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ВАВИЛОВСКИЙ ЖУРНАЛ ГЕНЕТИКИ И СЕЛЕКЦИИ

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# К 135-летию академика Н.И. Вавилова: актуальные исследования коллекций генетических ресурсов растений

Уважаемые читатели!

25 ноября 2022 г. исполняется 135 лет со дня рождения нашего выдающегося соотечественника Николая Ивановича Вавилова!

Предлагаем вашему вниманию выпуск «Вавиловского журнала генетики и селекции», в котором в честь юбилея великого ученого представлены обзорные и оригинальные статьи в развитие тех направлений комплексного и разностороннего изучения культурных растений, которые были начаты Н.И. Вавиловым в ВИР в 1920–1930-х гг. с использованием традиционных и новейших методов исследования мирового уровня.

Сегодня с применением современных методов и подходов работу с генетическими ресурсами растений в тесном сотрудничестве с ВИР им. Н.И. Вавилова ведет широкий круг исследователей по всей стране. Авторами статей текущего выпуска являются сотрудники Федерального исследовательского центра Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР) из Санкт-Петербурга, а также Краснодарского края и Приморского края (филиалы ВИР), сотрудники Федерального селекционно-технологического центра садоводства и питомниководства (Москва), Федерального исследовательского центра «Фундаментальные основы биотехнологии» РАН (Москва), Федерального научного центра овощеводства (Москва), Северо-Кавказского федерального научного центра садоводства, виноградарства, виноделия (Краснодарский край), Омского аграрного научного центра (Омская область), Сибирского федерального научного центра агробиотехнологий РАН (Новосибирская область), Федерального исследовательского центра Институт цитологии и генетики СО РАН и его филиала – Сибирского НИИ растениеводства и селекции, а также Курчатовского геномного центра ИЦиГ СО РАН (Новосибирская область), Санкт-Петербургского государственного университета, Саратовского национального исследовательского государственного университета им. Н.Г. Чернышевского, Новосибирского национального исследовательского университета, Томского государственного университета и Дальневосточного федерального университета.

Рациональная стратегия комплексного изучения коллекций генетических ресурсов растений основана на структурировании и дифференциации генофонда. В качестве примера этого этапа комплексных исследований и рационального использования генетических коллекций может служить работа Е.В. Семеновой с коллегами по фенотипированию образцов гороха в условиях Краснодарского края на Адлерской опытной станции ВИР. Оценка генофонда гороха по четырем качественным, десяти количественным и четырем фенологическим признакам в течение трех лет в экологических условиях Адлерской опытной станции позволила дифференцировать изученный генофонд по направлениям использования (зерновое, кормовое и овощное). Структурирование генофонда и представленные данные, которые характеризуют каждый образец по комплексу признаков, определяющих направление использования, позволят рационально подходить к выбору как исходного материала для селекции, так и образцов для дальнейших генетических исследований. Еще одним примером комплексной фенотипической оценки генофонда для его дальнейшего рационального использования служит оригинальное исследование М.Г. Евдокимова с соавторами, целью которого было выявление перспективных генетических источников для создания сортов твердой яровой пшеницы в Западной Сибири. В результате выделены источники хозяйственно ценных признаков по таким показателям, как натура и качество зерна и устойчивость к основным заболеваниям в регионе возделывания. Кроме того, выделены 25 образцов пшеницы, резистентных к возбудителю стеблевой ржавчины агрессивной угандской расы Ug99.

Неотъемлемой частью комплексной оценки генетических ресурсов растений является хемотипирование. Особую значимость данная оценка имеет в случае изучения плодовых и ягодных культур. По результатам исследования С.М. Мотылевой с коллегами даны оценки плодов ремонтантной малины по содержанию макро- и микроэлементов в условиях выращивания в средней полосе России. Образцы с наибольшим накоплением макро- и микроэлементов в ягодах рекомендованы в качестве генетических источников для дальнейшей селекции малины на улучшение минерального состава плодов. В работе Т.В. Шеленги с соавторами осуществлено метаболомное профилирование плодов образцов черной смородины и жимолости с применением газожидкостной хроматографии, сопряженной с масс-спектрометрией. Это позволило высказать рекомендации по источникам как для пищевого назначения, так и для экстракции биологически активных веществ. В качестве источника биологически активных веществ интерес представляет многолетнее травянистое растение перилла. Проведенный М.П. Разгоновой с соавторами анализ полифенольных соединений в листьях растений из генофонда данной культуры позволил выявить широкий спектр биологически активных соединений (кумарин умбеллиферон, тритерпен сквален, стеаридоновая кислота, ряд высокомолекулярных карбоновых кислот и др.).

В 1920-х гг. Николай Иванович Вавилов организовал впервые в нашей стране масштабные генетические исследования культурных растений и их диких родичей. За столетие произошла колоссальная трансформация методик, используемых для генетического анализа. Задачи, поставленные Н.И. Вавиловым по изучению генетических ресурсов растений, сегодня решаются при помощи молекулярных и геномных подходов. Для выявления доноров ценных генов, а также определения характера наследования того или иного признака у изучаемых сортов используются диагностические ДНК-маркеры. Это значи-

тельно упрощает и ускоряет как генетический анализ, так и пребридинговую и селекционную работу по признакам с моно- и олигогенным контролем.

Так, И.И. Супрун с коллегами выполнили молекулярно-генетическую идентификацию генов, детерминирующих признаки качества плодов яблони (потребительская привлекательность, лежкоспособность и транспортабельность плодов) и устойчивость к парше. Выделенные образцы, несущие приоритетные для селекции аллели искомых генов, являются комплексными донорами, а также ценным селекционным материалом для создания сортов с повышенными характеристиками качества плодов и устойчивостью к парше. В работе А.В. Кулаковой с соавторами выделены перспективные ДНК-маркеры для генотипирования сортов томата с целью отбора доноров устойчивости к различным стрессовым факторам. Исследование коммерческих сортов яровой мягкой пшеницы Восточной и Западной Сибири в работе С.Э. Смоленской с соавторами при использовании методов фенотипирования и генотипирования на основе ДНК-маркеров, специфичных для генов яровизации мягкой пшеницы *Vrn*, позволило выделить три группы сортов, у которых яровой тип развития и сроки контролируются при помощи одного, двух и трех генов *Vrn* соответственно. Установленное аллельное состояние генов *Vrn* и сведения о сроках спелости изучаемых сортов позволят в дальнейшем целенаправленно вести маркер-контролируемый отбор по группам спелости, что важно для учета экологической зональности (тайга, подтайга, лесостепь, степь) в регионах возделывания пшеницы.

Иные подходы применяются в отношении количественных признаков. Преобладающая часть локусов, контролирующих такие признаки, до сих пор остается не изученной. Для их выявления проводят полногеномный анализ ассоциаций на основе фено- и генотипирования генофонда. Так, например, в работе И.Н. Леоновой и Е.В. Агеевой, нацеленной на картирование локусов, ассоциированных с устойчивостью к полеганию у яровой мягкой пшеницы, выявлено 26 значимых ассоциаций в хромосомах 1B, 2A, 3A, 3D, 4A, 5A, 5B, 5D, 6A и 7B. Полученные результаты позволяют предположить, что районы хромосом 3A и 6A могут содержать кластеры генов, влияющих как на устойчивость к полеганию, так и на высоту растения. Результаты могут иметь значение для разработки методов создания устойчивых к полеганию сортов с помощью маркер-ориентированной и геномной селекции.

Отдельное направление современных работ с генетическими ресурсами растений представляют исследования в сфере биотехнологии, которые включают в себя применение методов генной и клеточной инженерии и геномного редактирования. Последний подход, как показано в обзоре А.Б. Щербаня, позволяет более эффективно вовлекать в селекционные программы дикие родичи культурных растений, применяя методы доместикации *de novo*. Важное значение в работах биотехнологического направления имеет повышение эффективности процессов (например, каллусообразования, регенерации, гаплоиндукции и др.), с которыми напрямую связан успех достигаемых практических результатов. Этим вопросам посвящены публикация Г.В. Хафизовой и Т.В. Матвеевой, а также обзорная статья А.В. Ульянова с соавторами.

Комплексные исследования коллекций генетических ресурсов растений, результаты которых представлены в выпуске, проводились в рамках Федеральной научно-технической программы развития генетических технологий на 2019–2030 гг., грантов Российского научного фонда, а также научно-исследовательских работ государственных заданий федеральных государственных бюджетных учреждений.

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# Phenotypic traits differentiating the genetic resources of pea (*Pisum sativum* L.) by the type of use

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**Abstract.** The paper presents an analysis of the data obtained for pea accessions from the VIR collection studied at the Adler Experiment Station in the setting of the Krasnodar Territory in 2017–2019. It was for the first time that these accessions were studied for a set of phenotypic traits. The object of the study was a sample of 494 pea accessions originated from 43 countries and 18 regions and territories of the Russian Federation. The work was carried out in compliance with the methodological guidelines developed at VIR. Statistica 13.3 software was employed for statistical data processing. An assessment of four qualitative, 10 quantitative and four phenological traits in the accessions made it possible to differentiate them by the type of use, that is, as dry, forage and garden peas. The varieties differing in the type of use significantly differed by the values of such traits as stem length, number of pods per plant, number of nodes to the first flower, number of flowers in the inflorescence, the maximum number of seeds per pod, pod length, and a narrower pod of forage pea compared to that of dry and garden peas. The average values of these traits were recorded for the peas with different types of use. The maximum difference was noted between garden and forage pea varieties. Dry pea varieties occupied an intermediate position. The complex of phenotypic traits identified determines the differences between three types of pea use, which is important when selecting the initial material for breeding appropriate varieties.
Key words: pea; VIR collection; trait variability; correlation; ANOVA; PCA.

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# Фенотипические признаки, определяющие дифференциацию генофонда гороха (*Pisum sativum* L.) по направлениям использования

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**Аннотация.** Поддержание жизнеспособности и изучение образцов коллекции гороха ВИР осуществляется на опытных станциях института в разных агроклиматических зонах страны. Каждый образец коллекции включен в трехлетний цикл полевого изучения по комплексу селекционно значимых признаков. Фенотипический скрининг коллекции позволяет осуществить оценку признаков, выявить характер их изменчивости и ранжировать образцы по значениям признаков в качестве исходного материала для селекции. Один из основных принципов дифференциации генофонда гороха – разделение образцов по направлениям использования: зерновому, кормовому и овощному. Каждое из этих направлений требует специализированного исходного материала. Между тем четко очерченного перечня различий между фенотипами растений каждого из этих направлений не существует. Более того, имеется множество сходных признаков, порой затрудняющих отнесение сорта к той или иной категории использования. В настоящей статье приведены анализ и обобщение данных трехлетнего фенотипирования образцов коллекции в условиях Краснодарского края на Адлерской опытной станции. Объектом исследования служили 494 коллекционных образца, происходящих из 43 стран и 18 областей и краев Российской Федерации. Образцы оценивали по четырем качественным, десяти количественным и четырем фенологическим признакам. Статистическая обработка данных полевой оценки позволила выявить, что образцы означенных направлений достоверно различались по комплексу признаков: длина стебля; число бобов на рас-

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тении; число узлов до первого цветка; число цветков в кисти; максимальное число семян в бобе; длина и ширина боба. Определены средние значения этих признаков для всех направлений использования. Овощные сорта максимально отличаются от кормовых. Зерновые занимают промежуточное положение. Выявленный комплекс фенотипических признаков, определяющий различия трех групп направлений использования гороха, упростит работу с коллекцией, в частности подбор исходного материала для селекции соответствующих сортов.
Ключевые слова: горох; коллекция ВИР; изменчивость признаков; корреляции; дисперсионный анализ; метод главных компонент.

## Introduction

The VIR collection of peas has accumulated the global diversity of *Pisum sativum* L. and contains more than 8 thousand accessions from 93 countries of the world. The collection is structured in accordance with the botanical and agroecological classification, the status of the accessions reflecting the degree of breeding process completeness, and is ranked in accordance with the value of biological and agronomic traits, etc. In order to use the gene pool of peas in the national economy, it is most important to differentiate it according to the types of use as dry, forage, and garden peas.

There is no clearly delineated list of differences between plant phenotypes belonging to each of these three groups. Moreover, there is a multitude of common, so-called "overlapping" traits, which sometimes make it difficult to attribute a variety to a particular group. Breeding efforts aimed at improving varieties of all types of use have common tasks, e. g., high yield, high protein content in seeds, resistance to lodging, plant architectonics suitable for mechanized harvesting, and resistance to pathogens. However, there are complexes of traits characteristic of each group of economic use, some of which are inherent only in one particular group.

Dry peas (*P. sativum* L. subsp. *sativum*), which are used as a food crop, are characterized by white flowers, smooth seed surface, mainly with a yellow and yellow-pink seed coat (green and gray-green also possible). For good digestibility, the seed coat must be thin (Khangildin, 1972). Dry pea breeding is aimed at high seed productivity, high harvesting index (that is, seed yield in relation to the cut pea mass), and high protein content.

Forage pea varieties have differently colored flowers and seeds of different color, mostly dark and speckled. The breeding of mown forage pea varieties is aimed at obtaining high green mass volume, high rate of its accumulation, plant tallness and high leafiness, low percentage of fiber and high protein content in the green mass (Adamova, 1975). Therefore, when breeding forage varieties, it is better to use the forms with the traditional leaf type and indeterminate type of growth (Pea…, 2019). Low 1000 seed weight (less than 100 g) is desirable for forage varieties, as it makes it possible to reduce the weight of the sown seed. Among the varieties of dry and forage peas, there exist transitional forms, which can be called grain-forage varieties.

Garden pea varieties are white-flowered, with brain-like (wrinkled), predominantly green seeds, and large pods. They are required to be uniform in flowering and fruit formation, as well as to have a high yield of green peas in relation to the vegetative mass, which implies a relatively low plant height. One of the main aspects to be pursued in garden pea breeding is the improvement of the carbohydrate complex, which determines the taste of green peas in fresh and canned form. This is a high sugar content (6.5–8.5 %) along with a relatively low accumulation of starch (4–5.5 %) containing a high percentage of amylose (Samarina, 1970; Alikina (Putina) et al., 2016; Putina et al., 2018). Starch grains of garden peas have a specific complex structure with the predominance of small fragments.

A clear differentiation of pea varieties by the type of use is crucial for the characterization of the initial material and its targeted use. This is especially important when diverting from the breeding of universal varieties to breeding varieties for specific use. The identification of criteria for distinguishing leguminous crop varieties by different types of use by phenotypic traits is carried out in VIR systematically (Vishnyakova et al., 2011, 2013; Burlyaeva, Malyshev, 2013; Burlyaeva et al., 2014).

The annual regeneration of accessions from the VIR collection and their phenotypic assessment in the field for a number of biological and agronomic traits makes it also possible to reveal the differences within the gene pool according to a variety of parameters along with obtaining the data on the assessed traits. The present paper was aimed at outlining the range of traits that determine the subdivision of the gene pool of pea (*P. sativum* L.) according to areas of economic use.

## Materials and methods

**Material.** The collection accessions were studied for three years (2017–2019) at the Adler Experiment Station, a branch of VIR. The study included 494 accessions of peas (*P. sativum* L.) (203 of dry, 217 of garden, and 74 of forage peas) of various types of use introduced in the collection from 43 countries and from 18 regions and territories of the Russian Federation received in the VIR collection since 2005. Ten countries were represented by ten or more accessions; these are Russia (112 accessions), USA (94), France (45), Australia (27), the Netherlands (26), Ukraine (18), China (16), Syria (14), Germany (12), and Canada (10). Within the Russian Federation, most of the accessions came from the Vologda Province (19 accessions), Oryol Province (14), Tyumen Province (14), Moscow Province (13), Krasnodar Territory (11), and Rostov Province (10).

**Methods.** The accessions were studied in compliance with the methodological guidelines (Vishnyakova et al., 2010). The seeds were sown in the third ten-day period of March on single-row plots 2.5 m long (~1 $m^2$). A description of phenological and morphological characteristics was carried out during the vegetation period. The plants were harvested as they matured, from early June through early August. They

were gathered in bundles and assessed for their main morphological, biological and economic traits. Some parameters were evaluated in points, in accordance with the "International Comecon List of Descriptors for the Genus *Pisum* L." (Makasheva et al., 1986), while for some the measurement was made in grams, pieces and days.

The list of the assessed traits includes:

- Qualitative indicators (points): seed color, leaf morphotype, presence/absence of the parchment layer in the pod valve, fusion of the seed stalk and testa;
- Quantitative traits: stem length (cm), number of flowers per inflorescence (pcs), pod length (cm), pod width (cm), number of nodes to the first flower (pcs), yielding ability (seed yield per plot, g), seed productivity per plant (g), 1000 seed weight (g), number of pods per plant (pcs), resistance to pea weevil (*Bruchus pisorum* L.) as percentage of the healthy seeds mass out of the total mass;
- Phenologial dates (sowing, emergence, flowering, ripening). Duration has been calculated for the following interphase periods (days): from sowing to emergence (SE), from emergence to flowering (EF), from flowering to ripening (FR), and the growing season duration – from emergence to ripening (ER).

