± 1.04	0.56 ± 0.39	0.22 ± 0.20	0.78 ± 0.49
F_1 (Novosib. 18 × Sigma)	1.74 ± 0.31	1.39 ± 0.68	0.35 ± 0.30	1.74 ± 0.88
F_2 (Novosib. 18 × Sigma)	1.00 ± 0.2	0.40 ± 0.36	0.40 ± 0.40	0.80 ± 0.70
F_1 (Novosib. 75 × Lut. 111/09)	0.69 ± 0.60	0.00	0.00	0.00
F_2 (Novosib. 75 × Lut. 111/09)	1.17 ± 0.38	0.33 ± 0.29	0.17 ± 0.29	0.50 ± 0.00
F_1 (Novosib. 31 × Lut. 111/09)	0.00	0.00	0.00	0.00
F_2 (Novosib. 31 × Lut. 111/09)	0.00	0.00	0.00	0.00
F_1 (Zagora Novosib. × Lut. 111/09)	0.35 ± 0.34	0.35 ± 0.34	0.00	0.35 ± 0.34
F_2 (Zagora Novosib. × Lut. 111/09)	3.00 ± 1.05	1.80 ± 0.35	0.20 ± 0.35	2.00 ± 0.35
F_1 (Zagora Novosib. × Obskaya 2)	7.08[1] ± 3.94	0.00	2.92[1] ± 1.13	2.92 ± 1.13
F_2 (Zagora Novosib. × Obskaya 2)	3.80 ± 1.28	0.40	1.40[1] ± 1.08	1.80 ± 1.24
Mean	3.74	1.45	0.63	2.08
$LSD_{0.05}$	2.50	1.72	0.42	1.88
$LSD_{0.10}$	2.11	1.45	0.36	1.58

Note. N – neoplasms; GP – green plantlets; AP – albino plantlets; A – anthers; TP – total plantlets; Novosib. – Novosibirskaya; Lut. – Lutescens.
[1] Differences from the mean are significant at $p = 0.05$; [2] differences from the mean are significant at $p = 0.10$.

due to secondary embryogenesis or an ELS developing into polyembryoids (structures with several shoot growth points (Seldimirova, 2009; Pershina et al., 2020)). Both mechanisms produce clones or sister plants.

Albinism acts as a limitation for DH production in *in vitro* androgenesis. Our experiment showed the prevalence of green plantlets in the total number of plantlets in varieties as follows: Obskaya 2, Lutescens ShT-335, F_1 hybrids Novosibirskaya 15 × Lutescens ShT-335, Novosibirskaya 15 × Lutescens 111/09, Novosibirskaya 16 × Lutescens 111/09, Novosibirskaya 18 × Sigma, Zagora Novosibirskaya × Lutescens 111/09, and F_2 hybrids Novosibirskaya 18 × Lutescens 111/09, Novosibirskaya 75 × Lutescens 111/09, Zagora Novosibirskaya × Lutescens 111/09 (see the Figure). It follows



Percentage of green and albino plantlets per 100 neoplasms in *in vitro* androgenesis of wheat varieties and F_1–F_2 hybrids.

No. 3 – (Novosibirskaya 15 × Lutescens ShT-335); No. 2 – (Novosibirskaya 15 × Lutescens 111/09); No. 7 – (Novosibirskaya 16 × Lutescens 111/09); No. 9 – (Novosibirskaya 18 × Lutescens 111/09); No. 61 – (Novosibirskaya 18 × Sigma); No. 23 – (Novosibirskaya 75 × Lutescens 111/09); No. 14 – (Novosibirskaya 31 × Lutescens 111/09), No. 26 – (Zagora Novosibirskaya × Lutescens 111/09); No. 24 – (Zagora Novosibirskaya × Obskaya 2); $LSD_{0.05}$ (GP/100N) = 19.51; $LSD_{0.05}$ (AP/100N) = 7.81.

from the analysis of variance that around 50 % of albinism cases are genotype-related (see Table 2).

There are several factors increasing the chance of albinism, including genotype, donor growth conditions, cultivation conditions, medium composition, incompatibility of nuclear and plastid genomes, and plastid DNA deletions or mutations (Nielsen et al., 2015; Zhao P. et al., 2017). The high significance of the genotype's effect on the number of albino plantlets is demonstrated in a number of papers (Lantos, Pauk, 2016; Castillo et al., 2019; Abd El-Fatah et al., 2020; Kanbar et al., 2020).

Genotypic dependency of albinism is associated with transcription activation of specific genes involved in chloroplast biogenesis at early stages (Mozgova et al., 2006; Canonge et al., 2021). Chloroplast DNA deletions were observed in albino plants, along with inhibited transcription of the nuclear genes coding for chloroplast-localized proteins, while levels of transcripts coding for proteins not present in chloroplasts were identical to those in green plants (Dunford, Walden, 1991).

To evaluate the prospects of using the studied varieties in further crosses, the heterosis effect in their hybrids was analyzed. Heterosis effect of *in vitro* androgenic responsiveness was described earlier, and its degree was shown to vary between genotypes (Ouyang et al., 1973; Ekiz, Konzak, 1994).

True (H_{tr}) and hypothetical (H_{hyp}) heterosis, inheritance indicator (Hp), and inbreeding depression (ID %) were calculated based on the number of neoplasms per 100 anthers, since, according to the analysis of variance, genotype significantly contributes to this value (73.44 %, see Table 2) and directly affects the subsequent *in vitro* androgenic responsiveness indicators. Maximum hypothetical heterosis was observed in Zagora Novosibirskaya × Obskaya 2, and minimum, in Novosibirskaya 31 × Lutescens 111/09 (Table 4). True heterosis characterizes stronger manifestation of the trait in F_1 compared to the best parent.

Maximum true heterosis of 100 % was observed in Novosibirskaya 15 × Lutescens 111/09, negative heterosis was observed in hybrids with Novosibirskaya 31 demonstrating androgenic non-responsiveness. Significant negative H_{tr} was also observed in Novosibirskaya 16 × Lutescens 111/09 and Zagora Novosibirskaya × Lutescens 111/09.

Analysis of the inheritance indicator showed positive heterosis for Novosibirskaya 15 × Lutescens ShT-335, Novosibirskaya 15 × Lutescens 111/09, Zagora Novosibirskaya × Obskaya 2. Intermediate inheritance was observed in combinations with Novosibirskaya 18. Negative dominance was observed in Novosibirskaya 16 × Lutescens 111/09, and negative heterosis, in Novosibirskaya 75 × Lutescens 111/09 and Zagora Novosibirskaya × Lutescens 111/09.

The degree of manifestation of *in vitro* androgenic attributes varies between F_1 and F_2 hybrids. The first generation outperformed the second one in neoplasms per 100 anthers in Novosibirskaya 15 × Lutescens 111/09, Novosibirskaya 16 × Lutescens 111/09, Novosibirskaya 18 × Lutescens 111/09, Novosibirskaya 18 × Sigma, Zagora Novosibirskaya × Obskaya 2. Inbreeding depression was observed in Novosibirskaya 15 × Lutescens ShT-335, Novosibirskaya 75 × Lutescens 111/09, Zagora Novosibirskaya × Lutescens 111/09 (see Table 4). Negative ID % value shows that F_1 hybrids outperform F_2 in terms of manifestation of the attribute.

To summarize the analysis of the inherited ability to produce structures from microspores in various combinations, it is

Table 4. Heterosis effect and inheritance indicator for the number of neoplasms per 100 anthers in nine common wheat combinations

Combination	Indicator			
	H_{hyp}, %	H_{tr},%	Hp	ID %
Novosibirskaya 15 × Lutescens ShT-335	113.22	53.31	2.90[1]	90.48
Novosibirskaya 15 × Lutescens 111/09	327.05	165.37	5.37[1]	–71.21
Novosibirskaya 16 × Lutescens 111/09	–74.84	–85.97	–0.94[3]	–77.01
Novosibirskaya 18 × Lutescens 111/09	–1.51	–37.24	–0.03[2]	–59.33
Novosibirskaya 18 × Sigma	–37.97	–66.60	–0.44[2]	–42.53
Novosibirskaya 75 × Lutescens 111/09	–61.24	–67.61	–3.11[4]	69.57
Novosibirskaya 31 × Lutescens 111/09	–100	–100	–*	–*
Zagora Novosibirskaya × Lutescens 111/09	–83.09	–87.08	–2.69[4]	757.14
Zagora Novosibirskaya × Obskaya 2	345.28	161.25	4.90[1]	–46.33

Note. H_{hyp}, % is the hypothetical heterosis; H_{tr},% is the true heterosis; ID % is the inbreeding depression; Hp is the degree of dominance; [1] positive heterosis; [2] intermediate inheritance; [3] negative dominance; [4] negative heterosis; * not available due to absence of neoplasms.

worth focusing on positive values observed for combinations with Novosibirskaya 15. These results agree with the previously obtained data on the responsiveness of F_1 and F_2 hybrids Obskaya 2 × Novosibirskaya 15 compared to parent varieties (Petrash et al., 2022). Studying the inheritance patterns in multiple combinations makes it possible to estimate positive *in vitro* androgenic responsiveness in hybrids to ensure effective pair selection for crosses under future breeding programs using doubled haploid technology.

Conclusion

The goal of the paper was to study the potential of the initial breeding material from the perspective of *in vitro* androgenesis in 10 different common wheat varieties and 9 combinations of F_1 and F_2, with a total of 28 genotypes analyzed. The androgenic indicators analyzed included the number of neoplasms (ELSs and calluses), green plantlets, albino plantlets, and the total number of regenerated plants.

As a result, the varieties showing *in vitro* androgenic responsiveness (Novosibirskaya 15) and non-responsiveness (Novosibirskaya 31) in the anther culture have been identified. Novosibirskaya 16 was characterized by low neoplasm regeneration ability. A significant heterosis effect was observed in hybrids Novosibirskaya 15 × Lutescens ShT-335, Novosibirskaya 15 × Lutescens 111/09, Zagora Novosibirskaya × Obskaya 2. Positive heterosis in terms of neoplasms per 100 anthers was observed in combinations with Novosibirskaya 15, and intermediate inheritance, in combinations with Novosibirskaya 18. Novosibirskaya 15 is recommended for inclusion into crosses as a variety ensuring high *in vitro* androgenic responsiveness in hybrids compared to the second parent. Doubled haploid technology made it possible to use the discussed hybrid material to produce DH lines, which are now being tested in the field.

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ORCID

N.V. Petrash orcid.org/0000-0002-7499-6803
T.N. Kapko orcid.org/0000-0003-1573-1618
V.V. Sovetov orcid.org/0000-0003-4497-9137

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Reconstruction and analysis of the gene regulatory network for cell wall function in *Arabidopsis thaliana* L. leaves in response to water deficit

A.R. Volyanskaya[1, 2], E.A. Antropova[1], U.S. Zubairova[1, 2], P.S. Demenkov[1, 3], A.S. Venzel[1, 3], Y.L. Orlov[1, 4, 5], A.A. Makarova[1], T.V. Ivanisenko[1, 3], T.A. Gorshkova[6], A.R. Aglyamova[6], N.A. Kolchanov[1], M. Chen[7], V.A. Ivanisenko[1, 2, 3] ✉

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Science, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
[3] Kurchatov Genomic Center of ICG SB RAS, Novosibirsk, Russia
[4] I.M. Sechenov First Moscow State Medical University of the Ministry of Health of the Russian Federation (Sechenov University), Moscow, Russia
[5] Peoples' Friendship University of Russia, Moscow, Russia
[6] Kazan Institute of Biochemistry and Biophysics, FRC Kazan Scientific Center of RAS, Kazan, Russia
[7] College of Life Sciences, Zhejiang University, Hangzhou, China
✉ salix@bionet.nsc.ru

Abstract. The plant cell wall represents the outer compartment of the plant cell, which provides a physical barrier and triggers signaling cascades under the influence of biotic and abiotic stressors. Drought is a factor that negatively affects both plant growth and development. Cell wall proteins (CWP) play an important role in the plant response to water deficit. The adaptation mechanisms of the cell wall to water loss are of interest for identifying important genetic factors determining plant drought resistance and provide valuable information on biomarkers for further selection aimed at increasing the yield of crop plants. Using ANDSystem, a gene network describing the regulation of CWPs under water restriction conditions was reconstructed. The analysis of the gene network and the transcriptome data analysis allowed prioritizing transcription factors (TF) based on their enrichment of differentially expressed genes regulated by them. As a result, scores were calculated, acting as indicators of the association of TFs with water deficit. On the basis of the score values, eight most significant TFs were selected. The highest priority was given to the TF GBF3. CWPs were prioritized according to the criterion of summing up the scores of transcription factors regulating these genes. Among the most prioritized CWPs were the *AT5G03350* gene encoding a lectin-like protein, *AT4G20860* encoding BBE-like 22 required for the oxidation of cellulose degradation products, and *AT4G37800* encoding xyloglucan endotransglucosylase/hydrolase 7. Overall, the implemented algorithm could be used for prediction of regulatory interactions between transcription factors and target genes encoding cell wall proteins in plants.
Key words: plant cell wall; drought; plants; differentially expressed genes; text mining; microarray; gene regulatory network.

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Реконструкция и анализ регуляторной генной сети функционирования клеточной стенки листьев *Arabidopsis thaliana* L. при ответе на водный дефицит

А.Р. Волянская[1, 2], Е.А. Антропова[1], У.С. Зубаирова[1, 2], П.С. Деменков[1, 3], А.С. Вензель[1, 3], Ю.Л. Орлов[1, 4, 5], А.А. Макарова[1], Т.В. Иванисенко[1, 3], Т.А. Горшкова[6], А.Р. Агъялмова[6], Н.А. Колчанов[1], М. Чен[7], В.А. Иванисенко[1, 2, 3] ✉

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
[3] Курчатовский геномный центр ИЦиГ СО РАН, Новосибирск, Россия
[4] Первый Московский государственный медицинский университет им. И.М. Сеченова Министерства здравоохранения Российской Федерации (Сеченовский Университет), Москва, Россия
[5] Российский университет дружбы народов имени Патриса Лумумбы, Москва, Россия
[6] Казанский институт биохимии и биофизики – обособленное структурное подразделение Федерального исследовательского центра «Казанский научный центр Российской академии наук», Казань, Россия
[7] Колледж наук о жизни, Чжэцзянский университет науки и техники, Ханчжоу, Китай
✉ salix@bionet.nsc.ru

Аннотация. Растительная клеточная стенка представляет собой внешний компартмент растительной клетки, который во многом обеспечивает физический барьер и запуск сигнальных каскадов при действии био- и абиотических стрессоров. Засуха негативно влияет как на рост, так и развитие растений. Белки клеточной стенки

(БКС) играют существенную роль в ответе растений на водный дефицит. Механизмы адаптации клеточной стенки к потере воды могут быть использованы для выявления важных генетических факторов, определяющих устойчивость растений к засухе, и предоставляют ценные данные о биомаркерах для дальнейшей селекции, направленной на повышение урожайности культурных растений. С помощью ANDSystem реконструирована генная сеть, позволяющая описывать регуляцию БКС в условиях ограничения полива. Анализ генной сети совместно с анализом транскриптомных данных позволил провести приоритизацию транскрипционных факторов (ТФ) по их обогащенности регулируемыми дифференциально экспрессирующимися генами. В результате были рассчитаны веса, являющиеся индикаторами ассоциации ТФ с водным дефицитом. По значениям весов отобраны восемь наиболее значимых ТФ. Наибольшим приоритетом обладал ТФ GBF3. Приоритизация БКС проведена по критерию суммирования весов транскрипционных факторов, регулирующих эти гены. К наиболее приоритетным БКС отнесены ген *AT5G03350*, кодирующий лектин-подобный белок; *AT4G20860*, кодирующий фермент берберинового моста BBE-like 22 (berberine bridge enzyme-like 22), необходимый для окисления продуктов распада целлюлозы, и *AT4G37800*, кодирующий ксилоглюкан эндотрансгликозилазу/гидролазу 7.
Ключевые слова: клеточная стенка растений; засуха; растения; дифференциально экспрессирующиеся гены; интеллектуальный анализ текста; микрочип; регуляторная генная сеть.

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Introduction

The plant cell wall is a complex structure composed of numerous biopolymers. The structure and composition of the cell wall change during plant development and are incredibly diverse not only between plant species but also between tissue types (Burton et al., 2010). Throughout their life cycle, plants are exposed to abiotic stresses such as drought, flooding, salinity, heavy metal pollution, nutrient deficiencies, and more. The plant cell wall provides a structural basis for supporting plant growth, serves as a source of various signals, and contributes to plant resistance to stressors.

Drought is a significant environmental problem, severely affecting plant growth, development, and yield. Plants subjected to water deficit exhibit morphological changes, in which proteins that are part of the cell wall play a critical role (Le Gall et al., 2015; Ezquer et al., 2020). However, the functions of these proteins, their regulation, and their interactions require further investigation.

Plant adaptation to drought has been demonstrated to be mediated by signaling pathways involving transcription factors (TFs) (Singh, Laxmi, 2015; Joshi et al., 2016). Therefore, studying the role of TFs as the primary regulators of water deficit-sensitive genes is particularly interesting. TFs regulate the expression of water deficit-sensitive genes in an abscisic acid (ABA)-dependent or ABA-independent manner (Yamaguchi-Shinozaki, Shinozaki, 2006). ABA-dependent positive regulators include the ABF/AREB (ABA-responsive element (ABRE)-binding proteins/ABRE-binding factors) family of the bZIP (basic leucine zipper) type, which recognizes ABA-sensitive elements (ABRE) in the promoters of ABA-induced genes (Choi et al., 2000). The ABA-dependent regulatory pathway also includes several other families of transcription factors, such as AP2/ERF, MYB, NAC, and bHLH. In contrast, key ABA-independent regulators are members of the DREB family (Fujita et al., 2011).

Reconstructing gene networks based on the analysis of transcriptomic data obtained under water deficit conditions can contribute to understanding the molecular-genetic mechanisms underlying the formation and functioning of the plant cell wall in drought resistance. Currently, approaches based on the automatic analysis of scientific publication texts are actively used for gene network reconstruction. Previously we have developed the cognitive ANDSystem tool based on artificial intelligence methods, which performs automatic extraction of knowledge from scientific publications and factographic databases (Ivanisenko et al., 2015, 2019, 2020, 2022a). ANDSystem has been applied to a wide range of tasks, including the interpretation of metabolomic data in the analysis of blood plasma from COVID-19 patients (Ivanisenko et al., 2022b) and the prioritization of genes associated with human diseases (Saik et al., 2016, 2018a, b, 2019; Yankina et al., 2018; Antropova et al., 2022). The ANDSystem technology has also been used to solve problems in the field of plant biology. For example, with the help of ANDSystem, the SOLANUM TUBEROSUM knowledge base (Saik et al., 2017; Ivanisenko et al., 2018), which contains associative gene networks of plants, was developed. The application of ANDSystem allowed the identification of important genes involved in the response to abiotic stresses caused by drought, soil salinity, and elevated cadmium concentration (Demenkov et al., 2021).

To date, several studies have been carried out on the reconstruction of gene networks describing the response of *Betula platyphylla* and barley to drought (Javadi et al., 2021; Jia et al., 2022). Gene networks have also been constructed that describe the biosynthesis of the secondary cell wall of *A. thaliana* and the interactions of TFs that regulate cell wall biosynthesis in rice (Taylor-Teeples et al., 2015; Zhao et al., 2019). However, these gene networks have not been focused on the involvement of the cell wall in response mechanisms to water deficiency.

Using the ANDSystem software package (Ivanisenko et al., 2015, 2019, 2020, 2022a), we reconstructed a gene network based on the analysis of transcriptomic data for *Arabidopsis thaliana* leaves under water deficit conditions (Perera et al., 2008; Ding et al., 2009; Kühn et al., 2014; Fang et al., 2016; Noman et al., 2019). The reconstructed gene network and transcriptomic analysis prioritized TFs and genes encoding cell wall proteins (CWP) based on their involvement in the

stress response during water deficit. The method of transcription factor prioritization contributed to isolating key regulatory proteins that are sensitive to the effects of water deficiency. The identification of key TFs made it possible to identify a list of target genes involved in the mechanisms of cell wall resistance to water deficit conditions. The final gene network containing priority genes included 8 TFs and 59 protein genes present in the cell wall according to the WallProtDB database (San Clemente, Jamet, 2015). According to the prioritization results, the *GBF3* gene encoding the TF made the most significant contribution to the regulation of cell wall genes. Among the cell wall genes, the lectin-like protein was the most important. The results reveal potential molecular-genetic mechanisms of the plant cell wall response to water deficit.

Materials and methods

Identification of *Arabidopsis thaliana* cell wall proteins. The WallProtDB plant cell wall proteomics database (http://www.polebio.lrsv.ups-tlse.fr/WallProtDB) (San Clemente, Jamet, 2015) was used for finding the *A. thaliana* cell wall proteins. WallProtDB contains proteins identified using mass spectrometry technology in the cell wall proteome. According to the WallProtDB data, all cell wall proteins were divided into nine functional classes: 1) proteins acting on cell wall carbohydrates, 2) oxidoreductases, 3) proteases, 4) proteins with protein or polysaccharide interaction domains, 5) structural proteins, 6) lipid metabolism-related proteins, 7) proteins presumably involved in signal transduction, 8) various proteins, and 9) proteins with unknown function (Jamet et al., 2008). Enzymes synthesizing cell wall components and forming necessary substrates are not included in the list, as they are localized in other compartments and are, therefore, not represented in the WallProtDB database.

Processing of transcriptomic data. Data on the differential expression of *A. thaliana* genes under limited watering conditions were taken from the DNA microarray experiments database from the NCBI Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) (Perera et al., 2008; Noman et al., 2019; Fang et al., 2016; Ding et al., 2009; Kühn et al., 2014). Bioinformatics analysis of transcriptomic data was performed in the R programming environment using Bioconductor packages (Gentleman et al., 2004). Reading of the CEL files containing probe identifiers and intensities was done using the readAffy() function from the affy package (Gautier et al., 2004). Data normalization, background noise correction, and gene expression level calculations were done using the affy package's rma() function. Differential gene expression analysis was performed using the limma package (Ritchie et al., 2015).

To identify differentially expressed genes (DEGs) across multiple experiments, consistently activated or consistently suppressed under water deficit conditions, binomial distribution (*p-value* = 0.05) was applied using the binomtest() function implemented in the scipy.stats library.

Reconstruction and analysis of the gene network. The gene network describing regulatory relationships of TFs with target genes associated with the cell wall response of *A. thaliana* leaves to water deficit was constructed using the ANDSystem software package (Ivanisenko et al., 2015, 2019, 2020, 2022a).

Prioritization of transcription factors and their target genes. The prioritization of transcription factors was carried out based on the score of the transcription factor (STF) values. The STF for a given transcription factor was equal to the number of DNA microarray experiments in which the list of cell wall genes regulated by this TF was enriched with DEGs. Enrichment was assessed using the hypergeometric distribution.

The prioritization of cell wall genes was carried out using the score of cell wall protein (SCWP), equal to the number of connections between the cell wall gene and TFs in the gene network.

Results

General analysis scheme

The overall workflow is shown in Figure 1. It consists of the stage of transcriptome data analysis for *A. thaliana* leaves under water deficit conditions (this analysis aims to determine stably DEGs), gene network reconstruction stage (at this stage, the cell wall gene regulatory network under water deficit conditions was reconstructed using automated text analysis methods for scientific publications, factographic databases, and differential gene expression data), and the stage of prioritizing genes based on their involvement in the response to stress caused by water deficit.

Differentially expressed genes under water deficit conditions

To determine the DEGs of *A. thaliana* under limited watering conditions, an analysis of data from five DNA microarray experiments from the NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/) was carried out (Table 1). All data were obtained on the Affymetrix Arabidopsis ATH1 Genome Array platform. In all experiments, the subject of the study was leaves, and the duration of days without watering ranged from 4 to 14 days.

A *p-value* threshold and a log fold change threshold (logFC) were used to determine differentially expressed genes: *p-value* < 0.05 and logFC > 1. Table 2 shows the number of DEGs for each of the five experiments. Expression data analysis across five experiments, performed using the binomial distribution, showed that changes in gene expression in two or more experiments could indicate that the gene is a stable DEG with a significance level of *p-value* < 0.05.

Gene network reconstruction

The input data for the gene network reconstruction consisted of 1073 *A. thaliana* gene identifiers TAIR, encoding CWP, obtained from the WallProtDB database (Supplementary Table 1[1]). Using ANDSystem, a regulatory network was reconstructed, containing interactions of these genes with transcription factors. For 692 genes, 599 potential TFs were identified. 381 CWP genes without interactions with TFs were removed from the network.