The results of quantitative traits assessment are given as average values for three years. Statistical processing employed Statistica 13.3 software. Correlations between quantitative traits of accessions were investigated. The one-way analysis of variance (ANOVA) was carried out for the type of use, presence of a parchment layer, fusion of the seed stalk and testa, and the two-factor ANOVA for the type of use and morphotype. For post hoc comparisons, Tukey's test for unequal samples was used. The sample structure was investigated by the principal component analysis (PCA). The study adopted a significance level of 5 %.

**Weather conditions during the study.** The Experiment Station is located on the northeastern coast of the Black Sea in the Krasnodar Territory with a humid subtropical climate, warm rainy winters and sunny summers. During the period of the study (2017–2019), 2017 was the coldest and wettest year, with the average temperature of 15.9 °C for April–June, and 403 mm of the total precipitation. The year of 2018 was the warmest, with the average temperature of 19.1 °C in April–June, and the total precipitation of 119 mm. The year of 2019 was characterized by an average temperature of 18.5 °C and precipitation of 233 mm for the above-mentioned period. Therefore, in general, the weather conditions at the station are quite consistent with the agroclimatic requirements for pea growing.

## Results and discussion

### Phenological data

The average duration of the period from emergence to the onset of flowering ranged from 27 to 53 days for dry peas, from 28 to 54 for garden peas, and from 34 to 58 days for forage pea accessions (Table 1). The earliest onset of flowering (on day 27) was recorded for the dry pea variety 'Nain de Mai' (k-10068) from France, and on day 28 for the garden pea varieties 'Salinero' (k-9811) from the Netherlands and 'Extra Rapide' (k-9137) from France. For the bulk of dry pea accessions (>80 %), the onset of flowering was recorded in the interval of 36–45 days from emergence. The distribution of garden pea varieties according to this indicator was more uniform. In forage pea accessions, flowering occurred later.

The most early ripening accession from the studied sample (k-9796, 'Alsweet', USA) belonged to the garden pea category and had the emergence to ripening period duration of 59 days, on an average, while the most late maturing accession (k-10174, 'Kormovoy-50', RF, Altai Territory) was a forage pea variety that matured in 84 days (Table 2). A high positive relationship between EF and ER in the entire sample ($r = 0.87$) should be noted.

**Table 1.** Numbers of accessions with different duration of the emergence to flowering period

| Type of use | Emergence to flowering period, days | | | | | |
|---|---|---|---|---|---|---|
| | <31 | 31–35 | 36–40 | 41–45 | >45 | Total |
| Dry peas | 4 | 10 | 59 | 105 | 25 | 203 |
| Garden peas | 23 | 33 | 78 | 74 | 9 | 217 |
| Forage peas | – | 2 | 18 | 21 | 33 | 74 |

**Table 2.** Numbers of accessions with different duration of the emergence to ripening period

| Type of use | Emergence to ripening period, days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | <60 | 60–64 | 65–69 | 70–74 | 75–79 | 80–84 | >84 | Total |
| Dry peas | 6 | 49 | 82 | 57 | 9 | 0 | 0 | 203 |
| Garden peas | 41 | 63 | 73 | 36 | 4 | 0 | 0 | 217 |
| Forage peas | 3 | 15 | 18 | 21 | 13 | 4 | 0 | 74 |

**Table 3.** Values of the studied traits in pea groups of different type of use

| Trait | Direction of use | | | |
|---|---|---|---|---|
| | Dry peas | Garden peas | Forage peas | All types |
| Number of accessions | 203 | 217 | 74 | 494 |
| Tendrillate leaf, % | 35.2 ± 3.4 | 16.4 ± 2.5 | 15.5 ± 4.3 | 24 ± 1.9 |
| Non-shattering of seeds, % | 20.7 ± 2.9 | 1.8 ± 0.9 | 21.6 ± 4.8 | 12.6 ± 1.5 |
| Emergence to flowering period (EF), days | 41.4 ± 0.3 | 38.1 ± 0.4 | 44.2 ± 0.6 | 40.4 ± 0.2 |
| Flowering to ripening period (FR), days | 26.0 ± 0.2 | 25.9 ± 0.2 | 25.6 ± 0.4 | 25.9 ± 0.1 |
| Emergence to ripening period (ER), days | 67.4 ± 0.3 | 63.9 ± 0.4 | 69.9 ± 0.7 | 66.2 ± 0.3 |
| Seed yield per plot, g | 178.5 ± 3.6 | 147.3 ± 3.5 | 163.7 ± 6.2 | 162.6 ± 2.4 |
| Seed productivity per plant, g | 8.7 ± 0.2 | 8.1 ± 0.2 | 8.0 ± 0.3 | 8.3 ± 0.1 |
| Resistance to pea weevil, % of healthy seeds | 69.6 ± 0.7 | 79.8 ± 0.6 | 72.5 ± 1.2 | 74.5 ± 0.5 |
| 1000 seed weight, g | 181.2 ± 2.4 | 158.9 ± 2.2 | 150.2 ± 5 | 166.8 ± 1.7 |
| Stem length, cm | 100.3 ± 2.4 | 84.4 ± 2.2 | 129.5 ± 4.6 | 97.7 ± 1.7 |
| Number of pods per plant, pcs | 12.5 ± 0.3 | 11.0 ± 0.3 | 14.6 ± 0.8 | 12.2 ± 0.2 |
| Number of flowers per inflorescence, pcs | 1.9 ± 0.0 | 1.7 ± 0.0 | 1.7 ± 0.0 | 1.8 ± 0.0 |
| Maximum seed number per pod, pcs | 6.3 ± 0.1 | 7.2 ± 0.1 | 6.6 ± 0.1 | 6.7 ± 0.1 |
| Pod length, cm | 6.9 ± 0.1 | 7.5 ± 0.1 | 6.7 ± 0.2 | 7.1 ± 0.1 |
| Pod width, cm | 1.5 ± 0.0 | 1.5 ± 0.0 | 1.3 ± 0.0 | 1.5 ± 0.0 |
| Number of nodes to the first flower, pcs | 14.2 ± 0.2 | 11.5 ± 0.2 | 15.3 ± 0.3 | 13.2 ± 0.1 |

**Comparison by ANOVA between groups of varieties of different type of use**

An analysis of trait values by one-way ANOVA (Table 3) showed that the accessions of different types of use manifested significant differences concerning the majority of the studied traits, except for FR ($p = 0.636$).

The leaf morphotype is a significant trait determining suitability of a variety for mechanized harvesting. Most modern varieties are semi-leafless (with tendrillate leaf type) (*afaf* genotype). In the studied sample, this morphotype was significantly more common in dry peas (35.2 %) than in garden (16.4 %) and forage peas (15.5 %). Such a distribution of accessions is quite consistent with the current state of pea breeding and the requirements to varieties of different types of use. This feature is not relevant for forage varieties, as was mentioned above. Large foliage that ensures abundant vegetative mass can be better achieved with the traditional leaf type. As for garden pea varieties, creation of semi-leafless ones began relatively late both abroad and in this country in comparison with cereals, and is in the process of development (Alikina (Putina) et al., 2016). The absence of significant innovations in the domestic breeding of garden peas is also evidenced by the recently revealed fact that both old and, to even a greater extent, new garden pea varieties are phenotypically less diverse than the foreign ones (Sinjushin, Anisimova, 2020).

The trait of seeds non-shattering due to the fusion of the seed stalk and testa was rarely observed in garden peas (in 1.8 % of varieties) compared with dry (20.7 %) and forage peas (21.6 %). This is explained by the fact that the trait was introduced into pea varieties to prevent seed shedding when ripe pods crack as they dry out (Zelenov, 2013). This is important for dry pea varieties used for both food and feed purposes. Garden pea varieties are harvested at technical ripeness, long before the possible cracking of the pods, which makes this feature not relevant. In addition, the stalk being firmly adhered to the seed spoils the appearance of canned peas.

The ER period duration averaged 66.2 days for all accessions, while all groups were significantly different. The longest duration of the ER period was recorded for forage varieties (69.9 days), medium for dry (67.4 days), and the shortest for the garden pea group (63.9 days). These figures correspond to the purpose of the varieties: the maximum accumulation of the vegetative mass in forage varieties requires a longer period, and garden peas require the minimum period for achieving their technical ripeness. In our opinion, the ER period observed by us for garden peas can be shorter. We explain it by the fact that the sample contained quite many old garden pea varieties. In contrast to them, modern varieties are more early-ripening. For example, modern varieties created at the Krymsk Experiment Breeding Station have a growing season of 53 to 75 days, thereby providing a permanent supply of peas

for the long-term and uninterrupted processing (Besedin, 2014; Besedin, Putina, 2019; Putina, Besedin, 2020).

The ER period duration is associated with that of the EF period, which averaged 40.4 days: it was significantly shorter for garden peas (38.1 days) than for dry (41.4 days) and forage peas (44.2 days), which did not differ significantly between themselves. The FR period for all three groups did not differ and averaged 25.9 days.

The seed yield per plot averaged 162.6 g; dry peas with the highest yield (178.5 g/plot) significantly differed from garden peas (147.3 g/plot). Forage peas had a medium yield value of 163.7 g and did not differ significantly from other groups. The seed productivity per plant did not differ significantly according to Tukey's test and amounted to 8.3 g.

The pea weevil resistance in the studied sample averaged 74.5 % of healthy seeds. The highest value was demonstrated by garden peas (79.8 %), which was significantly higher than that of dry (69.6 %) and forage peas (72.5 %), which did not differ significantly between themselves. To a certain extent, a lower susceptibility of garden peas is ensured by their early maturity, which makes it possible to avoid the pea weevil flight in the beetle stage. The latter is known to follow a certain seasonal pattern.

The average 1000 seed weight (seed size) in the sample was 166.8 g. Dry peas were found to have the largest seeds (181.2 g), which were significantly bigger than those of garden (158.9 g) and forage peas (150.2 g), which did not differ significantly between themselves.

In addition, significant differences between the three groups were noted for the traits listed in Table 3 such as the stem length, the number of pods per plant, the number of nodes to the first flower, the number of flowers per inflorescence, the maximum number of seeds per pod, the pod length and width.

The two-factor analysis of the relationship between the type of use and the morphotype (tendrillate/traditional) employed 116 accessions of the tendrillate and 368 ones of the traditional morphotype. Ten accessions in the sample featured other morphotypes of the leaf: five had acacia-like (*tl*), one dissected leaflet (*af tac*$^{A}$), and four multiple imparipinnate leaf morphotypes (*af tl*).

The tendrillate morphotype was characterized by significant differences from the accessions with traditional leaf morphotype of the leaved forms, regardless of the type of use: by a greater proportion of accessions with non-shattering seeds (25.9 *vs.* 8.2 %) on an average, which can be explained by the fact that both traits were introduced into varieties in relatively recent times, therefore, the majority of seed shattering genotypes is inherent in varieties with the traditional morphotype. The tendrillate leaf varieties are characterized by a lower resistance to pea weevil (70.1 *vs.* 75.8 %) (Fig. 1), a shorter stem length (84.7 *vs.* 102.2), fewer pods per plant (10.8 *vs.* 12.6), and more flowers per inflorescence (2.0 *vs.* 1.7, except for the forage peas). The seed yield per plot did not differ significantly (159.6 g for the tendrillate pea, and 163.7 g for the traditional morphotypes).

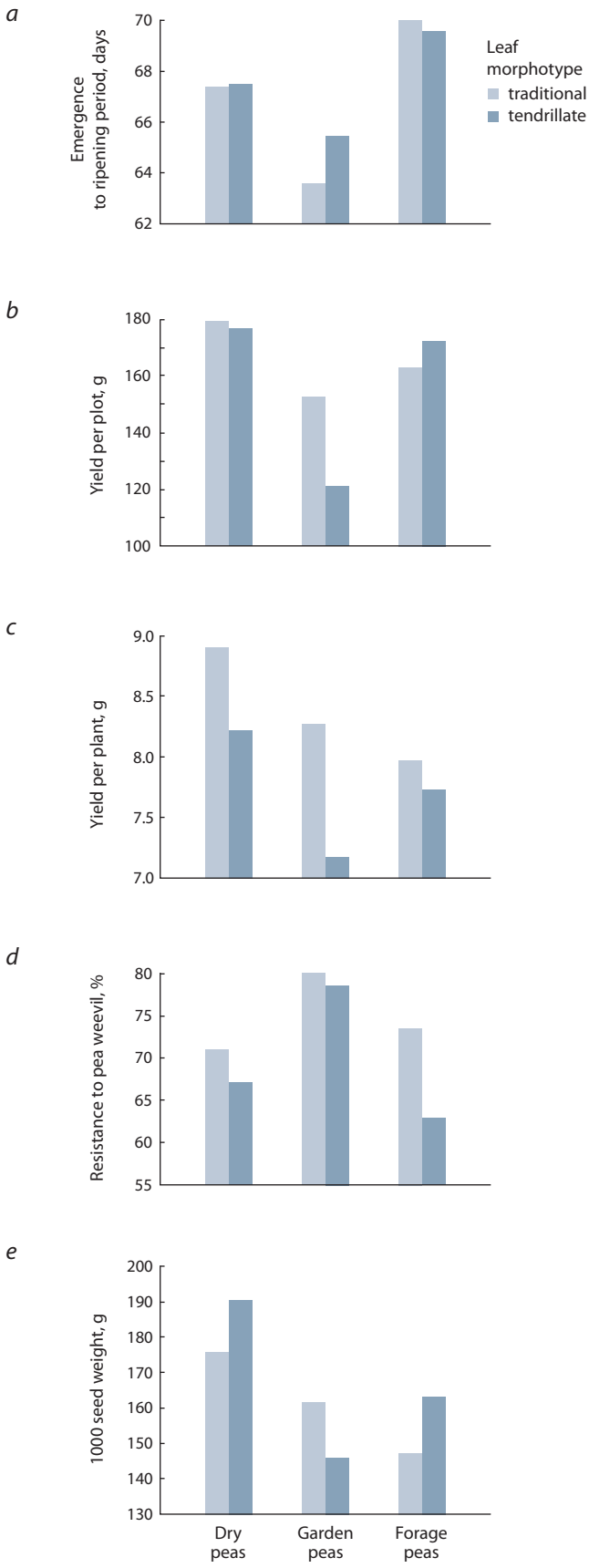


**Fig. 1.** Characteristics of pea accessions of different type of use with tendrillate and common leaf types.

*a*, emergence to ripening period; *b*, yield per plot; *c*, yield per plant; *d*, resistance to pea weevil; *e*, 1000 seed weight.

### Correlation analysis

The 3-year average values for the varieties were used to calculate the correlations of the economically important traits of the accessions with all the studied indicators (Table 4).

**Table 4.** Coefficients of correlation between economically important traits and other agrobiological indicators

| Trait | Emergence to ripening period (ER) | Yield per plot | Plant productivity | Resistance to pea weevil | 1000 seed weight |
|---|---|---|---|---|---|
| Sowing to emergence period (SE) | 0.00 | –0.41* | –0.36* | –0.07 | 0.06 |
| Emergence to flowering period (EF) | 0.87* | 0.01 | 0.05 | –0.29* | –0.12* |
| Flowering to ripening period (FR) | 0.53* | –0.03 | 0.05 | –0.12* | 0.01 |
| Emergence to ripening period (ER) | 1.00 | –0.01 | 0.01 | –0.31* | –0.09* |
| Yield per plot | –0.01 | 1.00 | 0.78* | –0.15* | 0.33* |
| Seed productivity per plant | 0.05 | 0.78* | 1.00 | 0.00 | 0.29* |
| Resistance to pea weevil | –0.31* | –0.15* | 0.00 | 1.00 | –0.18* |
| 1000 seed weight | –0.09* | 0.33* | 0.29 | –0.18* | 1.00 |
| Stem length | 0.33* | 0.32* | 0.35* | –0.27* | –0.15* |
| Number of pods per plant | 0.21* | 0.28* | 0.29* | –0.17* | –0.30* |
| Number of flowers per inflorescence | 0.20* | 0.00 | –0.07 | –0.20* | 0.00 |
| Maximum seed number per pod | –0.06 | –0.07 | 0.05 | 0.36* | –0.28* |
| Pod length | –0.01 | –0.04 | 0.16* | 0.18* | 0.26* |
| Pod width | –0.13* | 0.16* | 0.28* | 0.00 | 0.57* |
| Number of nodes to the first flower | 0.61* | 0.21* | 0.18* | –0.44* | 0.03* |

* Coefficients with 0.05 significance level.

Medium and strong correlations, i. e. those with the correlation coefficient ($r$) with the module greater than 0.3, have been analyzed.

Economically important characters include such quantitative traits as the growing season duration (ER), yield, seed productivity per plant, 1000 seed weight, and resistance to pea weevil. The relationships between traits in the groups of different type of use was the same for most characters, which makes it possible to characterize the sample as a whole (see Table 4).

The yield per plot positively correlated with the productivity per plant ($r$ = 0.78), 1000 seed weight ($r$ = 0.33), and the stem length ($r$ = 0.32), while there was a negative correlation with the sowing to emergence period ($r$ = –0.41). The first three relations are obvious, while the last is apparently explained by the fact that the long-emerging seeds have lower germination energy, which is an indicator characterizing simultaneousness and uniformity of seedlings emergence, hence good uniformity and survival of plants, which ensure their productivity.

On an average, plant productivity in the sample was positively associated with plant length ($r$ = 0.35) and negatively with SE ($r$ = –0.36). However, the ways of its formation were different in varieties of different types of use: in dry peas, the coefficient of relationship between seed productivity and the number of pods was $r$ = 0.31. In garden peas, the coefficient of relationship with the stem length was $r$ = 0.53, and 0.49 with the number of pods. In forage varieties, the coefficient of relationship with the stem length was $r$ = 0.40; it was 0.32 with the number of seeds per pod, 0.48 with the pod length, 0.47 with the pod width, and $r$ = 0.48 with 1000 seed weight.

The ER period duration correlated more with that of EF ($r$ = 0.87) than with the FR period duration ($r$ = 0.53).

The relationship between the stem length and the growing season ($r$ = 0.33) is explained by the fact that the bulk of the varieties are indeterminate; the longer a plant lives, the longer

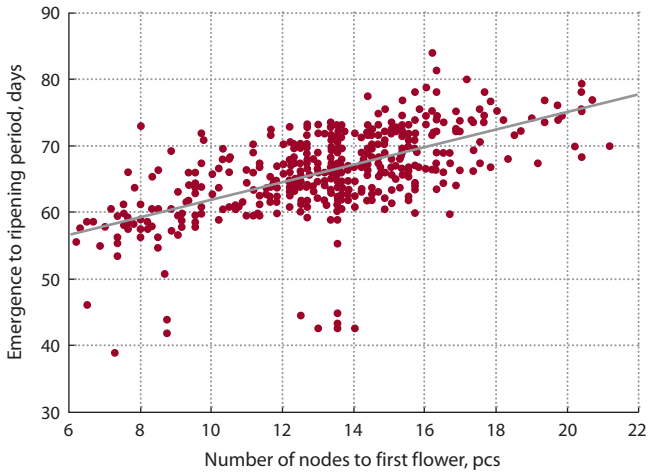


**Fig. 2.** The emergence to ripening period duration as a function of the number of nodes to the first flower.

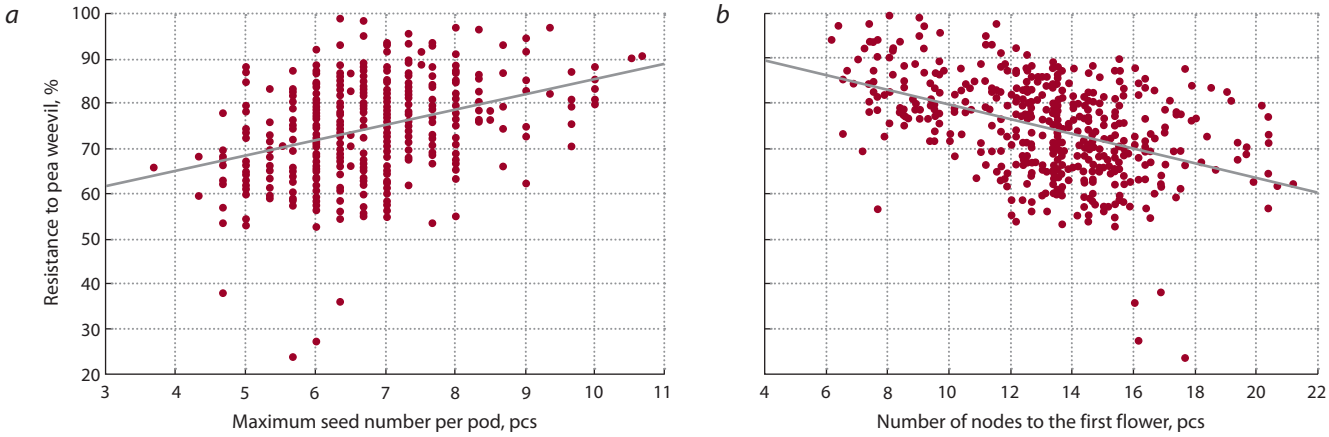


**Fig. 3.** Resistance to pea weevil as a function of seed number per pod (*a*) and number of nodes to the first flower (*b*).

it is. A correlation between the ER period and the number of nodes to the first flower (NN) was found to be $r = 0.61$, which confirms the role of NN as an indicator of early maturity (Makasheva et al., 1986). With an increase in NN by one node, the ER period increases by 1.3 days (Fig. 2). This regularity can be expressed by the formula $ER = 48.8 + 1.3 \times NN$.

In general, pea weevil resistance in the sample was positively associated with the number of seeds per pod ($r = 0.36$), and according to the type of use, $r$ was 0.32 for dry, 0.22 for garden, and insignificant 0.01 for forage peas. Pea weevil resistance was negatively associated with NN ($r = –0.44$); according to the type of use, $r$ was –0.29 for dry, –0.40 for garden, and insignificant (–0.05) for forage peas. The bigger the number of unproductive nodes, i. e. the later a variety ripens, the fewer the number of healthy seeds due to a greater damage by the pest. Early ripening accessions avoid the flight of insects; therefore, they get less damaged (Fig. 3). The coefficient of relationship with the growing season duration (ER) according to the type of use was $r = –0.18$ for dry peas, –0.32 for garden, and 0.02 for forage peas.

The percentage of healthy seeds was higher when seeds were green (79.3 %), smaller for yellow seeds (68.8 %), in both dry (73.7 *vs.* 68.5 %) and garden peas (80.4 *vs.* 71.9 %). This was also evidenced by the fact of a stronger pea weevil resistance in garden pea varieties with predominantly green seeds.

There was a positive relation between 1000 seed weight and the pod width ($r = 0.57$). This dependence was observed in accessions of all types of use and demonstrated the strongest correlation, that is, with $r = 0.43$ for dry peas, 0.65 for garden, and 0.71 for forage peas. With an increase in the pod width (PW) by 1 cm, 1000 seed weight (W1000) increases by an average of 109 g. This dependence can be expressed by the formula $W1000 = 8.7 + 109.0 \times PW$.

Seed productivity per plant is one of the most important traits for the pea yield structure and, together with 1000 seed weight, it determines the individual productivity of plants. This trait is known as one of the most variable in different crops, including peas.

The highest coefficient of year-to-year variation was observed for the seed yield per plot (55.5 % per sample, on an average), while the yield per plant was slightly more stable (36.6 %). The number of pods (28.4 %) and the stem length (14.4 %) demonstrated a greater stability. The number of flowers per inflorescence (2.2 %), NN (4.1 %), pod length (4.4 %), pod width (4.5 %), the maximum number of seeds per pod (7.2 %), and 1000 seed weight (10.3 %) were most stable over the years.

Polymorphism within the sample was subjected to the principal components analysis (PCA). According to the scree criterion, four factors, which explain 69.6 % of the total variance, were distinguished (Table 5).

The first factor (explaining 24.5 %) is associated with the ER period duration and such associated characters as the stem length and the number of nodes to the first flower. It can be called the plant vegetation factor. The second factor (18.2 %) is associated with the yield per plot, seed productivity per plant, 1000 seed weight, and the pod width. The third factor (13.9 %) is the pod length, while the fourth (12.1 %) is the maximum number of seeds per pod.

The first factor distinguishes the groups of garden and forage type of use (Fig. 4, *a*), which are opposed in terms of the ER period duration, stem length, and NN. Factors 2 and 3 determine no visual differences between types of use, and according to the fourth factor, the garden type accessions with the maximum number of seeds per pod are contrasted to dry peas with the minimum number of seeds (see Fig. 4, *b*).

Previously, we studied a sample of 112 pea accessions from the VIR collection in the conditions of the Leningrad Province and carried out a discriminant analysis of the obtained data, which made it possible to identify the traits by which an accession can be attributed to a particular group of economic use (Semenova, Sobolev, 2009). The traits that were most significant for the statistical attribution of an accession to a variety of use type were such qualitative traits as the seed and pod shape, the presence of anthocyanin in the flower, as well as the range of variability of such quantitative traits as the number of pods, the number of productive nodes per plant, and 1000 seed weight. Like in the present study, the largest seeds were observed in dry pea varieties, smaller ones in garden peas, and the smallest in forage varieties (195.9, 184.3

**Table 5.** Four factor loadings in PCA

| Trait | Factor 1 | Factor 2 | Factor 3 | Factor 4 |
|---|---|---|---|---|
| Sowing to emergence period (SE) | –0.337 | –0.403 | 0.423 | 0.297 |
| Emergence to flowering period (EF) | **0.727** | –0.201 | 0.505 | –0.216 |
| Emergence to ripening period (ER) | 0.694 | –0.206 | 0.505 | –0.181 |
| Yield per plot | 0.407 | **0.703** | –0.293 | 0.110 |
| Seed productivity per plant | 0.358 | **0.773** | –0.190 | –0.076 |
| Percentage of seeds not damaged by pea weevil | –0.516 | 0.059 | –0.220 | –0.486 |
| 1000 seed weight | –0.089 | 0.600 | 0.324 | 0.613 |
| Stem length (average) | **0.710** | 0.193 | –0.214 | –0.202 |
| Number of pods per plant (average) | 0.611 | –0.054 | –0.422 | –0.151 |
| Number of flowers per inflorescence (average) | 0.235 | –0.234 | 0.264 | 0.190 |
| Maximum number of seeds per pod | –0.327 | 0.176 | 0.236 | **–0.790** |
| Pod length | –0.326 | 0.491 | 0.600 | –0.401 |
| Pod width | –0.224 | 0.692 | 0.423 | 0.134 |
| Number of nodes to the first flower (average) | **0.814** | –0.004 | 0.298 | 0.000 |
| Expl. Var | 3.553 | 2.546 | 1.940 | 1.701 |
| Prp. Totl | 0.254 | 0.182 | 0.139 | 0.121 |

Note. The highest loads of the factors are boldfaced.

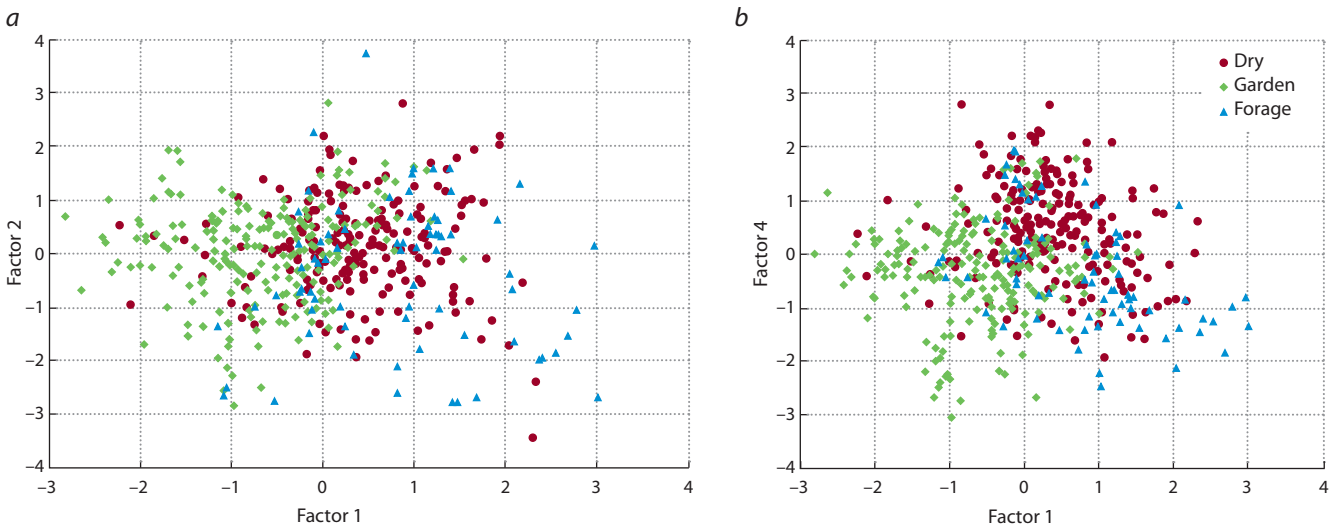


**Fig. 4.** Distribution of 494 pea accessions within the domain of factors 1–2 (*a*) and 1–4 (*b*).

and 150.5 g, respectively, in the current study, and 181.2, 158.9 and 150.2 g in the previous). Interestingly, the average number of pods per plant was the same in both experiments, i. e. 12.2 pcs, with the largest number for forage peas (14.6 in the present study and 16.5 in the cited work), medium for dry pea varieties (12.5 *vs.* 11.4), and the smallest for garden peas (11.0 *vs.* 8.6). Similar results with the current ones were obtained on the basis of the "number of seeds in a bean", despite the fact that the average number was calculated in the cited work, and we have the average of the maximum number of seeds in a bean. The highest value was noted in vegetable varieties (5.0 in the cited and 7.2 in this work), the average in fodder varieties (4.8 and 6.3) and the minimum in cereals (4.4 and 6.6).

The similarity of the results obtained from the studies carried out in a wide range of ecological and geographical conditions in both experiments indicated that the listed traits can be regarded as differentiating ones when attributing pea accessions to one or another type of economic use.

The RAPD marking of the above-mentioned phenotyped sample of 112 accessions revealed the genetic proximity of varieties within the limits of different types of use, and their distance from each other. The dendrogram of genetic kinship shows the tightly grouped garden pea varieties, and compactly located forage varieties, while both groups were considerably remote from each other. Dry pea varieties, which show genetic affinity to both groups, were initial for both of them (Vishnyakova et al., 2011). Like in the present study, it was established that both dry and forage pea varieties contain transitional forms that occupy an intermediate position and can be called grain-fodder varieties.

In the work of French scientists who studied a sample containing 148 modern pea varieties of mainly West European origin and primitive forms using 121 protein markers and PCR analysis, the sample was also differentiated by the types of use into dry, forage, and grain fodder peas. It was possible to trace the main tendencies in the West European breeding over the past twenty years of the 20th century, such as an increase in seed size, predominance of white-flowered and semi-leafless forms, and an increase in cold resistance required for sowing in autumn, which is widely practiced in European countries (Baranger et al., 2004).

## Conclusions

A complex of phenotypic traits that significantly differed in pea varieties of different type of economic use (dry, forage and garden) has been revealed. These include the stem length, the number of pods per plant, the number of nodes to the first flower, the number of flowers per inflorescence, the maximum number of seeds per pod, pod length, and a narrower pod of forage peas compared to that of dry and garden peas. The average values of these traits were recorded for peas of all types of use. The largest number of distinctive traits was observed in garden pea varieties, which demonstrated their maximum difference from forage varieties. Dry pea accessions occupy an intermediate position and have a number of traits that overlap with those of forage ones.

A complete description of the material according to the features listed in the article was published in 2020 in the "Catalog of the VIR Global Collection", issue 910 (Semenova et al., 2020).

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# Promising genetic sources for the creation of varieties of durum spring wheat in Western Siberia

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**Abstract.** The study, expansion and preservation of the genetic diversity of the source material, and its purposeful use in hybridization is the basis for the creation of adaptive varieties of durum spring wheat that are resistant to biotic and abiotic factors of the environment of Western Siberia. The objects of research were samples of durum spring wheat. Over the years of research (2000–2020), about 3 thousand samples were worked out from the world gene pool of various countries and regions: from the collection of the VIR, the gene pool from Mexico (CIMMYT) within the framework of the agreement and cooperation program (2000–2007), from 2000 to the present time under the program of the Kazakh-Siberian Spring Wheat Breeding Network (KASIB), from other scientific institutions of Russia in exchange activities. Using generally accepted techniques, the obtained material was studied for a complex of traits: yield, adaptability, grain quality, resistance to diseases. In the cycle of studying the gene pool from CIMMYT, 50 genotypes were identified in terms of yield at the level of the Omskaja jantarnaja standard, 276 grains by test weight, 131 samples by pasta color, 131 samples by resistance to hard smut, and 112 by resistance to powdery mildew. Almost all samples were not affected by leaf rust. The study set showed high sensitivity to extreme conditions and most forms of interest in quality and disease resistance were low-productive in our environment. In KASIB nurseries, 29 samples were identified in terms of yield and adaptability, 29 samples in terms of grain quality, 21 in terms of resistance to diseases, including 8 resistant to stem rust. In the set of varieties received from the VIR, 15 genotypes were adaptive, 16 had high grain quality, 11 were resistant to stem rust. In the breeding material, 17 samples of the local population resistant to stem rust (6 of them were comprehensively resistant) and 25 race-resistant to Ug99 were identified. The genotypes identified as a result of research are of interest as sources of valuable traits.
Key words: durum wheat, breeding, variety, sample, genotype, yield, grain quality, disease resistance.