In the next step, we selected TFs considering their involvement in biological processes related to plant responses to drought. In ANDSystem, four biological processes were

[1] Supplementary Tables 1–5 and Supplementary Figure are available at: https://vavilovj-icg.ru/download/pict-2023-27/appx34.xlsx

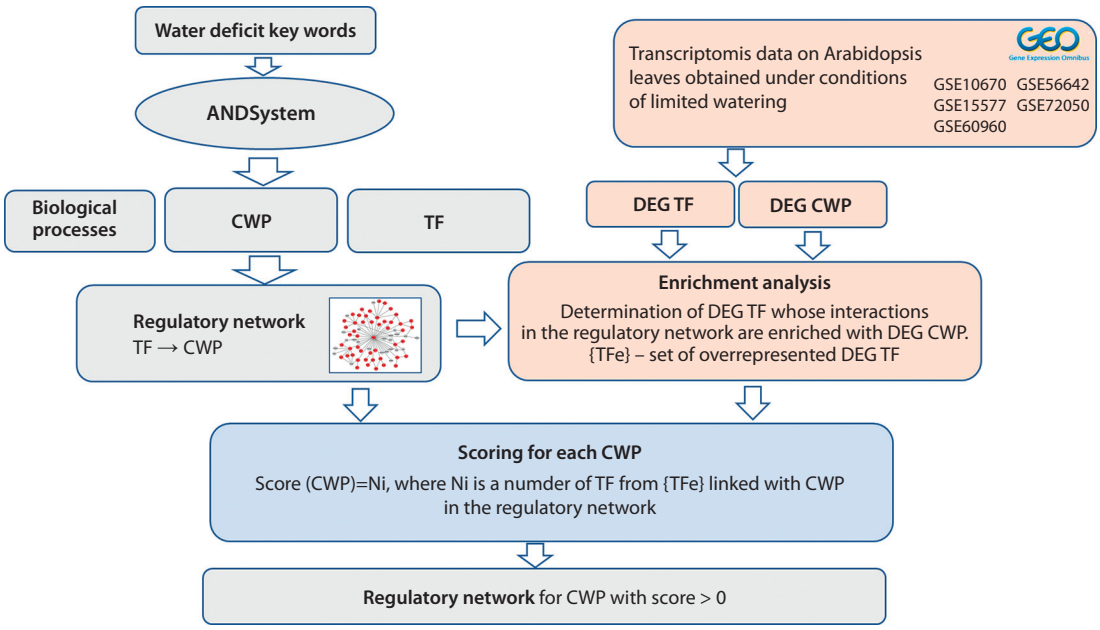

Fig. 1. A pipeline for reconstructing a regulatory gene network describing the regulation of expression of *A. thaliana* cell wall proteins in drought response.

DEG, differentially expressed genes; CWP, cell wall protein; TF, transcription factors.

Table 1. Publicly available DNA microarray data for *A. thaliana* leaves used in this study

GEO ID	Development stage	Days without watering	Cell source	Ecotype	Reference
GSE10670	2 months	7 days	Leaves	Col-0	Perera et al., 2008
GSE56642	1 month	14 days	Leaves	Col-0	Noman et al., 2019
GSE15577	1 month	6 days	Leaves	Col-0	Fang et al., 2016
GSE72050	1 month	5 days	Leaves	Col-0	Ding et al., 2009
GSE60960	1 month	4 days	Leaves	Col-0	Kühn et al., 2014

Table 2. Number of identified differentially expressed genes in the experiments

Study (GEO ID)	Number of DEGs	Number of TF genes among DEGs	Number of CWP genes among DEGs
GSE10670	3158	21	113
GSE56642	6017	33	185
GSE15577	3753	12	140
GSE72050	1959	11	105
GSE60960	1603	13	51

represented, with their names containing the keywords "drought" and "water" in combination with "tolerance" and "deprivation." These processes included the response to water deprivation, obsolete drought tolerance, drought recovery, and response to water. Fifty-six TFs were associated with these processes in ANDSystem (Fig. 2).

These 56 TFs were found to regulate 425 CWP genes (Suppl. Table 2). As a result of the analysis of *A. thaliana* leaves, it was shown that 23 TFs (Suppl. Figure) and 146 CWP genes demonstrated a stable unidirectional change in expression (Suppl. Table 3). However, not all CWP genes among the targets of the 23 TFs in the gene network were stably unidirectional DEGs. Therefore, we assessed the importance of TFs for the plant response to drought based on the enrichment analysis of their targets in the CWP DEG gene network. We assumed that the more CWP DEGs are among the targets of a transcription factor, the more significantly the transcription factor is associated with the plant's response to water deficit.

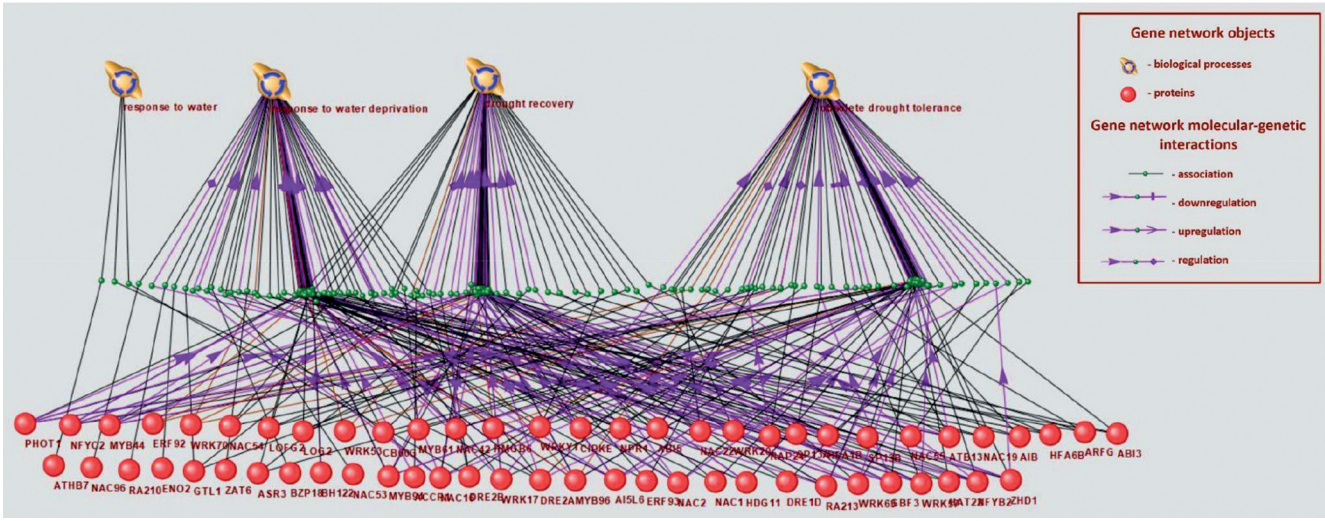

Fig. 2. Associative network of transcription factors related to drought.

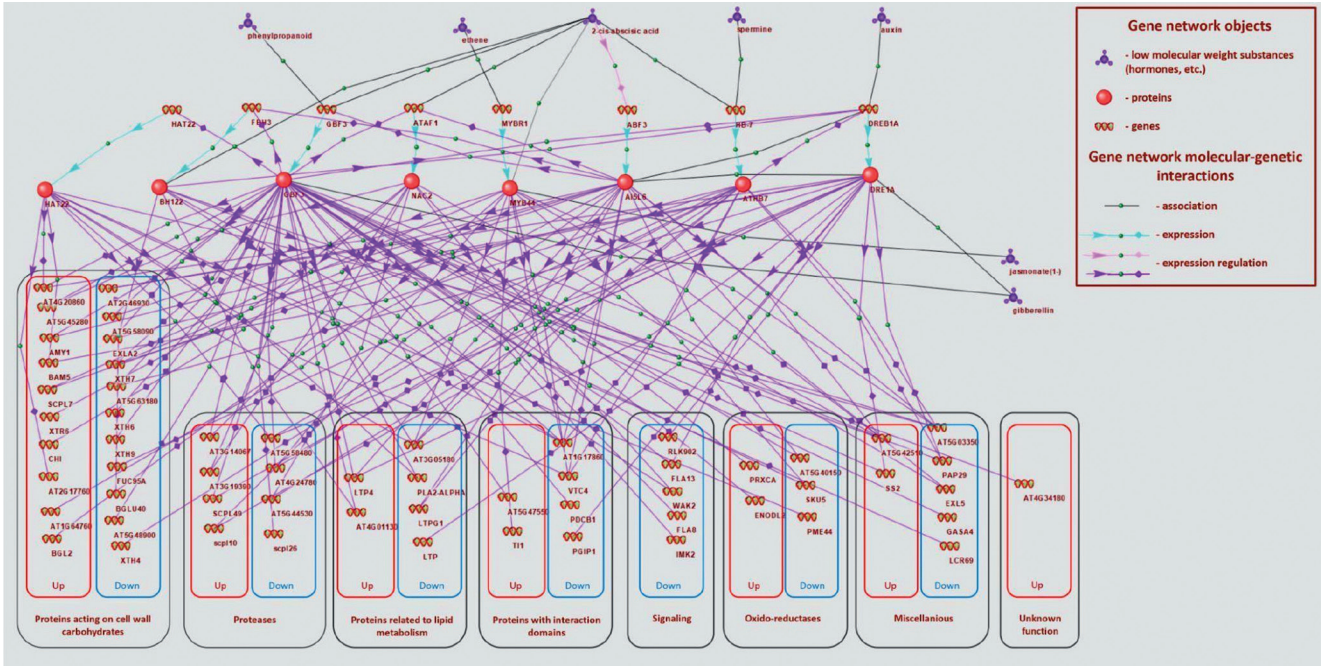

Fig. 3. Gene network regulating the cell wall of *Arabidopsis thaliana L.* in response to water deficit and connection with key hormones.

Prioritization of transcription factors and their target genes and reconstruction of the resulting gene network

The prioritization of TFs and CWP genes for the response to drought was based on the STF and SCWP criteria, which characterized both their differential expression and their connections with DEGs in the gene network (see methods). The values of these indicators were calculated for the participants of the gene network and are presented in Supplementary Tables 4 and 5. The highest STF and SCWP values corresponded to the highest priority.

Priority TFs were selected based on the statistically significant enrichment of their target genes encoding CWPs in DEGs in at least one of the transcriptomic experiments. Thus, according to this criterion, out of 23 TFs, 8 priority TFs were identified. The identified transcription factors belonged to the TF families HD-ZIP, bZIP, ERF, NAC, and MYB. All TFs were removed from the network to obtain the resulting gene network, except for those identified as priority TFs. Cell wall protein genes not connected to TFs were also removed. After filtering, the gene network contained 8 TFs and 59 CWP genes (see Suppl. Tables 4 and 5). We were also interested in analyzing the possible regulation of the identified TFs by active low-molecular-weight compounds (polyamines and hormones). For this purpose, the gene network was expanded with interactions of TFs with metabolites (Fig. 3).

Discussion

The scientific literature actively studies the genetic regulation of plant cell wall functioning under drought conditions. To date, a large amount of information has been accumulated on the molecular-genetic events of plant responses to water deficit, including data from differential gene expression experiments. Applying an approach based on the reconstruction of gene networks allows for integrating disparate knowledge to describe the molecular-genetic mechanisms of complex cell processes. Here, we reconstructed a gene network using ANDSystem and prioritized its participants based on their importance for the response of *A. thaliana* to drought conditions. The reconstructed gene network contains eight transcription factor genes and their protein products, six low-molecular-weight compounds (hormones and polyamines), and 59 genes encoding cell wall components regulated by the identified TFs (see Fig. 3). These 59 genes belong to 8 of the nine functional groups according to the classification presented in Supplementary Table 5. One functional class (structural protein group) is not represented among the identified genes.

The most prioritized transcription factor

Interestingly, all eight identified genes encoding TFs showed expression activation (see Suppl. Table 4). Among them, GBF3 regulates the transcription of the most significant number of genes encoding CWP. At the same time, it was stably expressed in all analyzed *A. thaliana* transcriptomes in this study obtained under water restriction conditions. There is also evidence that overexpression of GBF3 in *A. thaliana* led to improved resistance to osmotic stress, salinity, and drought, in addition to conferring insensitivity to ABA (Ramegowda et al., 2017).