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# Перспективные генетические источники для селекции яровой твердой пшеницы в Западной Сибири

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**Аннотация.** Изучение, расширение и сохранение генетического разнообразия исходного материала и его целенаправленное использование в гибридизации являются основой для создания адаптивных сортов твердой яровой пшеницы, устойчивых к биотическим и абиотическим факторам среды Западной Сибири. Объектами исследований служили образцы твердой яровой пшеницы. За годы исследований (2000–2020) проведен скрининг более 3000 образцов из мирового генофонда разных стран и регионов: из коллекции ВИР, банка генетических ресурсов CIMMYT (Мексика) в рамках договора и программы сотрудничества (2000–2007 гг.), с 2000 г. по настоящее время по программе Казахстанско-Сибирской сети по улучшению яровой пшеницы (КАСИБ), из других научных учреждений России в порядке обмена селекционным материалом. Полученный материал был изучен с использованием общепринятых методик по комплексу признаков: урожайности, адаптивности, качеству зерна, устойчивости к болезням. При исследовании в 2000–2007 гг. генофонда из CIMMYT по урожайности на уровне стандарта Омская янтарная было выделено 50 генотипов, по натуре зерна – 276, по цвету макарон – 131, по устойчивости к твердой головне – 131, мучнистой росе – 112. Почти все образцы не поражались бурой ржавчиной. Изученный набор показал высокую чувствительность к экстремальным условиям; большинство форм, представляющих интерес по качеству и устойчивости к болезням, были низкопродуктивными в наших условиях. В питомниках КАСИБ по урожайности и адаптивности выделено 29 образцов, по качеству зерна – 29, устойчивости к болезням – 21, в том числе к стеблевой ржавчине – 8. В наборе сортов, поступивших из ВИР,

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выявлено 15 адаптивных генотипов, 16 – с высоким качеством зерна, 11 – устойчивых к стеблевой ржавчине. При оценке селекционного материала выделено 17 образцов, устойчивых к стеблевой ржавчине местной популяции (6 из них комплексно устойчивы к бурой, стеблевой ржавчине, мучнистой росе), и 25 резистентных к расе Ug99. Выделенные в результате исследований генотипы представляют интерес как источники ценных признаков.
Ключевые слова: твердая пшеница; селекция; сорт; образец; генотип; урожайность; качество зерна; устойчивость к болезням.

## Introduction

Hybridization with targeted selection of parental forms remains one of the most important ways to create durum wheat varieties. Therefore, the study of the source material is the main factor in successful breeding. The doctrine of the source material was developed by K.A. Flaksberger (1934), N.I. Vavilov (1935) and was further developed in the works of many researchers.

The main bank of genetic resources is the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) with its branches and bases in various climatic zones of the country, the number of which, unfortunately, has greatly decreased in recent years (Lyapunova, Andreeva, 2020). From 2000 to 2007, a large number of samples was received from the International Center for the Improvement of Maize and Wheat (CIMMYT, Mexico) within the collaboration under the agreement and cooperation program; from 2000 to the present time, samples have been received under the program of the Kazakh-Siberian Network for the Improvement of Spring Durum Wheat (KASIB). At the same time, the basis for the creation of varieties is the breeding material obtained with the involvement of samples from CIMMYT and exposed to natural selection in local soil and climatic conditions.

In recent years, interest in local and ancient varieties of durum wheat has increased (Pagnotta et al., 2005; Kan et al., 2014; Peneva, Lyapunova, 2020), as they are characterized by unique features and, above all, resistance to a number of adverse environmental factors that have a major impact on plant survival, and to some races of local populations of fungal and bacterial diseases.

In the last century, many works were devoted to the evaluation of the source material carried out in the conditions of Siberia, the Volga region, Ukraine, Kazakhstan, Uzbekistan and other regions of the former Soviet Union (Evdokimov, 2006). In recent years, the trend has been reflected in the works of domestic scientists engaged in the directions of selection to increase yields, adaptability, grain quality and disease resistance (Ziborov, Rozova, 2012; Evdokimov et al., 2017; Malchikov et al., 2018; Mukhitov, Timoshenkova, 2018; Samofalova et al., 2018; Dorokhova, Kopus, 2020; Rozova et al., 2020; Malchikov, Myasnikova, 2021;Yusov et al., 2021).

The need to study the collection material in Siberian conditions lies in the fact that the behavior of the genotype in different environmental conditions is far from the same. At the same time, the study of the source material should be carried out taking into account the main directions of breeding: further increase in yield and adaptive potential, quality of grain and pasta, resistance to diseases and stability of agronomically important traits. For the Omsk region, with sharp fluctuations in meteorological factors during the growing season and by year, such stability is of paramount importance.

Preservation, study and replenishment of the gene pool with new forms is relevant in the purposeful screening of source material in breeding programs (Likhenko et al., 2014). This will make it possible to make a certain contribution to the creation of varieties that meet the requirements of agricultural production and the implementation of the scientific program “Bread of Russia” in 2022–2027, which is aimed at accelerating, stabilizing the selection process and, ultimately, ensuring the country’s food security.

The main aim is to identify promising sources of agronomically important traits for the creation of varieties of durum spring wheat in the conditions of Western Siberia.

## Material and methods

The objects of research were samples from the VIR collection. From 2000 to 2007, a large number of samples was received from CIMMYT within the collaboration under the agreement and cooperation program, from 2000 to the present time samples have been received through the Kazakh-Siberian Network (KASIB). In recent years, varieties and breeding material have been obtained from other scientific institutions of Russia (Altai Research Institute of Agricultural Sciences, Samara Research Institute of Agricultural Sciences, Research Institute of Agricultural Sciences of the South-East, Voronezh FASC named after Dokuchaev) as part of an exchange.

The principle of the approach to the development of the material was as follows: after the first year of study, samples with low values for a set of indicators were rejected, and the selected genotypes were further tested in the second year. For three years, only promising samples were tested. The number of genotypes studied was more than 3 thousand.

A significant part of the gene pool was from North America – Mexico, USA, Canada; Russia, CIS countries – Kazakhstan, Azerbaijan; Ukraine; European countries – Italy, Spain, Portugal, France; a small number of samples came from the Middle East – Turkey, Israel, Yemen; Central, East and South Asia – Iran, China, India; North Africa – Algeria, Morocco, Tunisia, Ethiopia; South America – Chili (Table 1). The bulk of the material from the North American

**Table 1.** Volume of study of the collection material of spring durum wheat (2000–2020)

| Region | Years | | | | Total |
|---|---|---|---|---|---|
| | 2000–2005 | 2006–2010 | 2011–2015 | 2016–2020 | |
| Russia | 232 | 219 | 227 | 159 | 837 |
| CIS countries | 151 | 50 | 91 | 57 | 349 |
| Europe | 6 | 68 | 22 | 6 | 102 |
| Middle East | 5 | 5 | 4 | 12 | 26 |
| North Africa | 0 | 14 | 10 | 1 | 25 |
| North America | 2853 | 569 | 88 | 14 | 3524 |
| South America | 0 | 2 | 0 | 0 | 2 |
| Total | 3247 | 927 | 442 | 249 | 4865 |

continent came from Mexico (CIMMYT). In 2000–2007, with an annual intake of 3 nurseries (IDYN – International Durum Yield Nursery, EDUIT – Elite Durum Unrepricield Yield Treals, IDSN – International Durum Screening Nursery), the total volume was 2711 samples. Under the KASIB program, 210 genotypes were studied, and 186 genotypes were studied from the VIR revenues. In addition, samples were studied at the final stages of the selection process (preliminary and competitive variety testing).

To study the gene pool of durum wheat, a collection nursery was annually laid in the breeding stationary of the durum wheat breeding laboratory in accordance with the guidelines of the VIR (Merezhko et al., 1999), as well as a nursery of competitive and environmental tests according to the GSU methodology (Methodology of State Variety Testing…, 2019). In 2000–2008, the Omskaya jantarnaya variety was used as a standard, and since 2009 an additional standard has been introduced – the Jemthujina Sibiri.

Sowing was carried out on plots with an accounting area of 2–3 $m^2$ (collection), 10 $m^2$ (competitive, environmental test) in 2–4 repetitions with the SSFC-7 planter. Harvesting of plots was carried out by the combine HEGE 125.

Indicators of the test weight of grain, pasta properties, susceptibility to major diseases were determined by generally accepted methods (Kolmakov, 2007; Koishybayev, 2018). To assess susceptibility, the CIMMYT scale was used: 0 – immune, there are no signs of the disease; R – stable, chlorous spots are formed, occupying up to 5–10 % of the leaf surface (on the Stekman scale, 1 point); MR – medium resistance, pustules are small, there are chlorotic zones occupying no more than 10–25 % (2 points); MS – medium susceptibility, pustules are small, occupy up to 40–50 % of the leaf surface (3 points); S – high susceptibility, pustules are large, occupy up to 50–100 % of the leaf surface (4 points).

Mathematical processing of the results was carried out according to B.A. Dospekhov (2012) using a package of applied statistical programs Microsoft Excel. The parameters of ecological plasticity were calculated according to S.A. Eberhart, W.A. Russel in the presentation of V.A. Zykin and co-authors (Zykin et al., 2011). Analysis of principal components (Principal compatible analysis, PCA) was carried out using the R version 4.0.3 package.

## Results and discussion

### Yield and adaptability

The studied samples in nurseries from CIMMYT in terms of yield were significantly inferior to the Omskaya jantarnaya standard. The average yield in nurseries ranged from 51.6 to 87.5 %. The number of genotypes at or above the standard level in kennels 32 IDYN, 37 IDYN, 38 IDYN, 35 EDUYT was 1–2, in 33 IDYN, 34 IDYN, 36 IDYN – 3–4, in 30 EDUYT, 34 EDUYT, 36 EDUYT – 5–6, and only in 32 EDUYT – 18 samples. In nurseries 35 IDYN, 31 EDUYT, 33 EDUYT, not a single sample formed a yield at the level of the Omskaya jantarnaya variety.

In terms of yield and adaptability in these nurseries, Anade 1/Tarro 1//Lican (32 IDYN), Nehama 15/Brisina 2//Plata 9 deserve attention (30 EDUYT), SN Turk MI83-84/Nigris5; GA//2* Chen/Altar 84; Cado/Boomer 33; Dipper 2/Bushen 3; Himan 9/Lotus 1; Crake 10/Rissa; Chen/Altar 84/3/Hui//Poc//Bub/Rufo/4/Fnfoot (32 EDUYT), Cndo/Vee//7*Plata 8/3/Plata_L/Snm//Plata 9; Vanrrikse 14/Plata 6//Green 17; Plata 22/3/Magh 72/D67.2//FGO (34 EDUYT), Arment//Srn_3/Nigris 4/3/Canelo 9.1 (35 EDUYT), Minimus_6/Plata 16; Ajaia_16//Hora/JRO (36 EDUYT). Among those presented in Table 2, 15 genotypes combine yield with high nature, 5 with the color of pasta, 8 with resistance to hard smut, 6 to powdery mildew, 25 to leaf rust.

Among the studied varieties of the VIR collection in 2000–2003, the following were identified in terms of yield: k-59881, k-60388, k-60364, k-60366, k-60413, k-61303, the following samples had an advantage and the color of the

**Table 2.** Characteristics of the high-yielding samples from CIMMYT nurseries

| Samples | Yield to standard, % | Test weight, g/l | Pasta color, ball | Damage, % | | | Nursery, year |
|---|---|---|---|---|---|---|---|
| | | | | Hard smut | Powdery mildew | Leaf rust | |
| Anade 1/Tarro 1//Lican | 105.9 | 784 | 3.5 | 1.4 | 25.0 | 0 | 32 IDYN 2000–2001 |
| Nehama 15/Brisina 2//Plata 9 | 114.5 | 745 | 3.7 | 3.9 | 60.0 | 0 | 30 EDUYT 2000–2001 |
| Dipper 2/Bushen 3 | 107.9 | 725 | 3.7 | 0 | 50.0 | 0 | |
| Corm/Rufo//Ru3/Rissa/4/Yazi/5/Gutros 1 | 106.3 | 727 | 4.0 | 2.1 | 50.0 | 0 | |
| Dipper 2/Bushen 3 | 103.0 | 802 | 3.6 | 6.3 | 50.0 | 31.2 | 34 IDYN 2002 |
| Plata 1/SNM//Plata 9 | 101.0 | 774 | 3.5 | 5.9 | 20.0 | 27.4 | |
| Bejah 6/SLA2 | 103.0 | 798 | 3.9 | 0 | 60.0 | 0 | 32 EDUYT 2002 |
| Cado/Boomer 33 | 114.0 | 814 | 3.6 | 8.5 | 50.0 | 0 | |
| Chen/Altar 84/3/Hui//Poc//Bub/Rufo/4/Fnfoot | 103.0 | 800 | 4.1 | 12.3 | 50.0 | 0 | |
| Crake 10/Rissa | 109.0 | 800 | 3.8 | 0 | 60.0 | 0 | |
| Dipper 2/Bushen 3 | 113.0 | 786 | 3.8 | 0 | 20.0 | 0 | |
| GA//2* Chen/Altar 84 | 118.0 | 785 | 3.5 | 9.4 | 30.0 | 0 | |
| Kucuk | 109.0 | 793 | 4.1 | 0 | 30.0 | 0 | |
| Himan 9/Lotus 1 | 114.0 | 795 | 3.8 | 7.2 | 60.0 | 0 | |
| SN Turk MI83-84/Nigris5 | 133.0 | 784 | 3.9 | 0 | 30.0 | 0 | |
| SN Turk MI83-84/Nldkls5 | 112.0 | 787 | 4.2 | 0 | 20.0 | 0 | |
| Plata 22/3/Magh 72/D67.2//FGO | 104.0 | 758 | 3.2 | 5.5 | 20.0 | 0 | 34 EDUYT 2004 |
| Plata_L/Snm//Plata_9/3/Tilo_L/Lotus_4 | 99.0 | 787 | 3.5 | 12.5 | 50.0 | 0 | |
| Cndo/Vee//7*Plata 8/3/Plata_L/Snm//Plata 9 | 104.0 | 763 | 3.6 | 9.2 | 20.0 | 0 | |
| Vanrrikse 14/Plata 6//Green 17 | 102.0 | 784 | 3.5 | 6.4 | 40.0 | 0 | |
| Green 18/Bushen 4//Porto 3 | 96.6 | 745 | 3.1 | 7.5 | 30.0 | 0 | 37 IDYN 2005 |
| Arment//Srn_3/Nigris 4/3/Canelo 9.1 | 101.0 | 770 | 3.2 | 0 | 50.0 | 0 | 35 EDUYT 2005 |
| Auk/Guil//Green/3/Adamar/4/Rascon 37/Tarro 2 | 100.0 | 761 | 3.2 | 8.2 | 40.0 | 0 | |
| Bcris/Bicum//Llareta Inia/3/Dukem 12/2*Rascon_21 | 97.0 | 730 | 3.2 | 9.5 | 60.0 | 0 | 38 IDYN 2007 |
| Minimus 6/Plata 16 | 124.3 | 769 | 3.3 | 4.2 | 40.0 | 0 | 36 EDUYT 2007 |
| Ajaia 16//Hora/JRO | 111.4 | 780 | 3.3 | 9.4 | 30.0 | 0 | |
| Ajaia 16//Hora/JRO | 107.1 | 764 | 3.2 | 8.2 | 40.0 | 0 | |
| Omskaya jantarnaya standard | 100.0 | 771* | 4.0* | 13.5* | 50.0* | 1.0* | |
| $LSD_{05}$ for yield in nurseryes 8.2–9.8 % | | | | | | | |

* Average data on kennels are given.

pasta: k-59881, k-60388, k-60364. All these specimens were resistant to lodging, due to the optimal ratio of anatomical features of the stem. In the 2007–2008 cycle, short-stemmed samples from Europe, the United States and Canada were tested. Due to the shortened lower internodes, they are highly resistant to lodging, their disadvantages are low drought resistance and yield. However, 4 samples k-62658, k-63126, k-63160, k-64353 formed a yield at the level of the Omskaya

**Table 3.** Characteristics of the highest productivity samples from the VIR collection

| Number of catalogue | Origin | Yield to standard, % | Pasta color, ball | Test weight, g/l | Long of stem, cm | Years of study |
|---|---|---|---|---|---|---|
| Omskaya jantarnaya | Standard | 100.0 | 3.9 | 775 | 90.0 | 2000–2008 |
| k-59881 | Russia | 103.1 | 3.9 | 768 | 114.0 | 2000–2003 |
| k-59888 | | 98.8 | 3.6 | 773 | 126.0 | |
| k-60364 | Ukraine | 100.5 | 3.8 | 776 | 108.0 | |
| k-60366 | Kazakhstan | 100.5 | 3.4 | 765 | 99.0 | |
| k-60388 | Russia | 108.8 | 3.7 | 758 | 110.0 | |
| k-60413 | Syria | 108.8 | 3.5 | 754 | 118.0 | |
| k-61303 | USA | 117.4 | 3.7 | 776 | 102.0 | |
| k-61631 | Canada | 93.8 | 3.5 | 770 | 60.0 | |
| k-61645 | Syria | 92.3 | 3.4 | 762 | 68.0 | |
| k-61645 | | 92.3 | 3.4 | 748 | 68.0 | |
| $LSD_{05}$ | | 8.4 | 0.13 | 10.3 | 8.5 | |
| k-62658 | USA | 101.2 | 3.4 | 761 | 64.6 | 2007–2008 |
| k-63126 | France | 104.3 | 3.1 | 772 | 54.6 | |
| k-63160 | | 105.4 | 3.2 | 753 | 39.8 | |
| k-64353 | Canada | 118.5 | 3.5 | 760 | 54.9 | |
| $LSD_{05}$ | | 10.2 | 0.10 | 12.5 | 5.3 | |
| Jemthujina Sibiri | Standard | 100.0 | 3.2 | 778 | 83.0 | 2009–2020 |
| k-6386 | USA | 81.3 | 3.3 | 760 | 80.0 | 2009–2012 |
| k-61619 | Ukraine | 105.4 | 3.0 | 769 | 78.0 | |
| k-63821 | | 87.7 | 3.6 | 770 | 76.0 | |
| k-64953 | Russia | 91.9 | 2.9 | 762 | 88.0 | |
| Sladunitsa | Ukraine | 91.0 | 3.2 | 777 | 85.0 | |
| $LSD_{05}$ | | 8.5 | 0.10 | 15.2 | 7.6 | |
| k-64488 | Russia | 95.9 | 4.2 | 763 | 109.0 | 2019–2020 |
| k-66294 | | 85.5 | 3.5 | 794 | 100.4 | |
| k-66519 | | 80.6 | 3.5 | 810 | 95.8 | |
| k-66675 | | 92.3 | 3.4 | 769 | 84.3 | |
| k-66886 | | 76.2 | 3.3 | 813 | 103.4 | |
| k-66887 | | 76.0 | 3.3 | 812 | 89.9 | |
| $LSD_{05}$ | | 9.8 | 0.12 | 11.3 | 8.3 | |

jantarnaya standard and above (with an increase of 1–18 %), but they do not represent breeding value in terms of grain quality (Table 3).

In 2009–2012, out of 62 genotypes, only one (k-61619) formed a yield above the Jemthujina Sibiri standard by 18.5 %, but by the test weight of the grain and the color of pasta, the indicators were low. When studied in 2019–2020, the most productive forms had a yield of 92–96 % in relation to the yield level of the Jemthujina Sibiri standard – k-64488, k-66675.

**Table 4.** Sources of high productivity and adaptability from KASIB nurseries

| Variety | Origin | Years of study | Nursery | Yield, Omsk, rank | Rank of adaptability | bi | $\sigma^2 d_i$ |
|---|---|---|---|---|---|---|---|
| 242.93 | Karabalyk AES | 2000 | KASIB 1 | 1 | 16 | 1.61 | 0.07 |
| Omskaya jantarnaya standard | Omsk ASC | | | 3 | 2 | 0.70 | 0.70 |
| G.430-88 | Karabalyk AES | 2001 | KASIB 2 | 2 | 2+ | 1.89 | 0.16 |
| Kargala 3 | Aktobe AES | | | 3 | 15 | 0.55 | 0.63 |
| Jemthujina Sibiri | Omsk ASC | 2003–2004 | KASIB 4–5 | 3 | 1+ | 0.97 | 0.13 |
| Kargala 30 | Aktobe AES | | | 1 | 3+ | 0.85 | 0.13 |
| G.94-9-1 | Omsk ASC | 2005–2006 | KASIB 6–7 | 3 | 3+ | 1.04 | 0.07 |
| G.94-94-13 | | | | 2 | 1+ | 0.93 | 0.22 |
| Subastrale 489 | Karabalyk AES | | | 2 | 2+ | 1.02 | 0.15 |
| Altyn dala | Karabalyk AES | 2007–2008 | KASIB 8–9 | 1 | 2+ | 1.06 | 0.08 |
| G.462 (Pamyaty Yanchenko) | Altai ARI (FASCA) | | | 3 | 3+ | 1.12 | 0.09 |
| G.97-49-1 | Omsk ASC | 2009–2010 | KASIB 10–11 | 3 | 1+ | 1.21 | 0.04 |
| G.98-42-1 | | | | 1 | 2+ | 1.06 | 0.09 |
| Kargala 69 | Aktobe AES | | | 2 | 5 | 0.89 | 0.14 |
| Omskiy izumrud | Omsk ASC | 2011–2012 | KASIB 12–13 | 1 | 3+ | 0.99 | 0.11 |
| Omskiy lazurit | | | | 2 | 5+ | 1.05 | 0.13 |
| G.677 | FASCA | | | 2 | 4+ | 1.08 | 0.06 |
| G.628 | FASCA | 2013–2014 | KASIB 14–15 | 1 | 14 | 1.01 | 0.07 |
| Omskiy corall | Omsk ASC | | | 3 | 3+ | 1.24 | 0.04 |
| G.748 | FASCA | 2015–2016 | KASIB 16–17 | 1 | 10 | 1.71 | 0.17 |
| L.1307 d54 | Samara ARI | | | 2 | 5 | 0.92 | 0.18 |
| Leuc.1469d-21 | | | | 3 | 1+ | 1.06 | 0.22 |
| G.03-20-18 | Omsk ASC | | | 6 | 2+ | 0.92 | 0.08 |
| G.178-05-2 | SPC GP named after Baraev | 2017–2018 | KASIB 18–19 | 3 | 3+ | 0.99 | 0.29 |
| G.05-42-12 | Omsk ASC | | | 4 | 4+ | 1.12 | 0.33 |
| L.1506-36 | Samara ARI | | | 2 | 1+ | 1.12 | 0.33 |
| G.08-67-1 | Omsk ASC | 2019–2020 | KASIB 20–21 | 2 | 5 | 1.12 | 0.07 |
| L.1970d5 | Samara ARI | | | 3 | 8 | 1.00 | 0.21 |
| G.924 | FASCA | | | 6 | 1+ | 1.10 | 0.30 |
| Melyana | Orenburg ARI | | | 1 | 9 | 0.98 | 0.09 |

Note. G – hordeiforme, Leuc. – leucurum, L – line. AES – Agricultural Experimental Station; ASC – Agrarian Scientific Center; ARI – Agricultural Research Institute; FASCA – Federal Altai Scientific Center for Agrobiotechnology; SPC GP named after Baraev – Scientific and Production Center of Grain Farming named after A.I. Barayev.

The Kazakh-Siberian Spring Wheat Improvement Network (KASIB), established in 1999, provides for the exchange of genetic material and the testing of samples over a vast territory of Russia and the Republic of Kazakhstan (43–55° N, 55–85° E) with an annual precipitation range of 250–500 mm. The main advantage of this project is that within one year when tested in different ecological points, and there are 6–8 of them for durum wheat, it is possible to evaluate genotypes by a complex of traits: adaptability, drought resistance, stability and purposeful inclusion of them

in the breeding process as sources of the main economically valuable traits.

Table 4 presents the most productive varieties and lines in the conditions of Omsk that formed a high average yield for all points of variety testing of the KASIB network, created in Russia and Kazakhstan. Among them, 18 have a rank of 1–3 in terms of average yield and are adaptive forms. According to the Eberhart–Russell test, genotypes 242.93, G.430-88, (Karabalyk Agricultural Experimental Station, AES), G.97-49-1, Omskiy corall (Omsk Agrarian Scientific Center, ASC), G.748 (FASCA) are intense – bi = 1.24–1.89, extensive include Omskaya jantarnaya (Omsk ASC), Kargala 3, Kargala 30, Kargala 69 (Aktobe AES) – bi = 0.55-0.89. Variance deviations from the regression line ($\sigma^2$di) indicate that they form a stable yield (see Table 4).

**Grain quality and pasta properties**

Among the CIMMYT material by test weight of grain 276 samples were allocated, by the color of pasta – 131 samples. The studied set showed a high response to extreme stressors and most of the forms of interest in grain quality and resistance to diseases in the conditions of the Omsk region were low-productive. Therefore, 56 genotypes are of paramount importance in terms of grain and pasta quality. By test weight grains and pasta quality in CIMMYT nurseries deserve attention those in 32 IDYN – Topdy 18/ Focha 1//Altar 84 (test weight 807 g/l, 4.1), Dipper 2/ Bushen 3, Rascon 37/2* Tarro 2; in 30 EDUYT – Ajata/ Bichena, Yavaus/Tez//Altar 84, Wizza 23/Cona, Fulvous 1/ Meowl 13, Dusky 12/Bushen 4, Cham 3/Comdk//Ajata; in 34 IDYN – Dipper 2/Bushen 3, Yel/Bar/3/Garza/AFN, Rascon 39/Tilo 1; in 32 EDUYT – Chen/Altar 4/3/Hui/…, Eupoda 3/Suv 2//Minimus, Kucuk, SN Turk MI83-84/ Nldkls5; in 36 IDYN – Tarro 1/2* Yual 1/Ajata 13, Duck 2// Cham 3/3/Canelo 9; in 34 EDUYT – samples Plata 1/SND// Plata 9, SN Sturk M 183-84503/Lotus 14, GS/CRA/SBA 81; in 38 IDYN – 1A.1D5+10-/2*WB881, Skest/Krm//Sla/3/…; in 36 EDUYT – Ajaia 12/F3Local, Stot//Altar 84/ALD, Rascon 21/3/Mque. A detailed description of the above sources is presented in the Suppl. Material 1[1].

By test weight grains and the color of the pasta, of interest as sources are the samples from the VIR: k-59881, k-59889, k-60388, k-60364, k-6386; by nature – k-63281, Sladunitsa; according to color estimates, pasta – k-61117, k-62657, k-64353, k-64355, k-64354, k-17985, k-60410.

In KASIB nurseries, grains are valuable by test weight – Kargala 1538 (Aktobe Agricultural Experimental Station), Altyn Dala, Sharifa (Karabalyk Agricultural Experimental Station), Lan (Kazakh Research Institute of Agriculture and Plant Growing), G.178-05-2, Line 250-06-14 (SPC GP named after Baraev), G.94-24-12, G.96-160-8 (Omskaya stepnaya), Omskiy izumrud, G.98-42-5 (Omskiy zircon), G.00-96-8 (Omskiy lazurit), G.04-85-4 (Omskiy corall), G.00-178-4 (Omskaya birjusa), G.05-42-12, G.08-67-1 (Omsk ASC), G.677, G.829, G.864 (FASCA), Line 653d-44, L.1469d-21, G.1591-21, Line 1970d-5, Line 2021d-1 (Samara ARI), Luch 25, Line D-2165 (Research Institute of Agricultural Sciences of the South-East), Melyana (Orenburg ARI). According to the pasta color assessment, Omsk varieties and lines are allocated – G.94-24-12, Omskaya stepnaya, Omskiy zircon, Omskiy lazurit, G.05-42-12, Omskiy izumrud, G.08-67-1, Altai – G.677, G.864, Samara ARI – Line 653d-44, Saratov – Luch 25, Kazakhstan – G.178-05-2 (Suppl. Material 2). Of great importance are the genotypes of Omskiy zircon, Omskiy lazurit, G.05-42-12, G.864, Line 653d-44, forming a grain with a high test weight and color of pasta.

**Resistance to biotic factors**

Currently, one of the directions of ecological farming is the creation of immune varieties for pesticide-free technologies. Selection for disease resistance is a rather time-consuming and complex aspect since each pathogen has an extensive set of physiological races and evolves quite quickly, often ahead of the selection process of the new variety. Therefore, the search for new resistance genes is one of the most important in the strategy of plant protection.

In CIMMYT nurseries, for resistance to hard smut, 131 genotypes (0–1.0 %) were revealed, to powdery mildew – 112 (6–7 points). Almost all samples were not affected by leaf rust. Among the samples that have an advantage in other parameters, 54 were resistant to hard smut, leaf rust, 38 – to powdery mildew. The most interesting are the forms that are resistant to 2–3 diseases. These include Srn 2// Yavaus/Hui/3/ (36 IDYN), Malmuk 1/Serrator, Kucuk 2/ Pata 2 (34 EDUYT) that showed immunity to hard smut, powdery mildew, and leaf rust (damage grade 0). Of greatest interest are genotypes that combine resistance with high rates of grain test weight and pasta color. First of all, we should highlight the samples Dipper 2/Bushen 3, Chen/ Altar 84/3/Hui//Poc//Bub/Rufo/4/Fnfoot (32 IDYN); Lhnke/ Rascon//Cona, Fulvous 1/Mfowl 13/3/Stot//Altar 84/Ald (30 EDUYT); Rascon 39/Tilo 1, Yel/Bar/3/Garza/AFN/ (34 IDYN); Srn 2//Yavaus/Hui/3/, Cndo/Primadur//Hai (36 IDYN); Ajaia 4/Yebas, SN Turk MI83-84, Tarro l/Yuan, SN Turk MI83-84 03/Lotus, Plata 20/Fillo// (34 EDUYT) (Suppl. Material 3). Genotypes Fulvous 1/Meowl 13// Altar 84, Chen//Altar 84… carry resistance genes *Lr23*, *Sr B*, *Sr E* transmitted from the cultivar Altar 84 (McIntosh et al., 2008).

All the forms distinguished in terms of grain quality and disease resistance were actively involved in the breeding process. Only in the period from 2001 to 2006, with the participation of Mexican forms, crosses were carried out on 215 hybrid combinations. The share of hybrid combinations with Mexican samples in these years was 31.6–53.4 %. In 2007, a selection was made from the hybrid combination Omskaya jantarnaya//Pod 11/Yazi (31 EDUYT), which,

[1] Supplementary Materials 1–4 are available in the online version of the paper: http://vavilov.elpub.ru/jour/manager/files/Suppl_Evdokimov_27_7.pdf

**Table 5.** Characteristics of isogenic lines on resistance to stem rust, 2019

| Isogenic line (origin lines) | Gene | Degree of damage, % | Infection type |
|---|---|---|---|
| Einkorn | *Sr21* | 20 | MR |
| T. monococcum/8*LMPG-6 DK13 | *Sr21* | 70 | MS |
| Exchange CI 12635 | *Sr23* | 10 | R |
| Agatha (CI 14048)/9*LM PG-6 DK16 | *Sr25* | 10 | MR |
| Eagle Sr26 | *Sr26* | 10 | MS |
| Kota RL471 | *Sr28* | 20 | MS |
| Selection from Webster F3:F4 #6 | *Sr30* | 20 | MS |
| Seri 82 | *Sr31* | 10 | MR |
| (Benno)/8*L MPG-8 DK42 | *Sr31* | 30 | MS |
| Trident | *Sr38* | 10 | R |
| RL 5711 | *Sr39* | 20 | MR |
| RL 6087 | *Sr40* | 20 | MR |
| Fleming | *Sr6*, *Sr24*, *Sr36*, *1RS-Am* | 20 | MR |
| Chris | *Sr7a*, *Sr12*, *Sr6* | 20 | MR |
| Standard of susceptibility | – | 90 | S |

subsequently, in 2018 was transferred to the State Test under the name of the 'Omskiy corall' variety, and included in the State Register of Breeding Achievements in 2021. However, these lines are of interest as a starting material for further breeding process.

In Western Siberia, leaf rust, hard and dusty smut, powdery mildew were common among the diseases, and until recently there was no manifestation of stem rust. The first foci on spring soft wheat stem rust were discovered in 2007, from 2008 to 2014 it was observed annually to varying degrees, but the damage did not exceed 50 %, and epiphytotics of stem rust arose starting from 2015 (Rosseeva et al., 2019). In subsequent years, stem rust on durum wheat appeared regularly with a degree of damage from 70 to 100 % (Gultyaeva et al., 2020; Yusov et al., 2021). In recent years, epiphytotics of wheat stem rust have been noted in the northern regions of Kazakhstan and in the territories adjacent to the Omsk region of Russia. It was noted that the increase in the frequency of epiphytotics of stem rust is associated with the emergence of new virulent races of the causative agent of the disease and the cultivation of susceptible varieties of wheat (Rsaliev A.S., Rsaliev Sh.S., 2018).