Proteins acting on cell wall carbohydrates

In the reconstructed gene network, the largest group was represented by genes encoding proteins that act on cell wall carbohydrates (21 genes), 10 of which showed activation and 11 showed suppression of expression. This functional class includes expansins, glycosidases, and esterases. The highest priority among this functional class was given to the gene *XTH7* (AT4G37800), encoding xyloglucan endotransglucosylase/hydrolase 7. XTHs can hydrolyze and reconnect the molecules of xyloglucan – the key hemicellulose of primary cell walls (Rose et al., 2002). These enzymes are involved in cell wall remodeling during plant cell growth and response to various stressors. XTHs are encoded by a large multigene family, members of which are differentially expressed in various physiological situations having peculiarities in activity mode and regulation nuances (Zhang et al., 2017; Nazipova et al., 2022). According to the SCWP indicator, XTH7 is ranked third among the 59 considered cell wall genes (5 points, see Suppl. Table 5). As can be seen from Figure 3, this gene is regulated by TFs GTF3 and DREB1A (CBF3). The XTH7 enzyme and its activity have not been fully characterized yet. According to gene expression data, XTH7 is involved in salt resistance and ethylene-dependent apple softening (Zhang et al., 2017; Cai et al., 2023). It also participates in processes such as cell enlargement and restructuring. According to our differential expression analysis, it showed stable suppression, as a result of which its influence on limiting cell growth under drought conditions can be assumed. The other members of this functional class had priority ratings ranging from one to three points. The second-highest ranking gene is AT2G43570, encoding the enzyme endochitinase CHI (3 points, see Suppl. Table 5).

Proteases

The next most represented functional group was proteases, with eight genes. Subtilases are the most represented family of cell wall proteases (Jamet et al., 2008). Eight genes belonging to the protease functional group were identified in the regulatory gene network, with 4 showing activation of expression and 4 showing suppression. According to the SCWP indicator, three genes from this functional group scored three points: *AT2G23000*, encoding serine carboxypeptidase-like 10 (SCPL10), which is necessary for the biosynthesis of sinapoylated anthocyanins; *AT3G14067*, encoding subtilisin-like protease SBT1.4; and *AT5G44530*, encoding subtilisin-like protease SBT2.3 (see Suppl. Table 5). SBT1.4 is also called senescence-associated subtilisin protease due to its role in leaf aging. It has been shown that SCPL10 slows down the elongation of the main shoot, branching, and size of inflorescences (Martinez et al., 2015). According to our analysis, the expression of this gene increases under water deficit conditions, suggesting that SCPL10 may play a direct role in inhibiting plant growth under drought conditions. Other representatives of this group scored 1 to 2 SCWP points.

Proteins with interaction domains (with proteins or polysaccharides)

In our study, the highest SCWP score was obtained by the *AT5G03350* gene (7 points, see Suppl. Table 5), which belongs to the functional class of proteins having interaction domains with proteins or polysaccharides. This class includes lectins and enzyme inhibitors, such as polygalacturonase inhibiting protein, pectin methylesterase, and protease inhibitors. Among the six differentially expressed genes of this functional group identified under water deficit conditions, the expression of four genes was suppressed, while two genes demonstrated activation of expression. In a previous study conducted on 220 microarray samples of *A. thaliana* available in the GEO, it was also shown that under drought conditions, the *AT5G03350* gene, encoding salicylic acid-induced legume lectin-like protein 1, was suppressed 7.9 times (Shaik et al., 2013). It seems to be involved in *A. thaliana* responses to multiple environmental stresses (including cold, high light, oxidative, ozone, and wound) and SA-mediated processes occurring in the effector-induced immune response (Armijo et al., 2013; Biswas et al., 2022). Due to the unusual structure of the legume lectin domain, proteins of this family may have a wide range of carbohydrate-binding specificity (Sharma et al., 1997), which possibly determines their diverse functions (including involvement in symbiosis, defense mechanisms against bacterial infection, enhanced tolerance against insects, salinity, and stomatal closure) (Van Holle et al., 2017). According to our results, the *AT5G03350* gene is most significantly associated with TFs differentially expressed under water deficit conditions. These factors include HAT22, BH122, MYB44, ABF3, and ATHB7. Other representatives of this class scored between 1 and 3 SCWP points.

Oxidoreductases

In the reconstructed regulatory network, five oxidoreductase genes were identified; under water deficit conditions, the expression of two of these genes was activated, and three were suppressed. According to our study, the *AT4G20860* gene ranks second in priority among the 59 investigated cell wall genes, with an SCWP of 5 (see Suppl. Table 5). *AT4G20860* encodes berberine bridge enzyme-like 22 (BBE-like 22), which is necessary to oxidize cellodextrins (cellulose degradation products). Its role under limited irrigation conditions is unclear; however, it has been shown that *A. thaliana* with increased expression of the *AT4G20860* gene product is more resistant to the *Botrytis cinerea* fungus, presumably because oxidized cellodextrins are a less valuable carbon source (Locci et al., 2019). In the reconstructed gene network, *AT4G20860* may be regulated by TFs such as HAT22, GBF3, and ABF3 and is activated under water deficit conditions.

Other representatives of the oxidoreductase class – peroxidases – perform a dual function in plant cell walls: they contribute to the weakening of the cell wall by releasing hydroxyl radicals (OH-), which can cause polysaccharide scission (Schweikert et al., 2000) and increase wall rigidity by strengthening extensin cross-links and supporting lignification and suberization of the cell wall (Novaković et al., 2018).

Proteins related to lipid metabolism

In the reconstructed gene network, six genes encode proteins involved in the metabolism of cell wall lipids. According to the analysis we conducted, under water deficit conditions, the expression of two genes was enhanced, while the expression of four genes was suppressed. Various studies have shown that plants remodel lipid composition in response to drought (Gigon et al., 2004; Liu et al., 2021). In experiments on milk thistle, it was demonstrated that under drought conditions, PLA2-ALPHA (*AT2G06925*), which encodes a secretory phospholipase A2 enzyme, had reduced expression (Ghanbari Moheb Seraj et al., 2022), which is also evident in our results. Secreted PLA2s are low molecular weight calcium-dependent enzymes, which specifically hydrolyze the sn-2 position of phospholipids and can do that in an organized membrane (Mariani, Fidelio, 2019). They are involved in many cell wall-related processes; for example, *Arabidopsis* PLA2-ALPHA is required for the trafficking of PIN-FORMED auxin efflux transporters to the plasma membrane (Lee et al., 2010). The *PLA2-ALPHA* gene scored 4 points on the SCWP indicator (4th place in Suppl. Table 5), meaning it is significantly associated with regulatory factors differentially expressed under water deficit conditions (HAT22 and GBF3).

Three other genes from this category, AT1G27950 for LTPG1 (SCWP 3), *AT5G59310* for LTP4 (SCWP 2), and *AT2G15050* for LTP7 (SCWP 1) (see Suppl. Table 5) encode lipid transfer proteins. *AT1G27950* is a membrane-localized protein with a predicted GPI (glycosylphosphatidylinositol)-anchor domain. It extensively exports intracellular lipids (e. g., C29 alkane) to the surface to build the cuticular wax layer (Lee et al., 2009). *AT5G59310* and *AT2G15050* belong to non-specific lipid transfer proteins encoded by a large multigene family and occur only in land plants (Salminen et al., 2016). They are small proteins with a tunnel-like hydrophobic cavity that makes them suitable for binding and transport of phospholipids as well as galactolipids across membranes. LTPs are suggested to play a role in wax or cutin deposition in the cell walls (Salminen et al., 2016).

Signaling

Our study identified five genes encoding cell wall proteins involved in signal transduction. The expression of all considered genes was suppressed under water deficit conditions. Based on the SCWP indicator, among the genes of this functional group, the gene *AT2G45470* (see Suppl. Table 5) scored the highest number of points (3), encoding fasciclin-like arabinogalactan protein 8 (FLA8). Numerous plant FLAs are chimeric proteins that contain moderately glycosylated arabinogalactan protein and one to two fasciclin domains with characteristic highly conserved sequence stretches of around 15 residues and a conserved central YH motif. FLAs are non-structural components of the cell wall, might be linked to cell wall polysaccharides, and interact with various cell surface receptors involved in various plant development processes, including cellulose biosynthesis (Seifert, 2018). FLA8 itself has been poorly characterized. The *AT2G45470* gene is significantly associated with the transcription factor GBF3.

Another identified representative of the signal protein class is wall-associated kinase 2 (WAK2), encoded by the *At1g21270* gene, scoring 2 points on the SCWP. Alongside WAK1, WAK2 is a cell wall receptor with an intracellular protein kinase domain, a transmembrane domain, and an extracellular N-terminal domain capable of binding poly- and oligogalacturonans (Wagner, Kohorn, 2001). By binding pectins, WAK initiates signal transmission through mitogen-activated protein kinases (MAPK) for activation of vacuolar invertase and numerous other inducible proteins, regulating turgor pressure and, as a result, increasing cell size (Kohorn et al., 2006). Using antisense RNA, WAK2 is necessary for leaf cell expansion (but not for cell division) (Wagner, Kohorn, 2001). By interacting with polygalacturonan fragments formed as components of DAMP and PAMP under the influence of biotic and abiotic events, WAK can also trigger (via MAPK activation) a stress response. WAK expression is induced by injury, pathogen infection, and exposure to other stress factors such as ozone and heavy metals (Kohorn B.D., Kohorn S.L., 2012). A study conducted on sweet orange graft showed that WAK2 expression was suppressed under drought conditions in both sweet orange plants grafted on drought-tolerant and drought-sensitive rootstocks (Gonçalves et al., 2019). It can be assumed that under water deficit conditions, plants reduce WAK2 expression to lower turgor pressure, suspend leaf cell expansion, and induce other components of the stress response.

Miscellaneous

For this group, our study revealed seven genes – 5 with reduced and 2 with increased expression under water deficit conditions. Among them, based on the SCWP indicator, two genes scored 4 points each (5–6th places in Suppl. Table 5) – the downregulated *AT5G15230*, encoding the poorly characterized gibberellin-regulated protein 4 (GASA4), and the upregulated *AT5G42510*, encoding dirigent protein 1 (DIR1). DIR family

proteins are involved in lignin and lignin biosynthesis and play a role in plant response to biotic and abiotic stresses (particularly drought) that cause physical damage to the cell wall (Paniagua et al., 2017). It has previously been shown that the expression of several genes encoding DIR proteins is sensitive to water and cold stress and treatment with ABA. Moreover, in *Brassica* plants under water stress, the increased expression of DIR genes was temporally coordinated with an increase in lignin content (Thamil Arasan et al., 2013). In *Eucommia ulmoides* Oliv seedlings, it was shown that the expression level of *DIR1* increased almost 8-fold under osmotic stress within 6 hours and increased nearly three times under drought conditions within 12 hours (Li et al., 2021). The other representatives of this functional group each scored one point.

Hormones

Our reconstructed gene network also included hormones: ethylene, abscisic acid, auxin, jasmonate, gibberellin, and spermine – endogenous polyamine. These compounds affect transcription factors (altering the expression level or protein activity), subsequently leading to changes in the expression levels of target genes for transcription factors. Regulatory connections in the reconstructed network have been demonstrated under various conditions. Additional experiments are needed to explore whether they function under water deficit conditions.

For example, it was shown that upon infection of *A. thaliana* with aphids, the concentration of ethylene increases, which in turn induces the expression of the transcription factor MYB44 (Xia et al., 2014). Another transcription factor in our gene network, ABF3, is one of the key factors that transmit the abscisic acid signal and regulate the expression of target genes during water deficit (Yoshida et al., 2010). Under drought conditions, abscisic acid also induces the expression of the transcription factor ATHB7, which was observed within 30 minutes after experimentally induced stress, and *ATHB7* transcription continued to increase after 21 hours (Söderman et al., 1996). The expression of the transcription factor GBF3 is also activated by abscisic acid (Lu et al., 1996).

Polyamine spermine is essential for plants to respond to drought, as demonstrated in mutant *A. thaliana* plants knocked out for genes encoding spermine-synthesizing enzymes (Yamaguchi et al., 2007). Under water deficit conditions, the stomata of such plants remained open. Another low-molecular-weight compound in the gene network is gibberellin. Various studies have shown that reducing its level improves plant drought resistance (Shohat et al., 2021). Under cold conditions, the transcription factor DREB1A (CBF3) suppresses gibberellin accumulation (Zhou et al., 2017).

Based on the analysis of cell wall gene expression in different *A. thaliana* experiments under water deficit conditions, it can be noted that the plant's response to this abiotic factor involves changes in the expression of genes encoding proteins from almost all functional groups characteristic of the cell wall. The exception was the group of structural proteins, which may indicate that changes in the composition of cell wall structural components in response to water deficit do not occur or occur to a negligible extent. It can be observed that the expression of 23 examined genes is enhanced under these conditions, while that of 36 is weakened. In each functional group, there are both activated and deactivated genes, except for the group of genes encoding signaling proteins, in which the expression of all five examined genes was suppressed under water deficit conditions.

Conclusion

An analysis of five *A. thaliana* transcriptomes obtained under water deficit conditions was conducted. The implemented algorithm allowed to perform the prediction of potential regulatory interactions between transcription factors and target genes encoding cell wall proteins, which may play an important role in the response of *A. thaliana* to water deficit. Among the identified eight transcription factors regulating *A. thaliana* cell wall genes, GBF3 had the highest priority. Out of the 59 cell wall genes examined, the *AT5G03350* gene, encoding a lectin-like protein, was identified as the most prioritized for association with differentially expressed transcription factors under water deficit conditions. It is associated with transcription factors such as HAT22, BH122, MYB44, ABF3, and ATHB7. Also highly significantly associated with transcription factors are the *AT4G20860* gene, encoding BBE-like 22, which is necessary for the oxidation of cellulose degradation products (associated transcription factors – HAT22, GBF3, and ABF3), and *AT4G37800*, encoding xyloglucan endotransglucosylase/hydrolase 7 (transcription factors GTF3 and DREB1A), among others. Overall, the proposed algorithm that has been used to analyze the gene network of cell wall proteins can be applied to other model plant species.

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ORCID

A.R. Volyanskaya orcid.org/0009-0003-8472-4945
E.A. Antropova orcid.org/0000-0003-2158-3252
U.S. Zubairova orcid.org/0000-0002-0730-9145
P.S. Demenkov orcid.org/0000-0001-9433-8341
A.S. Venzel orcid.org/0000-0002-7419-5168
A.A. Makarova orcid.org/0009-0005-1844-7921
T.V. Ivanisenko orcid.org/0000-0002-0005-9155
N.A. Kolchanov orcid.org/0000-0001-6800-8787
V.A. Ivanisenko orcid.org/0000-0002-1859-4631

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InterTransViewer: a comparative description of differential gene expression profiles from different experiments

A.V. Tyapkin[1, 2], V.V. Lavrekha[1, 2], E.V. Ubogoeva[1], D.Yu. Oshchepkov[1], N.A. Omelyanchuk[1], E.V. Zemlyanskaya[1, 2]

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
ezemlyanskaya@bionet.nsc.ru

Abstract. Meta-analysis of transcriptomic data from different experiments has become increasingly prevalent due to a significantly increasing number of genome-wide experiments investigating gene expression changes under various conditions. Such data integration provides greater accuracy in identifying candidate genes and allows testing new hypotheses, which could not be validated in individual studies. To increase the relevance of experiment integration, it is necessary to optimize the selection of experiments. In this paper, we propose a set of quantitative indicators for a comprehensive comparative description of transcriptomic data. These indicators can be easily visualized and interpreted. They include the number of differentially expressed genes (DEGs), the proportion of experiment-specific (unique) DEGs in each data set, the pairwise similarity of experiments in DEG composition and the homogeneity of DEG profiles. For automatic calculation and visualization of these indicators, we have developed the program InterTransViewer. We have used InterTransViewer to comparatively describe 23 auxin- and 16 ethylene- or 1-aminocyclopropane-1-carboxylic acid (ACC)-induced transcriptomes in *Arabidopsis thaliana* L. We have demonstrated that analysis of the characteristics of individual DEG profiles and their pairwise comparisons based on DEG composition allow the user to rank experiments in the context of each other, assess the tendency towards their integration or segregation, and generate hypotheses about the influence of non-target factors on the transcriptional response. As a result, InterTransViewer identifies potentially homogeneous groups of experiments. Subsequent estimation of the profile homogeneity within these groups using resampling and setting a significance threshold helps to decide whether these data are appropriate for meta-analysis. Overall, InterTransViewer makes it possible to efficiently select experiments for meta-analysis depending on its task and methods.
Key words: transcriptome; data integration; auxin; ethylene; *Arabidopsis thaliana* L.

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InterTransViewer: сравнительное описание профилей дифференциальной экспрессии генов из разных экспериментов

А.В. Тяпкин[1, 2], В.В. Лавреха[1, 2], Е.В. Убогоева[1], Д.Ю. Ощепков[1], Н.А. Омельянчук[1], Е.В. Землянская[1, 2]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
ezemlyanskaya@bionet.nsc.ru

Аннотация. В настоящее время в связи со стремительным ростом количества полногеномных экспериментов по изучению изменения экспрессии генов в различных условиях все более широкое распространение получают методы метаанализа транскриптомных данных из разных экспериментов, так как интеграция данных может обеспечить большую точность в выявлении генов-кандидатов и позволяет тестировать новые гипотезы, которые невозможно было проверить в отдельных исследованиях. Для повышения информативности такой интеграции необходимо оптимизировать подбор экспериментов. В настоящей работе мы предлагаем набор количественных показателей для всестороннего сравнительного описания транскриптомных данных. Эти показатели легко могут быть визуализированы и интерпретированы. Они включают в себя количество дифференциально экспрессирующихся генов (ДЭГ), долю специфических (уникальных) ДЭГ в каждом наборе данных, попарное сходство экспериментов по составу ДЭГ, оценку однородности профилей дифференциально экспрессирующихся генов. Для автоматического вычисления и визуализации этих показателей мы разработали программу InterTransViewer. Мы применили InterTransViewer для сравнительного описания транскрипционных ответов на обработку фитогормонами у модельного растения *Arabidopsis thaliana* L., взяв в анализ 23 единообразно об-

работанных профиля дифференциальной экспрессии генов в ответ на ауксин и 16 профилей дифференциальной экспрессии, индуцированных этиленом или его предшественником – 1-аминоциклопропановой кислотой. Мы продемонстрировали, что комплексное рассмотрение характеристик отдельных профилей ДЭГ в контексте результатов попарных сравнений профилей по составу ДЭГ позволяет позиционировать эксперименты в контексте друг друга, оценивать тенденцию к их интеграции или сегрегации, генерировать гипотезы о влиянии весомых нецелевых факторов на исследуемый транскрипционный ответ. В результате это дает возможность выделять потенциально однородные группы экспериментов. Последующий анализ однородности этих групп профилей с помощью процедуры ресемплинга и установления порога уровня значимости помогает принять решение о целесообразности использования этих данных для метаанализа. В целом InterTransViewer позволяет эффективно формировать выборки экспериментов в зависимости от задачи и методов метаанализа.
Ключевые слова: транскриптом; интеграция данных; ауксин; этилен; *Arabidopsis thaliana* L.

Introduction

Analysis of differential gene expression under various conditions is one of the most promising approaches for studying the genetic regulation of traits (Stelpflug et al., 2016; Tello-Ruiz et al., 2016). The rapid increase in the number of experiments on whole-genome profiling of gene expression under different conditions and the availability of their results in functional genomics databases such as Gene Expression Omnibus (GEO) (Clough, Barrett, 2016) or BioStudies (Sarkans et al., 2021) open a wide space for comparative analysis of experimental results from different studies aimed at generalizing them across studies using meta-analysis (Cahan et al., 2007; Rung, Brazma, 2013; Keel, Lindholm-Perry, 2022). Such an approach allows not only to extract the most robust differentially expressed genes (DEGs) (Freire-Rios et al., 2020), but also to increase sample size to identify weak patterns (Bairakdar et al., 2023) or to test hypotheses that could not be investigated in individual studies (Sudmant et al., 2015; Winter et al., 2019).

For successful integration, data must meet several criteria (Cahan et al., 2007; Rung, Brazma, 2013; Yu, Zeng, 2018). First of all, experiments should be characterized according to the established minimum requirements for transcriptome experiments (Brazma et al., 2001; Brazma, 2009). In addition, the experiments should investigate similar hypotheses on the effect of the same factor. At the same time, one should avoid or correct the so-called batch effect, when non-target factors (biological characteristics of the object, experimental conditions, sample preparation protocol, choice of the data acquisition platform, etc.) affect the results of the experiment.

Simple data filtering by experimental conditions does not always ensure optimal selection of data for meta-analysis. On the one hand, a significant non-target factor may not be mentioned in the metadata, and formal matching of experimental conditions does not always rule out a batch effect. On the other hand, the results of experiments performed under non-identical conditions can be fairly well matched. Comparative description of transcriptome data from different experiments allows to optimize the choice of data and methods for data preprocessing. However, no standard has yet been developed for this procedure, and there is a significant lack of appropriate software tools, especially for graphical presentation of the results. For example, MetaQC program used for microarray quality assessment evaluates six quantitative metrics: (1) reproducibility of co-expressed groups of genes across experiments, (2) consistency of the co-expression pattern of known genes with databases of metabolic and signaling pathways (i. e. involvement of genes in the same process); (3–4) accuracy of detecting the enrichment of the DEG group in Gene Ontology terms (i. e., gene involvement in processes, association with cellular components or molecular functions) and their consistency across experiments; (5) accuracy of detection of known biomarkers; (6) consistency of DEG ranking between transcriptomes (Kang et al., 2012). However, MetaQC does not visualize these metrics, and some of the quality metrics rely on external databases and known markers rather than internal features of expression profiles, which can obscure insufficiently studied processes and complicate analyses for non-model species.

Another program, ViDGER, designed to simplify the interpretation of data from RNA sequencing experiments, provides a wide range of visualizations but does not offer a convenient means to compare DEG profiles (McDermaid et al., 2019). NetworkAnalyst 3.0 emphasizes the reconstruction of protein-protein interaction networks, but also provides the ability to visually compare gene lists using interactive heat maps, enrichment networks, Venn diagrams, and chord diagrams (Zhou et al., 2019).

In this paper, we propose a set of easily visualized and interpreted indicators for a comprehensive comparative description of DEG profiles. These indicators characterize individual differential expression profiles, their pairwise similarity, and their tendency to integrate or segregate. To automatically calculate and visualize these indicators, we developed the InterTransViewer program, which we applied to comparatively describe transcriptional responses to auxin (23 DEG profiles from 16 studies) and ethylene (16 DEG profiles from 8 studies) in *Arabidopsis thaliana*.

Materials and methods

Characteristics of individual differential expression profiles. In each hormone-induced transcriptome, we composed the DEG list. Next, we estimated (1) the number of DEGs, (2) the ratio of DEGs specific only for this DEG list to the total number of DEGs in the list, and (3) the ratio between the proportion of specific DEGs in the DEG list and the proportion of the transcriptome DEGs in the joint DEG list from all transcriptomes under study:

$$R_i = \frac{\delta_i \cdot N}{n_i},$$

where R_i is the ratio between two proportions for the DEG list i, δ_i is the proportion of DEGs specific for the DEG list i, n_i is the number of DEGs in the DEG list i, N is the number of DEGs in the joint DEG list for all transcriptomes under study. The calculated indicators are graphically represented

using mirrored histograms. Together with the metadata, they provide a first approximation for the similarity of DEG profiles and enable identification of potential outliers. For example, a too small or a too large number of DEGs or a high R value that do not correlate with specific experimental conditions or biological properties of the sample may indicate the influence of an unknown non-target factor or poor data quality.

Pairwise comparison of differential expression profiles by DEG composition. If a smaller DEG list is nested within a larger DEG list, and the deviation of the size of each DEG list from the mean is insignificant or correlated with specific experimental conditions or biological properties of the sample, we consider the results of the two experiments to be consistent. Therefore, to assess the similarity of any two DEG lists, we calculated the similarity index I as follows:

$$I = \frac{c}{\min\{a, b\} + c},$$

where c is the number of DEGs shared between the DEG lists, a is the number of DEGs present in the first and absent in the second DEG list, b is the number of DEGs present in the second and absent in the first DEG list. Thus, the similarity index I reflects the proportion of shared DEGs in the smaller DEG list. The similarity index can take values from zero to one, with zero corresponding to the absence of shared DEGs in two DEG lists, and one corresponding to full nesting of one DEG list in the other. DEG list similarity matrices are visualized as a heatmap, on the basis of which one can not only infer the similarity of expression profiles by DEG composition, but also identify individual groups of the most similar experiments.

Clustering of differential expression profiles. The similarity matrix described in the previous section compares the DEG lists without considering fold changes in gene expression. To identify groups of similar differential expression profiles, we used hierarchical clustering based on a matrix of Euclidean distances in the $\log_2$-transformed space of fold changes in gene expression ($\log_2$FC), without considering statistical significance of fold changes. To allow comparison of transcriptional response profiles from different experiments, fold changes were normalized to the range in each experiment and standardized for each gene beforehand. Hierarchical clustering was performed with the Bclast function from the shipunov v.1.17.1 (https://CRAN.R-project.org/package=shipunov) package, using the Ward.D2 method based on minimizing the sum of squares of the Euclidean distances between each object of the cluster and the cluster centroid.