The results of the evaluation of isogenic lines from the CIMMYT International Stem Rust Trap Nursery (ISRTN) in the field in 2019 with maximum damage showed that genes *Sr23* (Exchange), *Sr25* (Agatha(CI14048)/9*NMPG-6DK16), *Sr31* (Seri 82), *Sr38* (Trident) (degree of damage 10 %, infection type R–MR) are effective against the local stem rust population. Genes *Sr21* (*Einkorn*), *Sr26* (*Eagle Sr26*), *Sr39* (*RL 5711*), *Sr40* (*RL 6087*); pyramids of genes *Sr6*, *Sr24*, *Sr36*, *1RS-Am* (Fleming) and *Sr7a*, *Sr12*, *Sr6* (Chris) inhibit the damage (up to 20 %). The remaining lines were affected by 30–80 %, with the type of infection MS–S (Table 5). The susceptibility standard had a lesion rate of 90 % (infection type S). The high efficiency of the *Sr31*, *Sr38*, *Sr40* genes was previously identified in the conditions of Omsk by V.P. Shamanin and colleagues (2020). It should be noted that the effectiveness of genes *Sr21*, *Sr31* in different varieties was different. The Seri 82 variety showed resistance to the population, and the line (Benno)/8*L MPG-8 DK42, also carrying the *Sr31* gene, was affected. A similar picture was observed in the effectiveness of the *Sr21* gene, which was previously noted by L.P. Rosseeva and colleagues (Rosseeva et al., 2017).

The racial composition of stem rust populations varies considerably from region to region. In addition, the biotype composition of races is not of the same type. According to M.S. Hovmøller (2017), a comparative analysis of the TTTTF race isolated in Omsk differs significantly from that of the Sicilian race. This explains the differences in the efficiency of *Sr* genes in territorial and temporal space (Sochalova, Likhenko, 2013; Rosseeva et al., 2017).

Over the 19 years of the KASIB program's existence, 210 samples have been studied. As sources for resistance to the local population of stem rust, the following varieties have been distinguished: G.03-20-18, Omskaya jantarnaya, Omskiy izumrud, G.04-85-4 (Omskiy corall), G.05-42-12, G.08-67-1, G.08-107-5 (Omsk ASC), Kargala 28, Kar-

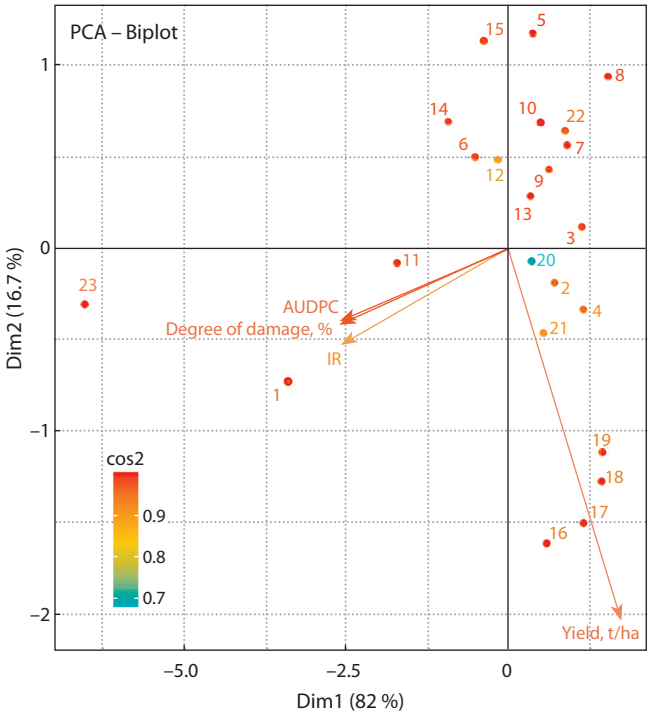


**Fig. 1.** Distribution of varieties and lines of durum wheat in the plane of the main components by the degree of stem rust damage in the field in 2019–2020.

1, Jemthujina Sibiri; 2, Omskiy izumrud; 3, Omskiy corall; 4, Triada; 5, Odysseo; 6, Soyana; 7, G.250-06-14; 8, Line 1927d; 9, G.07-115-1v; 10, G.08-76-1; 11, G.08-107-5; 12, G.0-68-1; 13, G.09-122-1; 14, G.10-32-4; 15, G.10-33-4; 16, G.11-48-12; 17, G.12-9-3; 18, G.16-8-2; 19, G.16-8-4; 20, G.16-8-5; 21, G.16-13-2; 22, G.16-13-4.

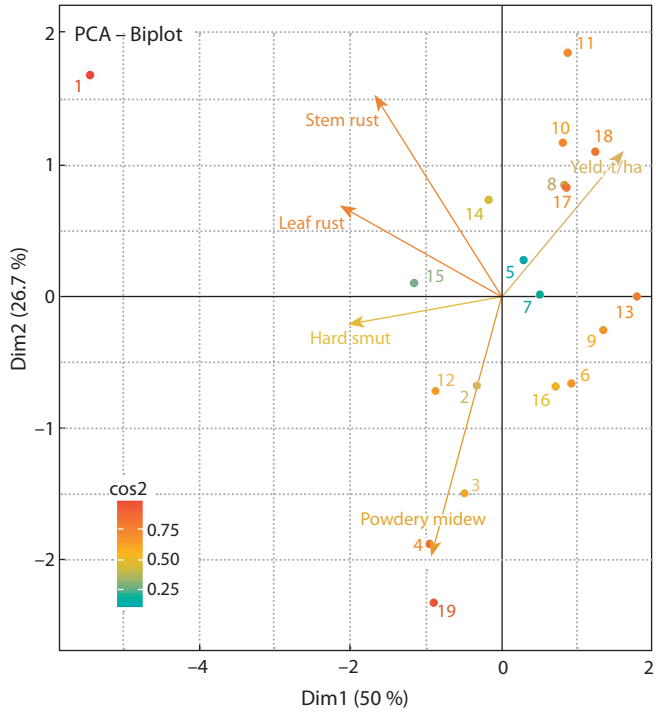


**Fig. 2.** Analysis of the main components of the main agronomically important traits of competitive and preliminary variety trial of spring durum wheat (in average for 2018–2020).

1, Jemthujina Sibiri; 2, Omskiy izumrud; 3, G.08-67-1; 4, G.09-122-1; 5, G.09-73-1; 6, G.10-32-3-1; 7, G.10-32-12; 8, G.10-63-1; 9, G.10-71-3; 10, G.11-45-2; 11, G.11-46-3; 12, G.11-97-3; 13, G.11-98-3; 14, G.12-11-1; 15, G.12-12-2; 16, G.11-92-2; 17, G.11-75-1; 18, G.12-31-1; 19, G 13-18-3.

gala 303, Kargala 1412, Kargala 1514, Kargala 1516/06 (Aktobe AES); Lines 688d-4, 1591d-21, 1560d-18 (Samara ARI); Durum 49, G.69-08-5, G.178-05-2, Line 250-06-14 (SPC GP named after Barayev); Line No. 9 from Karabalyk AES (Yusov et al., 2018).

Among the VIR samples, the breeding value for resistance to stem rust is: k-6386, k-6662, k-46983, k-60410, Iride, k-65353, k-65733, k-65734.

A comparative study of the varieties of ecological testing and lines created in the Omsk ASC showed that there are resistance forms, but the genetic control of resistance in them is not due to oligogenes (genes of vertical resistance). Varieties Omskiy izumrud, Omskiy corall, Triada, Odysseo, G.250-06-14, Lines 1927d, G.07-115-1v, G.08-76-1, G.09-122-1, G.12-9-3, Lines 2016-8-2, 2016-8-4, 2016-13-4; in accordance with the classification of A.A. Makarov and colleagues (Makarov et al., 2003) these are genotypes with high racially specific resistance, the resistance index of which is, on average, for 2019–2020, 0.21–0.40. They have a delayed development of the disease, and as a result, a low value of the AUDPC (area under the disease progress curve), which ranges from 542 in the Omskiy corall to 696 c.u. (conventional units) in the Omskiy izumrud, with the value of the standard variety Jemthujina Sibiri being (1626), susceptibility standards being (2230–2873 c.u.). The minimum value of the degree of stem rust damage (16.7 %) was noted in the Line of 1927d (Fig. 1). Their yields were above standard. These varieties have a pronounced nonspecific resistance, which is expressed by the delayed development of the disease and can persist for a long time. The genotypes of Soyana, G.08-107-5, G.09-68-1, G.10-32-4, G.10-33-4, G.11-48-12, G.16-8-5, G.16-13-2 have moderate racial-specific resistance.

The problem of resistance to diseases, including stem rust, has always been given special attention in the breeding programs of Omsk ASC, therefore, at present, varieties, promising samples and lines that are of interest primarily as sources of resistance to this pathogen have been created. At the final stages of the breeding process, 15 genotypes resistant to leaf rust, 11 to stem rust, 8 to hard smut, 10 to powdery mildew were identified. Highly productive breeding lines with a yield of more than 5.0 t/ha (Jemthujina Sibiri standard – 4.5 t/ha), with complex resistance to 3–4 diseases have been created: G.10-32-3-1, G.10-63-1, G.10-71-3, G.11-98-3, G.11-75-1, G.12-31-1 (Fig. 2).

Along with this, there is a danger and a threat of penetration from the countries of the Middle East and Central Asia of the malicious race of stem rust Ug99, which was first

**Table 6.** Entries resistant to stem rust race Ug99 (assessment in Kenya)

| Entries | Origin | Damage, % | Infection type | Year of assessment | Nursery |
|---|---|---|---|---|---|
| Durum 49 | SPC GP named after Barayev | 10 | MS | 2011 | KASIB 12 |
| G.748 | FASCA | 5 | MSS | 2015 | KASIB 16–17 |
| Lavina | SPC GP named after Barayev | 10 | M | | |
| L.1307d-54 | Samara ARI | 10 | MSS | | |
| G.950/99 | Karabalyk AES | 5 | MSS | | |
| Omskiy izumrud | Omsk ASC | 10 | MS | 2021 | KASIB 22 |
| Omskiy lazurit | | 10 | MS | | |
| G.11-77-3 | | 0.5 | MS | | |
| G.04-35-8 | Omsk ASC | 5 | RMR | 2011 | BN-3 |
| G.04-54-4 | | 5 | RMR | | |
| G.04-41-3 | | 5 | R | 2012 | |
| G.04-41-5 | | 5 | RMR | | |
| G.05-3-1 | | 10 | M | | |
| G.07-33-1 | | 10 | M | | |
| G.06-5-3 | | 5 | MSS | 2015 | |
| G.07-21-10 | | 10 | MSS | | |
| G.07-28-10 | | 10 | MSS | | |
| G.08-55-5 | | 0 | | | |
| G.08-94-3 | | 0 | | | |
| G.08-106-8 | | 10 | M | | |
| G.08-107-2 | | 10 | MSS | | |
| G.10-32-7 | | 5 | MR | 2016 | |
| G.07-41-4 | | 10 | MS | | |
| G.08-67-1 | | 10 | MR | | |
| G.09-51-1 | | 10 | MR | | |
| G.09-68-2 | | 10 | M | | |
| G.09-122-1 | | 10 | MS | | |
| G.10-32-4 | | 10 | MR | | |
| G.07-115-1 | | 10 | MR | 2020 | |
| G.11-49-1 | | 0.1 | MS | 2021 | |
| G.11-46-3 | | 1 | MS | | |
| G.11-98-3 | | 10 | MS | | |
| G.12-17-2 | | 0 | | | |
| Jemthujina Sibiri, standard | Omsk ASC | 50 | MSS | 2021 | KASIB 22 |

discovered on the African continent in Uganda and named after the place of its first discovery (Shamanin et al., 2015). A cause for concern is the TTKSK pathotype, which has high virulent properties and overcomes the effectiveness of many wheat resistance genes, including the *Sr31* gene (Singh et al., 2015). The effectivity of *Sr9e* durum wheat genes in Kronos (Li et al., 2021), *Sr13* in Cirilla (Laido et al., 2015) and Fielder (Zhang et al., 2017) in Africa and Kronos, Kofa, Medora, Scepter varieties in Canada (Simmons et al., 2011) have been shown. Effective under Canadian conditions, genes *Sr8* and *Sr14* have been identified in grade A9919-BY5C (Kumar et al., 2021).

In accordance with the program of international cooperation under the auspices of CIMMYT, breeding material created in the Omsk ASC, as well as samples and lines of KASIB, were sent to Kenya for evaluation in different years. In the kennels of KASIB, 7 genotypes showed resistance to the Ug99 race: Durum 49, Lavina (SPC GP named after Barayev), G.950/99 (Karabalyk AES), G.748 (FASCA), L.1307d-54 (Samara ARI), Omskiy izumrud, Omskiy lazurit, G.11-77-3 (Omsk ASC). When evaluating the breeding nursery, 27 numbers showed resistance to the Ug99 race. Among the immune forms are G.08-55-5, G.08-94-3, G.12-17-2 (Table 6).

## Conclusion

Based on the studies conducted in 2000–2021, when studying the gene pool of durum wheat from CIMMYT, 50 genotypes were identified at the level of the Omskaya jantarnaya standard in terms of yield, 276 grains by test weight, 131 samples by pasta color, 131 samples in terms of resistance to hard smut, and 112 samples to powdery mildew. Almost all samples were not affected by leaf rust. The studied set of samples are of interest due to their grain quality and disease resistance but have low productivity in the conditions of the southern forest-steppe of Western Siberia. 56 genotypes have been identified for resistance to hard smut, 54 – to leaf rust, 38 – to powdery mildew, in combination with other valuable features.

In KASIB nurseries, 29 samples have been selected for high yield and adaptability, 29 for grain quality, 21 for disease resistance, including 8 for resistance to stem rust. Among the varieties from the VIR collection, there are 15 adaptive genotypes, 16 with high grain quality, and 11 resistant to stem rust.

In the conditions of the Omsk region, effective genes for resistance to the local population of stem rust are *Sr23*, *Sr25*, *Sr26*, *Sr31*, *Sr38*. *Sr39*, *Sr40* genes; pyramids of genes *Sr6*, *Sr24*, *Sr36*, *1RS-Am* (Fleming) and *Sr7a*, *Sr12*, *Sr6* (Chris) restrain the damage (up to 20 %).

A new breeding material has been created that combines complex resistance to leaf, stem rust, hard smut, powdery mildew with high yields and good grain quality. When evaluating the breeding material, 17 numbers resistant to the local population of stem rust (6 of them have complex resistance) and 25 race-resistant to Ug99 were identified.

The genotypes identified as a result of research are of interest as sources of valuable traits. Part of the studied material is included in the scientific program of the “Bread of Russia”.

The studied gene pool of durum wheat, which includes a large set of varietal samples of various ecological and geographical origin, will contribute to the purposeful selection of parent pairs, in accordance with the principles of geographical remoteness and genetic divergence, developed by N.I. Vavilov (1935), which are still relevant at the present time.

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# Mineral composition of repair raspberry (*Rubus idaeus* L.) fruits

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**Abstract.** In recent years, raspberry breeding has shifted its emphasis from agronomic performance to characteristics related to the sensory qualities of the fruit and its potential health benefits. The therapeutic and preventive properties of raspberries are related to their biochemical composition. In this regard, the purpose of the work was to determine the content of macro- and micronutrients in fruits of different cultivars of repair raspberry using modern high-tech analytical methods and the selection of genetic sources of the analyzed elements for further breeding. The objects of the research were 17 cultivars of repair raspberry of different ecological and geographical origin from the genetic plant bioresource collection of FSBSO ARHCBAN. It was found that the ash residue of berries contains 12 major elements, which form the following descending series: K > P > Mg ≥ Mo > Ca > S ≥ Ni > Zn > Mn > Se > Fe ≥ Co. The largest proportion of ash residue in raspberry fruits is K. Depending on the cultivar, its quantity averaged from 12.81 wt % (Samorodok and Karamelka) to 22.37 wt % (Atlant). The minimum K content was observed in the ash of the Carolina cultivar (5.62 wt %), while in berries of this cultivar Mg (2.91), Ca (2.62) and Zn (0.14 wt %) accumulated above average. Among the group of early maturing cultivars, the cultivar Yubileinaya Kulikova stands out with a high content of Mo (4.63), Ca (2.19), Fe (0.25) and Co (0.21 wt %). The cultivar Pingvin is characterized by a high content of K (22.65) and Se (0.31 wt %). The medium maturity cultivar Samorodok is characterized by a higher content of P (4.08), S (0.47), Ni (0.51) and Zn (0.26 wt %). Among the late maturing cultivars, the cultivar Poranna Rosa stands out with the preferential accumulation of nine elements: Mg (2.98), P (4.42), S (0.36), K (20.34), Ca (1.71), Mn (0.14), Co (0.13), Se (0.21) and Mo (3.08 wt %). Correlation relationships between the elements have been established. Samples with the highest accumulation of macro- and microelements in berries represent genetic sources for further selection of raspberry for improvement of the mineral composition of fruits.
Key words: *Rubus idaeus* L.; cultivars; mineral composition; berries; energy dispersive spectrometry.

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# Минеральный состав плодов ремонтантной малины (*Rubus idaeus* L.)

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**Аннотация.** В последние годы акцент в селекции малины сместился с агрономических показателей на характеристики, связанные с сенсорными качествами плодов и потенциальной пользой их для здоровья. Терапевтические и профилактические свойства малины обусловлены ее биохимическим составом. В связи с этим целью работы было определение содержания макро- и микроэлементов в плодах различных сортов ремонтантной малины с помощью современных высокотехнологичных аналитических методов и выделение генетических источников анализируемых элементов для дальнейшей селекции. Объектом исследований служили 17 сортов малины ремонтантного типа различного эколого-географического происхождения из генетической биоресурсной коллекции растений ФНЦ Садоводства. Установлено, что в зольном остатке ягод содержатся 12 основных элементов, которые образуют убывающий ряд: K > P > Mg ≥ Mo > Ca > S ≥ Ni > Zn > Mn > Se > Fe ≥ Co. Наибольшую долю зольного остатка в плодах малины составляет K. В зависимости от сорта его количество в среднем изменяется от 12.81 мас.% (Самородок и Карамелька) до 22.37 мас.% (Атлант). Минимальное содержание K отмечено в золе сорта Carolina (5.62 мас.%), при этом в ягодах этого сорта выше средних значений накапливаются Mg (2.91), Ca (2.62) и Zn (0.14 мас.%). Среди группы сортов раннего срока созревания высоким содержанием Mo (4.63), Ca (2.19), Fe (0.25) и Co (0.21 мас.%) выделяется сорт Юбилейная Куликова. Сорт Пингвин характеризуется высоким содержанием K (22.65) и Se (0.31 мас.%). Сорт Самородок среднего срока созревания отличается

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повышенным содержанием P (4.08), S (0.47), Ni (0.51) и Zn (0.26 мас.%). Среди сортов позднего срока созревания по преимущественному накоплению девяти элементов выделяется сорт Poranna Rosa: Mg (2.98), P (4.42), S (0.36), K (20.34), Ca (1.71), Mn (0.14), Co (0.13), Se (0.21) и Mo (3.08 мас.%). Установлены корреляционные связи между элементами. Образцы с наибольшим накоплением макро- и микроэлементов в ягодах являются генетическими источниками для дальнейшей селекции малины на улучшение минерального состава плодов.
Ключевые слова: *Rubus idaeus* L.; сорта; минеральный состав; ягоды; энергодисперсионная спектрометрия.

## Introduction

Raspberries are one of the most popular berry crops in household farms and industrial production. In recent years, raspberry selection has shifted the focus from agronomic characteristics to characteristics related to sensory qualities of the fruit (Jennings et al., 2016) and potential health benefits (Mazzoni et al., 2016). At the same time, significant advances were made in the analytical chemistry of fruits. These new tools generate knowledge that can significantly accelerate the creation of new cultivars that meet consumer expectations in terms of sensory perception and the health benefits of eating fruit. In recent years, significant researches have identified environmental, biochemical, and genetic factors underlying the accumulation of certain compounds in raspberry fruits (Kowalenko, 2005; Dresler et al., 2015).

Raspberries are a source of biologically active compounds and minerals that have a positive effect on human health (Pereira et al., 2018; Eremeeva et al., 2019). Minerals belong to the vital components of nutrition (micronutrients) with a wide variety of physiological functions. They play an important role in plastic processes, the formation and construction of body tissues, in particular, the bones of the skeleton. Mineral substances are necessary for maintaining acid-base balance in the body, creating a certain concentration of hydrogen ions in tissues and cells, interstitial and intercellular fluids, as well as giving them osmotic properties that ensure the normal course of metabolism. Mineral elements have antioxidant properties, are involved in redox processes, in carbohydrate, protein, vitamin and fat metabolism, in the formation of bone tissue, regulate heat and gas exchange, hematopoiesis, growth, respiration, play an important role in immunobiological reactions, affect water-salt and acid-base balance (Salmanov, Isrigova, 2004; Nile, Park, 2014; Pochitskaya et al., 2017; Makuev et al., 2018). For example, Fe, being an indispensable component of blood, is involved in oxygen transport and oxidative metabolism (Emel'yanova, 2001). Ca is necessary for the formation of bone and connective tissue, is involved in the transmission of nerve impulses and muscle contraction (Erdman et al., 2012). Cu is a part of a number of important enzymes, normalizes cellular metabolism and catalyzes some of the reactions necessary for the normal functioning of the brains and nervous system. Mg is vital for energy metabolism. Mg and Mn are parts of enzymes, are involved in the metabolism of carbohydrates, amino acids and cholesterol (Ferlemi et al., 2016). Zn maintains an optimal concentration of tocopherol, plays an important role in the growth and development of plants, in the formation of the immune response, the function of the nervous system, promotes the absorption of vitamin A (Frassinetti et al., 2006). In the prevention and treatment of age-related diseases, antioxidant strategies based on nutrition are used, including the addition of antioxidants and trace elements in the prevention (Opara, Rockway, 2006).

Significant intervarietal differences in the mineral content of Na, K, Ca, Mg, Fe, Cu and Zn in raspberry fruits of different colors were established by the studies of Akimov et al. (2021). The quantitative and qualitative composition of mineral substances of fruits and berries depends on the botanical species, cultivar, soil and climatic conditions, methods of cultivation, etc. (Nilova et al., 2018). Despite the role of micronutrients, they have not received as much attention as vitamins, and this may be due to the fact that the safety range between deficiency and toxicity of some trace elements is relatively narrow.

Nevertheless, with the spread of knowledge about rational nutrition and the therapeutic and prophylactic properties of fruits and berries among the population, the demand for them, including raspberries, is growing, which is mostly satisfied by repair cultivars (Gambardella et al., 2016; Orzeł et al., 2016; Moreno-Medina et al., 2018; Evdokimenko, 2020). Despite the popularity of repair raspberries in industrial production, there is only fragmentary information about the mineral composition of its fruits in the scientific literature. Comparative studies of the mineral composition of the repair raspberries berries of the Federal Research Center of Horticulture collection have not previously been conducted. Consequently, the systematization of the content of macro- and microelements in the fruits of repair raspberry cultivars using modern high-tech analytical methods and the typification of samples of the *Rubus idaeus* L. collection is very relevant.

In this regard, the purpose of our work was to determine the content of macro- and microelements in the fruits of various cultivars of repair raspberries using modern high-tech analytical methods and to isolate the genetic sources of the analyzed elements for further selection.

## Materials and methods

The research was conducted in 2021 in the Laboratory of Biochemistry and Physiology of Plants of the Federal State Budgetary Institution of the Federal Research Center of Horticulture. The objects of study were the fruits of 17 repair cultivars of raspberries (*Rubus idaeus* L.) of various ecological and geographical origin, differing in terms of ripening, color and other economic and biological signs and properties (Table 1). The raspberries were grown on the site of the genetic collection of the Kokinsky experimental station of the Federal Research Center of Horticulture, located (53.154935° N, 34.123027° E), according to the generally accepted technology with late autumn mowing of stems (Kazakov et al., 2016).

The soils were gray forest, well cultivated, medium loamy. The depth of the arable layer was 26 cm, the humus content was 3.2 %, $P_2O_5$ was 35 mg per 100 g of soil, $K_2O$ was 13.5 mg per 100 g of soil, the reaction of the soil solution was slightly acidic (pH 6.1).

The scheme of planting on the site was single-row, the distance between the rows was 3 m, and between the plants it

**Table 1.** The characteristics of the objects of the research

| Sample name | Country of breeder | Ripening terms | Fruits color |
|---|---|---|---|
| The cultivars of the FRC of Horticulture | | | |
| Atlant | Russia | Late | Dark red |
| Evraziya | | Medium | Dark red |
| Zhar-Ptiza | | Late | Light red |
| Medvezhonok | | Early | Light red |
| Pingvin | | Early | Dark red |
| Poklon Kazakovu | | Medium | Dark red |
| Samorodok | | Medium | Dark red |
| Elegantnaya | | Medium | Red |
| Yubileynaya Kulikova | | Early | Red |
| The collection samples | | | |
| Karamelka | Russia | Late | Red |
| Brice | Great Britain | | Red |
| Carolina | USA | | Red |
| Enrosadira | Italy | | Red |
| Erika | Italy | | Red |
| Maravilla | USA | | Light red |
| Heritage | USA | | Dark red |
| Poranna Rosa | Poland | | Yellow |

was 0.5 m. During the season, one spring nitrogen fertilization was carried out (35 kg/ha a.i.). The intervals of the rows in the first half of the growing season were kept under pure steam, and after flowering under natural grassing.

A representative sample of mature raspberries with an average weight of 200 g was dried in a drying oven at a temperature of 50–60 °C. The dried samples were mineralized in a muffle oven Naberterm (Germany) at a temperature of 450 °C in accordance with the Russian State Standard GOST 26929-94 (2002). The resulting ash was dispersed by ultrasound at a frequency of 18 kHz for 15 min. A uniform layer of disperse was applied to a stage table covered with carbon tape.

The chemical composition of 12 main ash elements – Mg, P, S, K, Mn, Co, Fe, Ca, Zn, Ni, Se and Mo – was determined by energy dispersion spectrometry (EDS) on an analytical scanning electron microscope JEOL JSM 6090 LA in accordance with the technique (Motyleva, 2018). The resolution of the microscope was 4 nm, the accelerating voltage was 20 kV (image of secondary electrons). The working distance during the elemental analysis was 10 mm. The energy-dispersive microanalysis data were presented in accordance with standard protocols and included images of the microstructures of the sample under study, a table of weight data and spectral lines of the diagnosed elements. An example of an analysis report is shown in Figure 1.

The concentration of the desired elements was determined by the intensity of the spectral lines. The accuracy of chemical analysis was determined as follows: at the concentration of elements from 1 to 5 %, the accuracy was less than 10 %; at the concentration of elements from 5 to 10 %, the accuracy was less than 5 %; and at the concentration of elements more than 10 %, the accuracy was less than 2 %. In total, 10 sites of each sample were examined. The local analysis was 3 mm, and the scanning area was at least 12 μm.

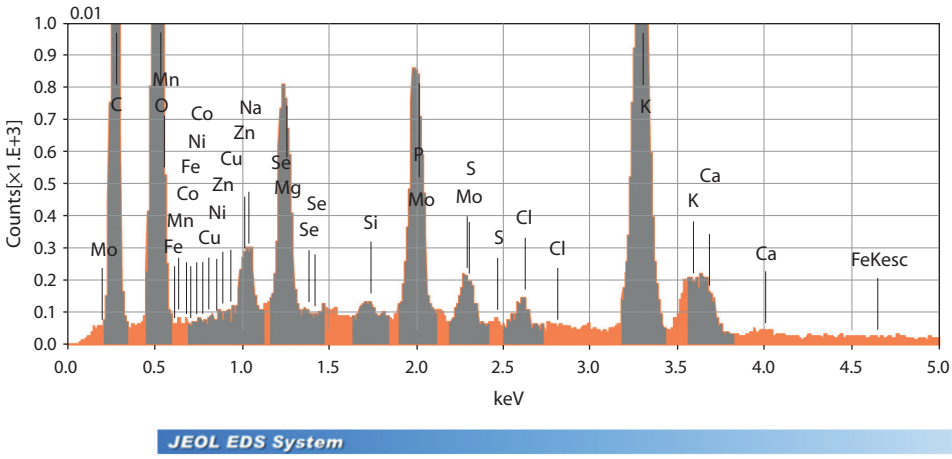
The results were expressed as average values ($n$ = 10) as standard deviation (SD). We used the statistical analysis of the Excel package (Microsoft Excel, v. 2016).

## Results and discussion

Raspberries are known to be rich in minerals (Pereira et al., 2018). 12 main elements that form a descending series have been identified: K > P > Mg ≥ Mo > Ca > S ≥ Ni > Zn > > Mn > Se > Fe ≥ Co. Among the macronutrients, K has had the highest concentration, which is observed in fruits and other berry crops – actinidia, blackberries, strawberries, blueberries (Motyleva et al., 2017; Pereira et al., 2018).

The highest value of K from 20.34 to 22.65 wt % was accumulated in 6 varietal samples – Poranna Rosa, Yubileynaya Kulikova, Zhar-Ptiza, Erika, Atlant and Pingvin. The lowest K content of 5.62 wt % was observed in the fruits of the cultivar Carolina (Table 2). In raspberries of early and late ripening periods, a 3–4 % higher accumulation of K was noted, which may be associated with the genotype of the cultivars.

Differences in the accumulation of K in raspberries with various fruits colors were revealed. In the berries of dark-colored and red cultivars of raspberries, the average content of K was 18.22 and 15.91 wt %, respectively. In light-colored berries (3 cultivars in total), the content of K ranged from 14.71 (Maravilla) to 21.88 (Zhar-Ptiza) and in yellow-colored berries it was 20.34 (Poranna Rosa) by weight %, respectively. Akimov et al. (2021) also mentioned the high content of K in yellow-colored raspberries of the cultivar Zheltuy Gigant. The




| Element | keV | mass % | Error % |
|---|---|---|---|
| Mg K | 1.253 | 3.42 | 0.31 |
| Mn K | 5.486 | 2.16 | 0.24 |
| S K | 1.739 | 0.48 | 0.25 |
| P K | 2.013 | 1.02 | 0.30 |
| K K | 3.312 | 23.89 | 0.11 |
| Ca K | 3.690 | 1.80 | 0.15 |
| Ni K | 4.508 | 0.64 | 0.22 |
| Fe K | 6.398 | 0.23 | 0.28 |

**Fig. 1.** The results of EDS-analysis. Spectral lines of diagnosed elements and a table of results.
keV is the energy of the X-ray radiation of the K-level; mass % is the weight part of the element; Error % – detection error recorded by the instrument.

**Table 2.** The content of K in the samples of *Rubus idaeus* L., wt % in ash

| Cultivar | K, wt % | max | min | SD | V, % | Cultivar | K, wt % | max | min | SD | V, % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Early ripening | | | | | | Late ripening | | | | | |
| Pingvin | 22.65 | 23.75 | 22.16 | 0.531 | 2.344 | Zhar-Ptiza | 21.88 | 22.88 | 20.31 | 0.756 | 3.454 |
| Yubileynaya Kulikova | 21.17 | 22.45 | 19.28 | 1.097 | 5.192 | Atlant | 22.39 | 23.24 | 21.08 | 0.669 | 2.987 |
| Medvezhonok | 15.91 | 16.88 | 14.11 | 0.791 | 4.978 | Karamelka | 12.81 | 14.01 | 12.15 | 0.603 | 4.694 |
| Average | 19.91 | | | | | Brice | 19.08 | 20.21 | 17.33 | 0.967 | 5.069 |
| Medium ripening | | | | | | Carolina | 5.62 | 16.61 | 14.88 | 0.658 | 4.207 |
| Elegantnaya | 16.81 | 17.98 | 15.384 | 0.601 | 3.582 | Poranna Rosa | 20.34 | 22.38 | 19.25 | 0.894 | 4.398 |
| Samorodok | 12.81 | 13.44 | 12.08 | 0.486 | 3.807 | Erika | 22.25 | 23.41 | 21.89 | 0.571 | 5.707 |
| Poklon Kazakovu | 17.18 | 18.21 | 16.42 | 0.468 | 2.721 | Enrosadira | 16.78 | 17.54 | 15.38 | 0.750 | 4.469 |
| Evraziya | 16.93 | 17.41 | 16.32 | 0.359 | 2.121 | Maravilla | 14.71 | 17.31 | 13.22 | 1.097 | 7.233 |
| Average | 15.93 | | | | | Heritage | 17.37 | 18.34 | 16.23 | 0.781 | 5.079 |
| | | | | | | Average | 18.62 | | | | |

Note. Average out of 10 measurements ± SD (standard deviation), V – the coefficient of variation.

content of K in raspberries of domestic and foreign selection cultivars was on average 18.28 and 16.54 wt %, respectively. However, the identified differences in the accumulation of K depending on the color of the berry require further comparative studies on a larger number of cultivars. The variation coefficient of K is low (V = 2.121–5.707 %), which indicates a stable intake of this element in raspberries. In the human body, K is necessary for the work of the heart muscle, maintaining acid-base and water balance. In ionic form, K increases the concentration of other ions and is found in all the organs of the human body (Meathnis et al., 1997).

The comparative content of macroelements P, Mg and Ca in raspberries is presented in Figure 2.

The content of P in raspberries varied from 1.59 wt % (Poklon Kazakovu) to 5.19 wt % (Enrosadira). The average content of P in raspberries, depending on the ripening terms, varied within: in berries of early ripening cultivars, its content was 4.29; of medium ripening, 3.27 and of late ripening, 4.81 wt % respectively; the differences were statistically significant at $p \leq 0.05$. In berries of foreign selection cultivars, P content is on average 1.5–2.0 % higher than in berries of domestic cultivars. P is involved in many physiological processes, including energy metabolism (in the form of ATP), regulation of acid-base balance, is part of phospholipids, nucleotides, nucleic acids, is necessary for bone mineralization (Avtsyn et al., 1991).

The differences in the content of Mg in raspberries were less expressed than in the content of P – from 1.05 wt % (Poklon Kazakovu) to 3.31 wt % (Zhar-Ptiza). The significant differences in the content of Mg in berries depending on the color of the berries and origin have not been established. In the human body, Mg is a coactor of many enzymes, including energy metabolism, it is involved in protein synthesis and is necessary to support homeostasis (Avtsyn et al., 1991).