Quantitative evaluation of homogeneity by DEG composition within a group of profiles. Let A be the set of genes identified as DEGs in at least one of the m analyzed DEG lists, and the number of these DEGs be $|A| = N$. The set A includes (1) DEGs, changes in the expression level of which in a given sample of m DEG lists are determined predominantly by the influence of a target factor, and (2) genes, changes in the expression level of which are significantly affected by non-target factors. Obviously, if we calculate the value of N_k for a subsample of k DEG lists ($k < m$) and then, adding one DEG list at a time to this subsample, calculate the values of N_{k+i}, then the value of N_{k+i} should not decrease as the i value grows. In this case, the more heterogeneous the set of DEG profiles (the more DEG lists formed under the influence of different non-target factors it contains), the stronger the growth of the N_{k+i} value will be.

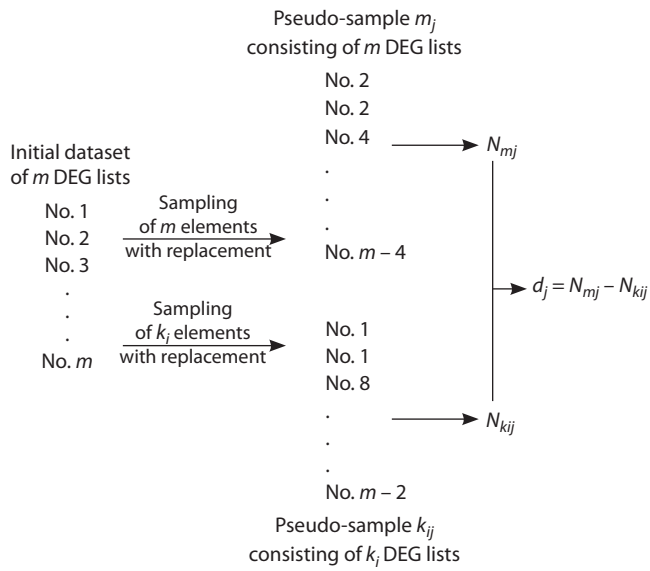

Fig. 1. The procedure for creating two pseudo-samples, each consisting of m and k_i ($k_i < m$) DEG lists selected randomly with replacement, and determining the difference d_j between the number of DEGs in at least one of the m and k_i DEG lists (N_{mj} and N_{kij}, respectively).

The subscript j denotes the serial number of the pseudo-sample. The operation was repeated 5000 times ($j \in \mathbb{N}$, $j = [1; 5000]$), thus generating a distribution of d values, which allowed to assess the significance of the difference of N values in the pseudo-samples. This procedure was repeated for each value of k_i ($k_1 = m - 1$; $k_{i+1} = k_i - 1$, where $i \in \mathbb{N}$, $i = [1; m - 1]$).

Using resampling, we created $m - 1$ sets of pseudo-samples of DEG lists: in one set i ($i \in \mathbb{N}$, $i = [1; m - 1]$), each pseudo-sample consisted of $k_i < m$ DEG lists ($k_1 = m - 1$, $k_{i+1} = k_i - 1$), to estimate at what value of k_i there would be a meaningful decrease in N_{ki} compared to N_m. To form a single pseudo-sample, from the original set of DEG lists consisting of m elements, we randomly selected k_i DEG lists with replacement (Fig. 1). For each pseudo-sample, we determined the number of genes N_{kij} identified as DEGs in at least one of the k_i DEG lists (index j denotes the number of the pseudo-sample in the same set). Simultaneously, we created a pseudo-sample of m DEG lists and calculated the corresponding value of N_{mj}, then calculated the difference $d_j = N_{mj} - N_{kij}$ (see Fig. 1). As a result of 5000 iterations ($j \in \mathbb{N}$, $j = [1; 5000]$), a variational series of these differences was generated. The confidence interval was determined using the percentile method (Rousselet et al., 2021). If a significant difference between N_m and N_{ki} was observed at some values of k_i, the analyzed set of profiles was considered heterogeneous. The distribution of d values was visualized as a histogram.

Implementation of the InterTransViewer program. The InterTransViewer program is implemented as an R script (v.4.1.2) and is available at (https://github.com/al-t1/InterTransViewer0/). InterTransViewer takes as input a table, in which the first column contains one grouping variable (gene identifiers, IDs) and each subsequent pair of columns contains $\log_2$-transformed gene expression fold change values (logFC) and the corresponding adjusted p values for each individual experiment. If the user preprocessed the raw transcriptome

data independently, such a table can be assembled using InterTransViewer's DEGweave function, which combines the results generated by the limma topTable function (for microarrays) and/or the DESeq2 results function (for RNA-seqs). It is advisable to perform preprocessing of raw data as uniformly as possible for each technology platform, and that the design of each experiment should include at least two biological replicates in both control and treatment trials. During the quality control step, it is recommended to pay particular attention to the data variation among replicates: for example, to employ the plotMDS function from the limma package for microarrays (Ritchie et al., 2015); fastQC and fastp for raw RNA-seq data (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; Chen et al., 2018) and to utilize the plotPCA function from the DESeq2 package for a count matrix (Love et al., 2014). It is essential that all differential expression profiles reflect the action of a single target factor. Technically, a DEG list is suitable for analysis with InterTransViewer if it has at least one DEG at the selected significance level, but it is recommended to have at least 10 DEGs in the DEG list.

The calculation of indicators for the comparative description of DEG profiles and their visualization are implemented as functions described in the InterTransViewer documentation. For example, the number of DEGs, the fraction of experiment-specific DEGs and the R_i ratio for all experiments can be obtained using the DEGsummary function and visualized as bar charts using the TotalSpecPlot and RmetricPlot functions. The GetSimMatrix function allows to obtain the similarity matrix *I*. The DE_bootstrap function allows resampling as described above. Hierarchical clustering is performed using the DE_clustering function. Finally, InterTransViewer generates a wide range of output data. For each transcriptome, two tables are generated containing a DEG list and a list of transcriptome-specific DEGs, both supplemented with the corresponding logFC and *p*-adj values.

InterTransViewer also outputs the following: the total list of genes that are DEGs in at least one experiment with the number of experiments, in which the gene is a DEG; the summary table generated by the DEGsummary function, and the corresponding histograms; the similarity matrix *I*, and the corresponding heatmap; the dendrograms obtained by clustering; tables and diagrams with resampling results to assess the homogeneity within groups of DEG lists.

Transcriptome datasets from publicly available sources. We collected all publicly available transcriptomic data on the treatment of *A. thaliana* with phytohormones auxin, ethylene, their precursors, or synthetic analogues. From those, we have selected transcriptomes of whole seedlings or individual organs of wild-type plants, in which hormone treatments were complemented by control experiments (mock treatment or no treatment). To allow subsequent comparative analysis, we performed uniform preprocessing of the raw data. Microarray data were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). RNA-seq data were extracted from the NCBI Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/). The genome sequence of *A. thaliana* and its annotation (TAIR 10) were downloaded from Ensembl Plants (https://plants.ensembl.org/index.html, release 52).

All microarray experiments found were performed using the ATH1 platform. Raw microarray data normalization and DEG calling were performed with the limma v.3.52.4 package (Ritchie et al., 2015). FastQC v.0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of RNA-seq data. Illumina reads were trimmed and quality filtered with fastp v.0.23.2 (Chen et al., 2018) using the following parameters: -q 20 -u 30 -5 -3 -W 4 -M 20. The reads were aligned to the *A. thaliana* genome with HISAT2 v.2.2.1 (Kim et al., 2019). SOLiD reads were aligned to the genome using TopHat (Kim et al., 2013). To quantify the number of uniquely mapped reads, we used the summarizeOverlaps function from the GenomicAlignments R package v.1.30.0 (Lawrence et al., 2013) with *A. thaliana* genome annotation. DEGs were called using the DESeq2 package v.1.34.0 (Love et al., 2014). For each dataset (both microarray and RNA-seq), we applied the Benjamini–Hochberg multiple hypothesis testing correction (Benjamini, Hochberg, 1995) to control the false discovery rate (FDR) for DEG calling. To detect DEGs, we used an FDR threshold of 0.05. As a result, we obtained 23 and 16 DEG lists for auxin and ethylene treatment, respectively. Each list contained at least 300 DEGs (see the Table).

Results and discussion

In this work, we applied InterTransViewer to comparatively characterize differential gene expression profiles in transcriptional response to phytohormones in *A. thaliana*. We selected 23 auxin-induced transcriptomes from 16 different studies and 16 transcriptomes induced by ethylene or its precursor ACC from 8 studies (see the Table and Materials and Methods).

Figure 2 schematically illustrates the metadata for each transcriptome. It can be seen that despite the similarity of the target factor, the experimental conditions are heterogeneous. In particular, there were differences in the chemical nature of the target factor, its concentration, the method and duration of treatment, the growing conditions of the plants, their age at the time of sample collection, the samples' nature, and the methods of expression profiling. Only two auxin-induced DEG profiles (No. 9 and 10) from two studies and three ethylene-induced DEG profiles (No. 1, 2, and 3) also from two studies were obtained under similar conditions according to the metadata. Thus, the aim of further comparative analysis was to investigate the homogeneity of phytohormone-induced transcriptomes depending on the conditions under which they were obtained.

Auxin- and ethylene-induced DEG profiles are variable in the number of DEGs

First, we characterized each DEG list using the DEGsummary function. Auxin- and ethylene-induced DEG profiles appeared to be heterogeneous in the number of DEGs: ranging from 410 to 11,966 in auxin-induced transcriptomes (median value 3205) and from 379 to 5253 in ethylene-induced ones (median value 1428) (Fig. 3, *a*, *b*). The deviation of the DEG numbers from the median value in most cases could be explained by specific experimental conditions. Thus, low numbers of auxin-sensitive DEGs were observed in the meristem and young flowers after short-term auxin treatment (No. 1; 586 DEGs) and in the root during long-term treatment (24 h) with low IAA concentration (1 μM) (No. 21, 686 DEGs). The reason for the low number of DEGs in the latter case is because the peak of transcriptional activity changes in response to auxin

Microarray and RNA-seq data used in this study

No.	Accession number	Type	Tissue, developmental stage	Treatment (concentration, time)	Number of replicates	Reference
			Auxin			
1	ERP021928	P	Meristem and young flowers up to and including stage 10	10 μM IAA, 0.5 h	3	Simonini et al., 2017
2, 4, 9	GSE18975	M	7 DAG seedlings	1 μM IAA, 0.5 h; 1 μM IAA, 1 h; 1 μM IAA, 3 h	3	Delker et al., 2010
3	SRP258689	P	Roots of 3 DAG seedlings	1 μM IAA, 1 h	3	Freire-Rios et al., 2020
5, 15	GSE3350	M	Roots without root apex of 3 DAG seedlings, grown on MS with 10 μM NPA	10 μM NAA, 2 h; 10 μM NAA, 6 h	2	Vanneste et al., 2005
6	GSE35580	M	Roots of 7 DAG seedlings	5 μM IAA, 2 h	3	Bargmann et al., 2013
7, 16	GSE42896	M	Roots without root apex of 3 DAG seedlings, grown on MS medium with 10 μM NPA	10 μM NAA, 2 h; 10 μM NAA, 6 h	3	De Rybel et al., 2012
8	GSE627	M	7 DAG seedlings	5 μM IAA, 2 h	3	Okushima et al., 2005
10	GSE58028	M	7 and 8 DAG seedlings	1 μM IAA, 3 h	3	
11	SRP033494	P	Roots of 7 DAG seedlings	5 μM IAA, 4 h	2	Chaiwanon, Wang, 2015
12, 18, 19, 21	GSE42007	M	Roots of 6 DAG seedlings	1 μM IAA, 4 h; 1 μM IAA, 8 h; 1 μM IAA, 12 h; 1 μM IAA, 24 h	3	Lewis et al., 2013
13	GSE7432	M	Roots of 3 DAG etiolated seedlings	1 μM IAA, 4 h	2	Stepanova et al., 2007
14	SRP102803	P	Roots of 3 DAG seedlings	1 μM IAA, 6 h	3	Omelyanchuk et al., 2017
17	GSE59426	M	Root apices of 3 DAG seedlings	10 μM IBA, 6 h	3	Xuan et al., 2015
20	GSE59741	M	Cauline buds of 21-28 DAG seedlings	1 μM NAA, 18 h	3	Müller et al., 2015
22	SRP074436	P	Shoot apical meristem region and axillary meristem region of 14 DAG seedlings	5 μM 2,4-D, 55 h	3	Mozgová et al., 2017
23	GSE179303	M	Leaves of similar sizes and developmental stages	23 mM 2,4-D, 72 h post treatment (spraying)	3	Romero-Puertas et al., 2022
			Ethylene			
11, 12	SRP118634	P	4 DAG seedlings	10 μM ACC, 2 h; 10 μM ACC, 4 h	3	Fu et al., 2021
1, 2, 6, 7	SRA063695	P	3 DAG etiolated seedlings	10 ppm ethylene gas, 4 h* 10 ppm ethylene gas, 12 h; 10 ppm ethylene gas, 24 h	3	Chang et al., 2013
3	SRP069072	P	3 DAG etiolated seedlings	10 ppm ethylene gas, 4 h	2	Zhang et al., 2016a
4, 8	SRP076862	P	Roots and shoots of 3 DAG etiolated seedlings separately	10 ppm ethylene gas, 4 h	2	Zhang et al., 2016b
5	SRP168223	P	3 DAG etiolated seedlings	Ethylene gas, 4 h	2	Zander et al., 2019
9	GSE7432	M	Roots of 3 DAG etiolated seedlings	10 ppm ethylene gas, 4 h	2	Stepanova et al., 2007
10	SRP126162	P	Roots of 6 DAG seedlings	10 ppm ethylene gas, 4 h	2	Feng et al., 2017
13, 14, 15, 16	GSE84446	M	Roots of 3 DAG seedlings	1 μM ACC, 4 h; 1 μM ACC, 8 h; 1 μM ACC, 12 h; 1 μM ACC, 24 h	3	Harkey et al., 2018

* In experiments No. 1 and 2, ethylene treatment was carried out under the same conditions.
The number of biological replicates available for each sample and used for DEG detection is indicated in the sixth column. For DEG calling, untreated control samples collected at the initial time point were used in auxin treatments No. 2, 4, 5, 7, 9, 16, 17, 22, 23, and in ethylene treatments No. 1, 2, 6, 7; otherwise, separate mock treated control samples were employed. R – RNA sequencing; M – microarray experiment; 2,4-D – 2,4-dichlorophenoxyacetic acid; NPA – naphthylphthalamic acid; IAA – indole-3-acetic acid; NAA – 1-naphthaleneacetic acid; IBA – indole 3-butyric acid; DAG – days after germination.

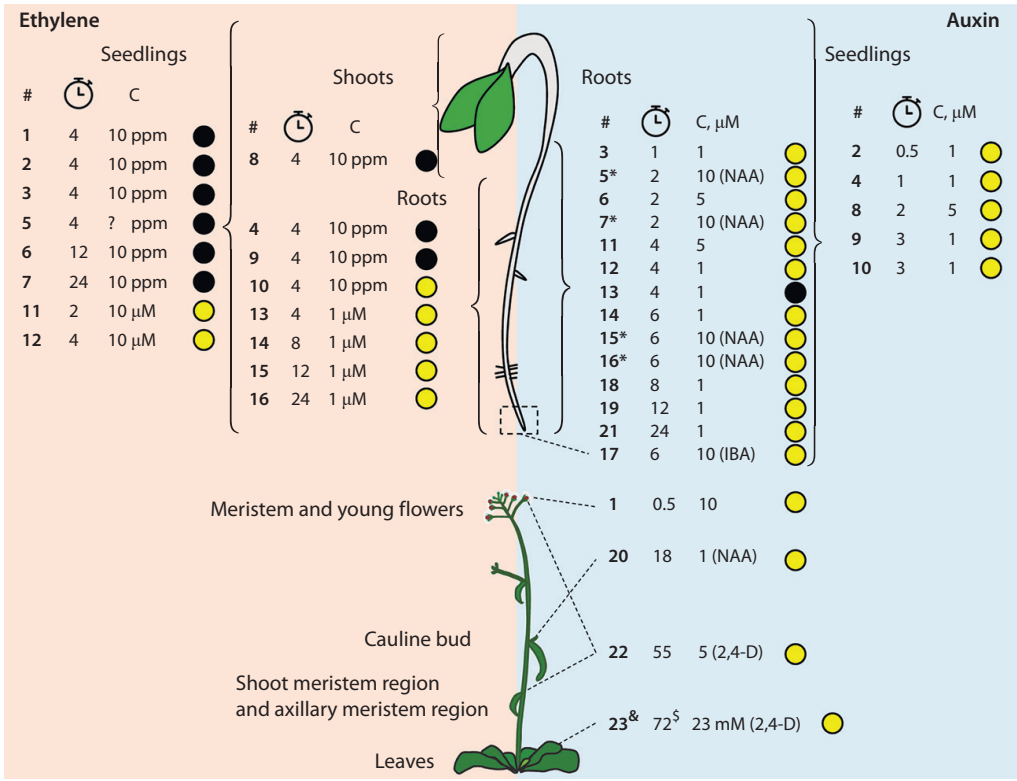

Fig. 2. Schematic representation of the experimental conditions, under which transcriptomes selected for comparative analysis were obtained.

The asterisk indicates the root segment between the root apical meristem and the root-hypocotyl junction. Ethylene was applied either as a gas (concentration in parts per million) or as its precursor ACC (μM). Auxin was applied as IAA, otherwise indicated. Treatment duration is indicated in hours. Numbers in bold denote the serial numbers of the experiments from Table. C – hormone concentration; black circles – etiolated seedlings; yellow circles – light-grown seedlings; [&] – spraying; [$] – post-treatment; ? – concentration of gaseous ethylene is not given in the primary source.

is observed at 2–8 h of treatment (Lewis et al., 2013). Treatment prolongation up to 12–24 h returns the transcriptional activity of most genes to the level observed in the control (no auxin treatment) samples, and the number of DEGs becomes close to the one detected in short-term (1 h) auxin treatments. A high number of DEGs (No. 22, 11,966 DEGs) was typical for prolonged treatment (55 h) of shoot apices and axillary meristems with 5 μM 2,4-D to induce callus initiation, which is accompanied by significant reprogramming of genome transcriptional activity (Xu et al., 2012). A fairly large number of DEGs was also found in shorter (4–6 h) treatments of seedlings with 5–10 μM IAA, which corresponds to the peak of transcriptional activity changes in response to auxin (Lewis et al., 2013). Notably, a large number of DEGs was observed in transcriptomes of whole roots or roots without root tips, both possessing a wide variety of tissues (No. 11, 15, and 16; 9461, 7692, and 11,905 DEGs, respectively). In the root tip (No. 17), on the contrary, the number of DEGs decreased to 4214, which can be explained by biological homogeneity of the sample (columella, stem cell niche and first progenitors of the initials).

Worth noting is the high value of the *R* ratio for the DEG profile of meristem and young flowers (No. 1), indicating that this auxin-induced transcriptome has a specific DEG composition compared to all others presented in the study. The reason for a significant deviation of the DEG number from the median in profile No. 10 (410 DEG) could be stress induced by a dramatic change in the seedling cultivation conditions, when the seedlings grown on agarized medium for 6–7 days were placed for a day in liquid medium with constant shaking before auxin treatment. In this case, auxin-sensitive genes associated with the stress response changed their expression both in the experimental and in the control groups. As a result, only genes unrelated to stress manifested as auxin-sensitive DEGs. At the same time, considering the slightly increased value of the *R* ratio for the DEG list No. 10 compared to the median, we can assume that the quality of these data is not high enough.

A low number of ethylene-sensitive DEGs was observed in roots of light-grown seedlings after a short-term (4 h) treatment with the ethylene precursor, ACC, at a low (1 μM) concentration (No. 13, 522 DEGs), which may be due to the insufficient treatment duration to implement a full response to ethylene. Treatment prolongation up to 8, 12, and 24 hours (No. 14, 15, 16) increased the number of DEGs approximately twofold in all cases (Harkey et al., 2018).

Thus, a complete response to ethylene and the number of DEGs close to the median value were observed for 8-hour and longer treatments. The low number of DEGs in the DEG list No. 9 (379 DEGs) can be linked to technical features of

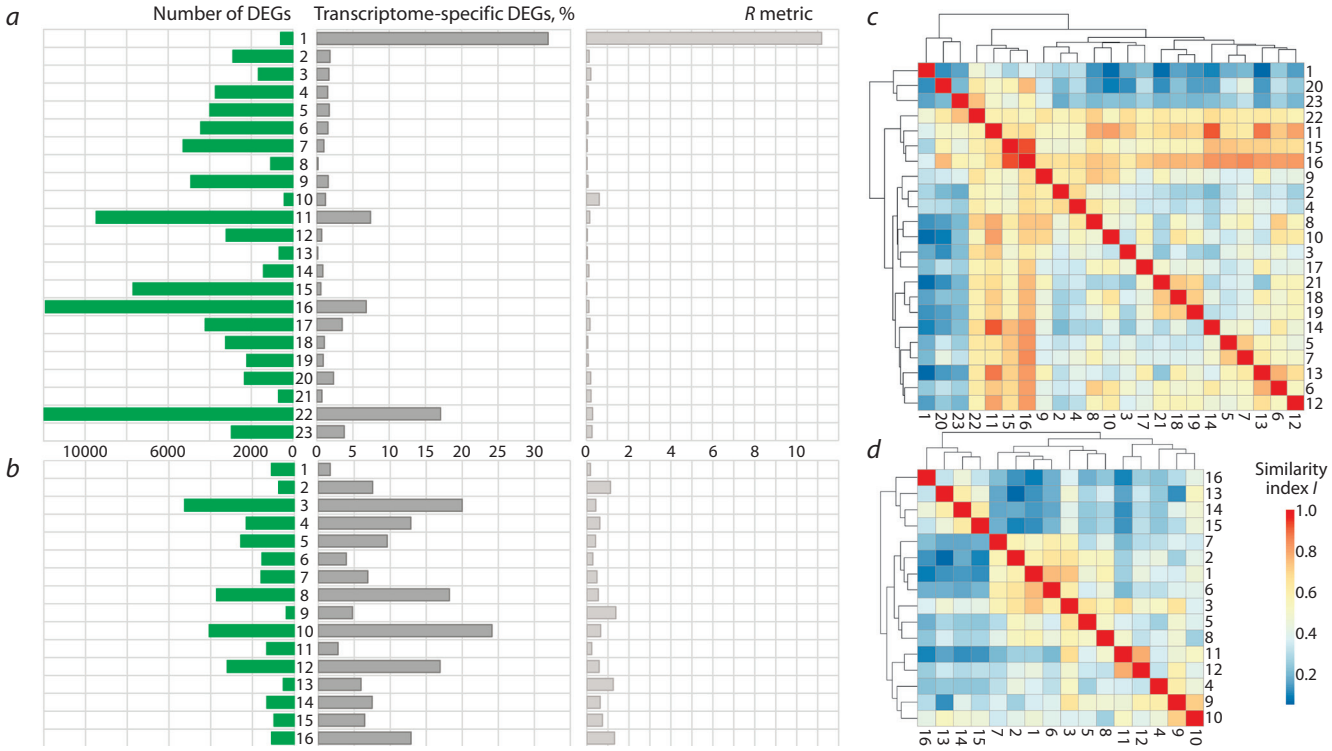

Fig. 3. Comparative description of the transcriptional response to auxin and ethylene under different conditions in *A. thaliana*.

a, *b* – number of DEGs and proportion of specific (unique) DEGs in the auxin (*a*) and ethylene (*b*) datasets, and *R* metric for each data set; *c*, *d* – pairwise comparison of auxin experiments (*c*) and ethylene experiments (*d*). The similarity index *I* reflecting the proportion of common DEGs in the smaller list is described in the Materials and Methods section. Experiment serial numbers correspond to those in Table.

the experiments, given the low number of DEGs in the auxin-induced profile No. 13 (657 DEGs) from the same study (Stepanova et al., 2007). Nevertheless, there is no reason to conclude that the quality of these data is low, since the observed deviations are not accompanied by a significant increase in the *R* ratio value. It is noteworthy that DEG numbers close to the median values were obtained in the experiments implemented with SOLiD RNA sequencing (No. 1, 2, 6, and 7) regardless of the treatment duration (Chang et al., 2013), as well as with Illumina sequencing of shoots (No. 4) and plants of the Ler (Landsberg erecta) ecotype (No. 5), but not Columbia, as in all other cases. In contrast, Illumina sequencing of etiolated shoots and roots yielded the numbers of DEGs greatly exceeding the median value (No. 3, 8, and 10; 5253, 3715, and 4067 DEGs, respectively).

Differential gene expression profiles in response to phytohormones in the samples from different plant parts differ in DEG composition

Next, we investigated the similarity of the DEG lists by DEG composition in more detail. Pairwise comparisons using GetSimMatrix confirmed the specific nature of the transcriptional response to auxin in the shoot meristem and young flowers (profile No. 1) compared to all other organs (see Fig. 3, *c*). Not surprisingly, a relatively high value of the similarity index for this DEG list ($I = 0.47$) was observed only with the one of shoot and axillary meristems (No. 22). Next, two groups of similar DEG lists represented the auxin response in whole seedlings and in the roots. The difference between seedling and root DEG profiles was also confirmed with DE_clustering, and it is intuitively clear, since the shoot is represented in the seedling along with the root (Fig. 4). Notably, with the detected intragroup similarity, there was still obvious variability among transcriptomes within each group (see Fig. 2, *c*). Finally, the DEG lists with more DEGs (No. 11, 15, 16, 22) showed a fairly high similarity index when compared in pairs with all others (see Fig. 3, *c*).

The qualitative similarity of large DEG lists with each other as well as with smaller DEG lists suggests their validity. Transcriptomic responses cauline leaf buds (No. 20) and leaves (No. 23) showed moderate similarity ($I \geq 0.42$) only to the large DEG lists. It can be hypothesized that treatment with high auxin concentrations (No. 11, 15, 16, 22) alters the expression of different groups of genes, each responding to low auxin concentrations only under certain conditions. In addition, the large number of DEGs in the late response may be due to a wide representation of secondary auxin response genes.

Pairwise comparisons of ethylene-induced transcriptomes revealed a discrete group (No. 13–16) from the study by A.F. Harkey et al. (2018) (see Fig. 2, *d*). They described gene expression changes in roots after treatment of seedlings grown under continuous light conditions with the ethylene precursor ACC. ACC is also thought to have ethylene-independent biological activity (Vanderstraeten et al., 2019), and light has a significant effect on shaping the transcriptional response to ethylene in *A. thaliana* (Shi et al., 2016a, b; Luo, Shi, 2019). We hypothesized that the chemical nature of the active compound and the light conditions during seedling growth could

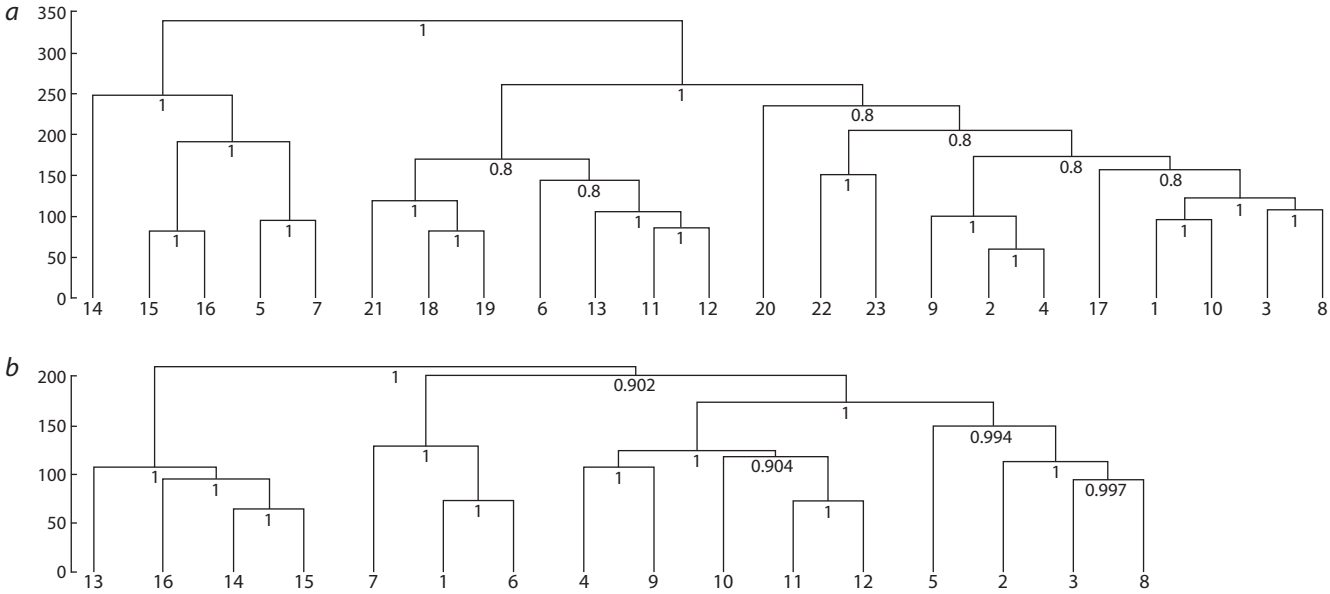

Fig. 4. Hierarchical clustering of auxin- (*a*) and ethylene-induced (*b*) transcriptomes using the Ward.D2 method.

act as significant non-target factors in this case. However, DEG lists No. 13–16 showed only moderate similarity to the ones from roots of ethylene-treated seedlings grown under long day (16 h) conditions (No. 10) (I = 0.59, 0.54, 0.52, and 0.49), and were quite different from ACC-induced transcriptomes of whole seedlings grown under 12 h day/12 h night conditions (No. 11 and 12) ($0.22 < I < 0.42$) (see Fig. 3, *d*). Thus, we cannot exclude that the isolation of profiles No. 13–16 may be due to a batch effect. The remaining profiles fell into two groups of similar DEG lists.

The first one included the transcriptional response to ethylene in the roots of seedlings regardless of light conditions, as well as in whole seedlings grown in the presence of light. The second group integrated the response to ethylene in etiolated seedlings or shoots. Thus, we confirmed the known fact that light plays an essential role in shaping the ethylene response (Shi et al., 2016a, b; Luo, Shi, 2019), but additionally we showed that this effect is observed in shoots but not in roots. Notably, hierarchical clustering using $\log_2$FC values showed that the time series for ethylene treatment of etiolated seedlings from (Chang et al., 2013) stands out as a separate group (see Fig. 4), which also raises the question of a possible batch effect.

The set of seven ethylene-induced transcriptomes is homogeneous in terms of DEG composition

The number of genes identified as DEGs in a set of transcriptomes (i. e. detected as DEG at least in one of the transcriptomes) essentially depends on the homogeneity of this set. In our case, 20,552 and 10,988 genes were identified as DEGs in at least one auxin- and ethylene/ACC-induced transcriptome, respectively. Given the size of the *A. thaliana* genome, which contains just over 30,000 genes, this is an unexpectedly large number of DEGs, which is markedly higher than the number of DEGs in individual experiments, and is likely explained by the dependence of transcriptome induction on experimental conditions. Quantification of the homogeneity of the DEG list sets by resampling (using the DE_bootstrap function) expectedly showed their heterogeneity in DEG compositions (Fig. 5, *a*, *b*).

At the same time, based on the results of pairwise comparison of DEG lists described in the previous section (see Fig. 3, *c*, *d*), we can suggest the potential homogeneity of auxin-induced DEG profiles in the root (No. 5–7, 12–14, 18–21) and ethylene-induced DEG profiles in etiolated seedlings/shoots (No. 1–3, 5–8). To test this hypothesis, we analyzed the corresponding sets of DEG lists using the DE_bootstrap function. While the set of auxin-induced root transcriptomes still showed heterogeneity (different durations of treatment probably caused differences in DEG composition), no significant differences in the number of ethylene-induced DEGs in etiolated seedlings were found. Thus, the set of ethylene-induced transcriptomes in etiolated seedlings/shoots (No. 1–3, 5–8), due to their homogeneity, can be reasonably used for meta-analysis (e. g., to better identify weak patterns).

Conclusion

Meta-analysis of transcriptomic data provides great opportunities for increasing the power of statistical analysis, if the data are homogeneous. However, reasonable selection of experiments for meta-analysis is often hampered by the lack of standards in this field and the absence of convenient software tools for comparative description of DEG lists, in particular, for the construction of user-friendly visualizations. In this work, we proposed a set of quantitative indicators for comparative description of DEG lists (n – number of DEGs; δ – proportion of DEGs specific for a given transcriptome; R – ratio describing the specificity of the transcriptional response; I – similarity index for a pair of transcriptomes based on DEG composition; assessment of the homogeneity of DEG lists) and implemented their calculation and visualization as the InterTransViewer program. We demonstrated that an integrated analysis of the

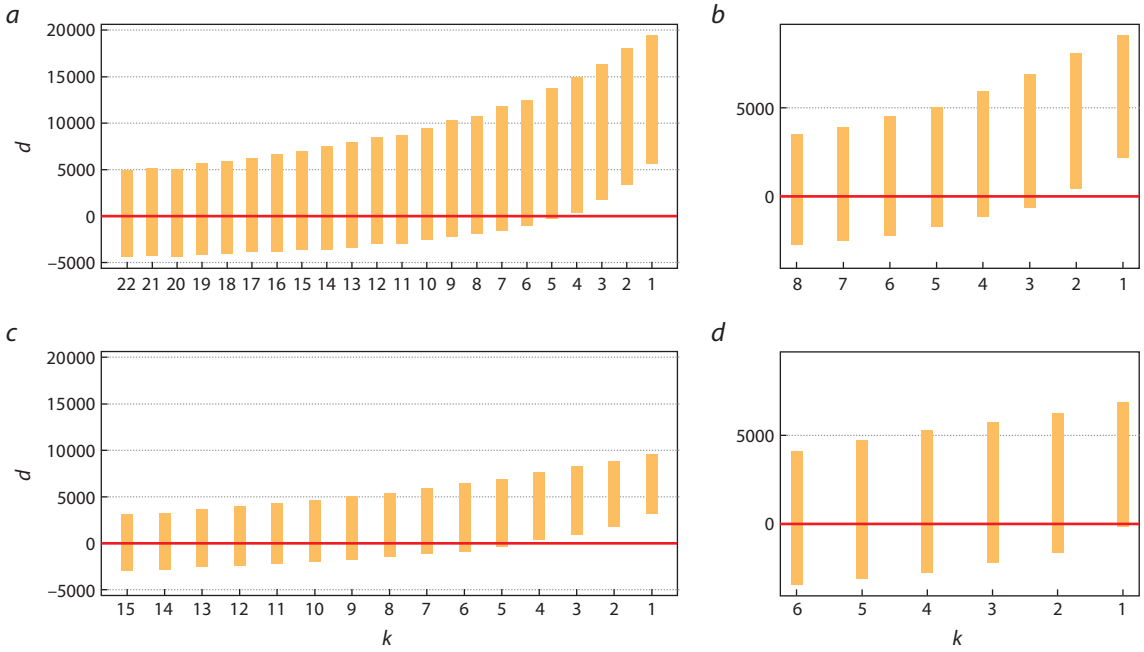

Fig. 5. Assessment of homogeneity of auxin- (*a*, *b*) and ethylene-induced (*c*, *d*) transcriptomes by resampling.

The bars represent the 95 % confidence interval of values $d = N_m - N_k$, where N_m is the number of genes that are DEGs in at least one of the DEG lists in the pseudo-sample of size m; N_k is the number of genes that are DEGs in at least one of the DEG lists in the pseudo-sample of size k, $m > k$. A detailed description of the procedure is presented in the Materials and Methods. *a* – results for $m = 23$ (all auxin-induced transcriptomes); *b* – results for $m = 9$ (auxin-induced transcriptomes No. 5–7, 12–14, 18, 19, 21); *c* – results for $m = 16$ (all ethylene-induced transcriptomes); *d* – results for $m = 7$ (auxin-induced transcriptomes No. 1–3, 5–8). The bold red line indicates the value of $d = 0$.

characteristics of individual DEG lists (n, δ, R) in the context of the results of pairwise comparisons of transcriptomes by DEG composition (both using the similarity index I and by clustering based on the fold changes in expression levels) allowed us to range the experiments in the context of each other, to assess the tendency for their integration or segregation, and to generate hypotheses about the influence of significant non-target factors on the transcriptional response. As a result, this made it possible to identify potentially homogeneous groups of DEG lists.

Subsequent analysis of the homogeneity of these groups using a resampling procedure and the establishment of a significance threshold allowed us to decide whether these data should be used for meta-analysis. Thus, InterTransViewer allows for efficient sampling of the induced transcriptomes depending on the meta-analysis aim and methods.

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ORCID

A.V. Tyapkin orcid.org/0000-0002-5969-3628
V.V. Lavrekha orcid.org/0000-0001-8813-8941
D.Yu. Oshchepkov orcid.org/0000-0002-6097-5155
E.V. Zemlyanskaya orcid.org/0009-0005-7316-7690

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