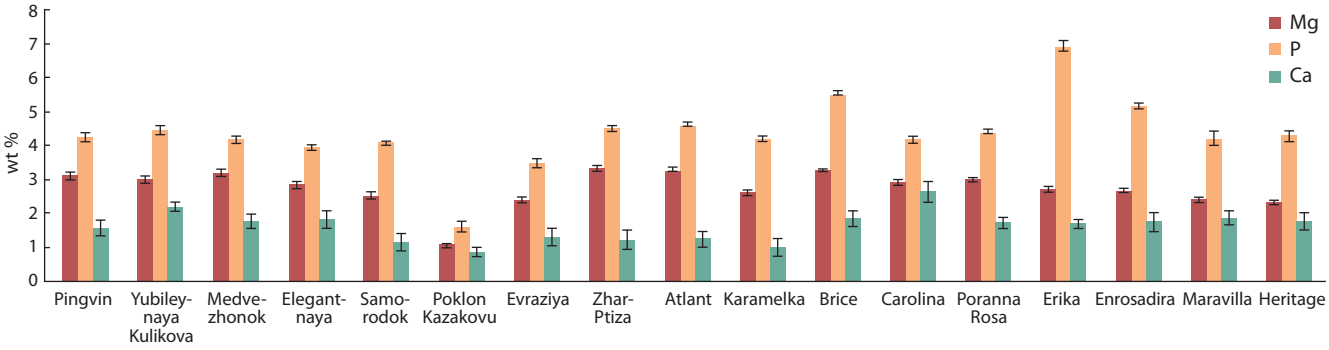



**Fig. 2.** The comparative content of Mg, P and Ca in *Rubus idaeus* L. berries.

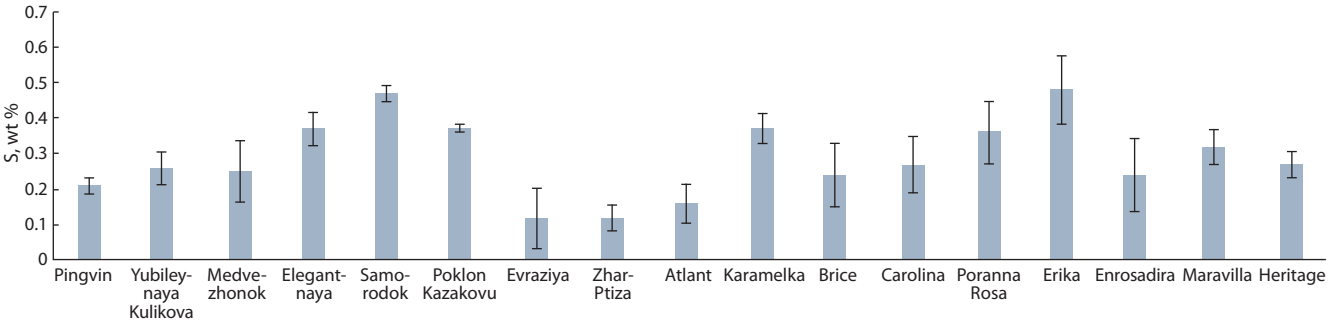



**Fig. 3.** The comparative content of S in the berries of *Rubus idaeus* L.

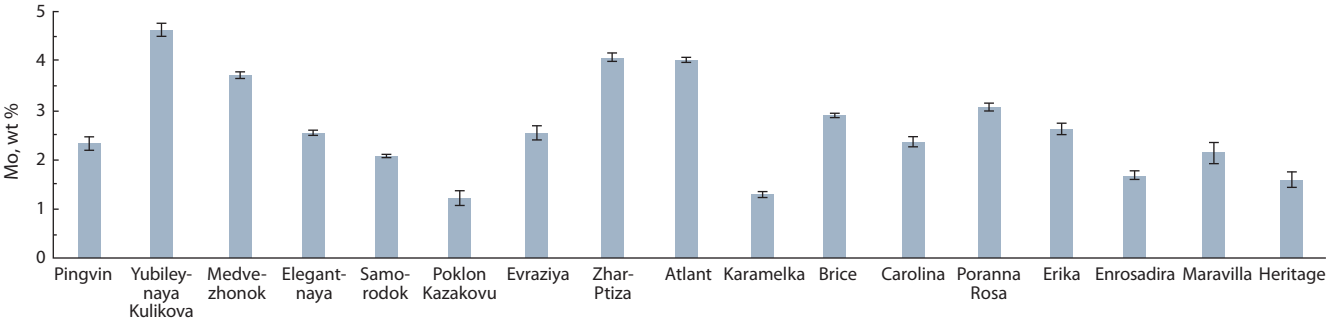



**Fig. 4.** The comparative content of Mo in the berries of *Rubus idaeus* L.

Ca ions are involved in blood clotting processes, as well as in ensuring constant osmotic pressure. It is involved in the processes of cell growth and development, it is a part of enzymes and affects metabolism and immunity (Gins et al., 2018). According to Jeong et al. (2008), the main elements in the composition of raspberries are K, P and Ca.

The content of S in raspberries ranged from 0.12–0.16 wt % (Evraziya, Zhar-Ptiza and Atlant) to 0.26–0.48 wt % (Yubileynaya Kulikova, Carolina, Erika, Samorodok) (Fig. 3).

Raspberries contain a group of trace elements – Mn, Fe, Co, Ni, Zn, Se and Mo. According to the results of our research, Mo in raspberries was contained in the concentrations comparable to Ca and ranged from 1.29 wt % (Poklon Kazakovu and Karamelka) to 4.63 wt % (Yubileynaya Kulikova) (Fig. 4). The high content of Mo was distinguished in the cultivars Atlant and Zhar-Ptiza – 4.01 and 4.07 wt %, respectively.

The content of trace elements Zn, Fe, Se and Co in raspberries did not exceed 0.35 wt %. The highest content of Se from 0.27 to 0.31 wt % was found in the berries of the cultivars Brice, Zhar-Ptiza, Atlant and Medvezhonok. The minimum content of this important trace element (0.04 wt %) was noted in the berries of the cultivars Poklon Kazakovu, Enrosadira and Maravilla (Fig. 5). The content of Zn in raspberries ranged from 0.06 (Enrosadira) to 0.25 wt % (Heritage and Samorodok).

The accumulation profiles of Fe and Co in the ash residue of raspberry fruits coincided. The maximum accumulation of these elements was noted in the berries of the cultivar Yubileynaya Kulikova (0.25 and 0.20 wt %) and Enrosadira and Brice (0.18 and 0.19–0.13 wt %). The average content of Fe from 0.11 to 0.15 wt % was found in the berries of the cultivars Karamelka, Maravilla, Heritage and Evraziya.

The proportion of Se in the raspberries of most cultivars was from 0.13 to 0.31 wt %. The maximum content of this trace element was found in raspberries of the cultivars Pingvin, Medvezhonok, Elegantnaya, Atlant, Zhar-Ptiza and Brice. The minimum content of Se (0.4 wt %) was found in the berries of the cultivars Enrosadira, Maravilla and Poklon Kazakovu. Among the cultivars with a high density of berries, the cultivar Atlant stood out, in the ash residue of which the content of K, Mn, Fe, Se and Mo was 1.3, 1.5, 3.8, 1.8 and 1.6 times more than in the berries of other late ripening cultivars. There is evidence that the increase in Se in food in Finland has clearly increased due to the use of fertilizers with the addition of Se (Ekholma et al., 2007).

K, Mg, Ca, Fe, Zn and Mn have been noted as the main elements that are found in red raspberries of the cultivar Willamette (Dragišić Maksimović et al., 2017). There is evidence that Zn and other elements from the group of heavy metals have antimicrobial effect (Daglia et al., 2011). Three key trace minerals, the role of which in antioxidant protection gradually attracts more and more attention, are Zn, Se and Fe. Over the past 20 years, a significant amount of evidence has been accumulated in favor of the role of these elements as cellular antioxidants (Powell, 2000). One of the ways in which Zn acts as an antioxidant is the induction of metallothioneins, a group of small molecule amino acid residues, the production of which is induced by Zn in many tissues, including the liver, intestines and kidneys. Metallothioneins have been shown to scavenge free radicals and bind certain oxidants in a relatively inert state and have been shown to act in this way under a variety of conditions, including radiation exposure, drug toxicity, ethanol toxicity, and mutagenesis (DiSilvestro, 2000). Se is an essential element of the antioxidant defense system of the human body, has an immunomodulatory effect, and participates in the regulation of the action of thyroid hormones (Nutrition hygiene…, 2021).

In the raspberry fruits of all samples, a sufficiently high, slightly varying from the genotype, content of Ni was found, which ranged from 0.35–0.38 wt % (Poklon Kazakovu, Karamelka, Evraziya, Poranna Rosa) to 0.44–0.58 and 0.76 wt % (Pingvin, Yubileynaya Kulikova, Medvezhonok, Brice and Heritage) respectively (Fig. 6). Ni is a transitional element widely distributed in the environment, air, water and soil. Its accumulation can occur from natural sources and anthropogenic activities. Although Ni is ubiquitous in the environment, its functional role as a trace mineral for animals and humans has not yet been recognized. The phytoextraction of Ni depends on the level of the concentration of Ni in the soil (Nordberg et al., 2007; Genchi et al., 2020).

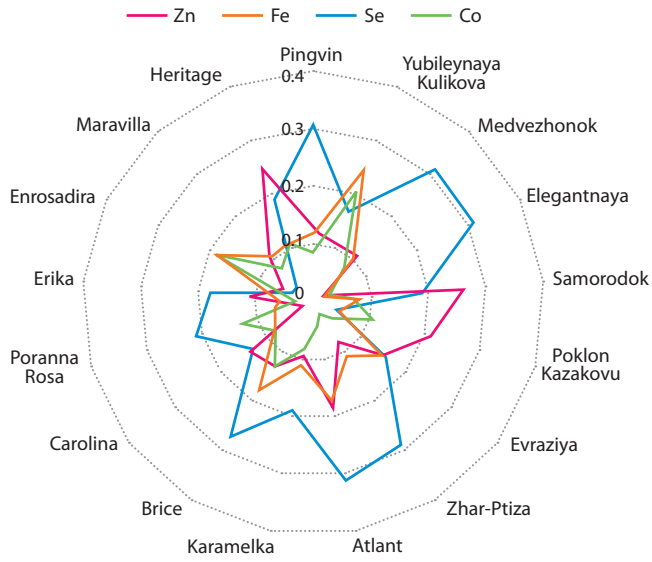



**Fig. 5.** The comparative content of trace elements (Zn, Fe, Se and Co) in the berries of *Rubus idaeus* L., wt %.

According to the total content of elements in the ash of fruits, the following cultivars were distinguished: Pingvin, Yubileynaya Kulikova, Medvezhonok, Elegantnaya, Zhar-Ptiza, Atlant, Brice, Poranna Rosa, Erika, Enrosadira and Heritage, in the ash residue of which 29–37 of weight % contained the determined elements.

The correlation analysis allows to determine the relationship between mineral elements (Table 3). The highest correlation exists between the elements S–Mg ($r$ = 0.9603), Co–S ($r$ = 0.9603), Se–Mg ($r$ = 0.8587) and Co–Ca ($r$ = 0.8577). The average correlation ($r$ = 0.61–0.73) was found between S–P, Mn–Ca, Co–Fe, Se–Ca and S, Mo–S, P and Fe. A low correlation ($r$ = 0.41–0.55) was noted between Ca–S, Mn–S, Fe–S, Ni–Co, Zn–Mn, Fe–Mg and Mo–Mg. There was practically no correlation ($r$ = 0.0085–0.0087) between Se–Ni and Mo–Ca.

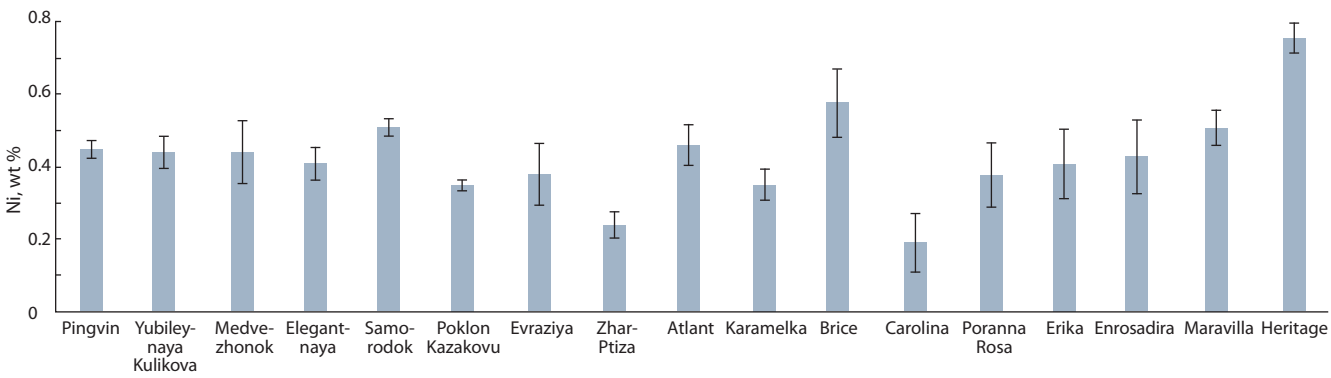



**Fig. 6.** The comparative content of Ni in the berries of *Rubus idaeus* L.

**Table 3.** The correlation matrix of mineral (ash) composition of *Rubus idaeus* L. berries

| Element | Mg | P | S | Ca | Mn | Fe | Co | Ni | Zn | Se |
|---|---|---|---|---|---|---|---|---|---|---|
| P | 0.5926 | | | | | | | | | |
| S | 0.9306* | 0.6698* | | | | | | | | |
| Ca | 0.4576 | 0.0736 | 0.4517 | | | | | | | |
| Mn | 0.5355 | 0.3277 | 0.5651 | 0.6195* | | | | | | |
| Fe | 0.3355 | 0.2926 | 0.4719 | 0.3917 | 0.2771 | | | | | |
| Co | 0.1358 | 0.2949 | 0.9603* | 0.8577* | –0.2391 | 0.6282* | | | | |
| Ni | 0.0501 | 0.2359 | 0.1558 | 0.4839 | 0.0484 | 0.4318 | 0.4309 | | | |
| Zn | 0.3417 | 0.1561 | –0.3505 | –0.2119 | 0.5241 | 0.4884 | 0.2198 | 0.4935 | | |
| Se | 0.8587* | 0.4229 | 0.7237* | 0.6435* | 0.4841 | 0.0584 | 0.5002 | 0.0085 | 0.4061 | |
| Mo | 0.4008 | 0.7075* | 0.6786* | 0.0087 | 0.3279 | 0.6449* | 0.2617 | 0.2835 | 0.2312 | 0.1605 |

* Essential at $p < 0.05$.

## Conclusion

In the fruits of repair raspberries, 12 mineral elements have been identified, the content of which varies depending on the genotype.

The genetic sources of high total accumulation of macro- and microelements in the berries are Pingvin, Yubileynaya Kulikova, Medvezhonok, Elegantnaya, Zhar-Ptiza, Atlant, Brice, Poranna Rosa, Erika, Enrosadira and Heritage.

In the selection it is proposed to use the cultivars Medvezhonok, Zhar-Ptiza and Atlant as the sources of increased content of Mg, Mo and Se; the cultivar Yubileynaya Kulikova as the source of accumulation of Ca, Mo and Fe; the cultivars Heritage, Samorodok and Atlant as the source of high content of Zn.

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# Metabolomic profiles of *Ribes nigrum* L. and *Lonicera caerulea* L. from the collection of the N.I. Vavilov Institute in the setting of Northwest Russia

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**Abstract.** Recently, the trend of using fruit and berry crops as ingredients for functional and dietary nutrition, the development and implementation of flavors, pigments, new medicines and dietary supplements has been actualized. Because the direction of use depends on the biochemical properties of fruits, which are determined not only by species and varietal characteristics, but also by reproduction conditions, the study of the biochemical composition of fruits grown in various regions of the world continues to be relevant. In this regard, the collection of N.I. Vavilov Institute (VIR), which has a wide diversity of fruit and berry crops, is of great interest for study. *Ribes nigrum* fruits have a balanced set of sugars, organic acids, essential oils, microelements, a high content of vitamins, anthocyanins, pectins. *Lonicera caerulea* fruits are characterized by high values of phenolic substances: bioflavonoids, hydroxycinnamic acids, flavonols, polyphenols, anthocyanins, as well as vitamins, carotenoids, iridoid glycosides and other natural antioxidants. The investigation of *L. caerulea* and *R. nigrum* fruit's accessions from the VIR collection using gas-liquid chromatography with mass spectrometry allows us to obtain new information about the biochemical characteristics of fruits, to identify *L. caerulea* and *R. nigrum* varieties with optimal economically valuable characteristics, to determine the specificity of *L. caerulea* and *R. nigrum* metabolomic spectra in the setting of Northwest Russia. As a result of the analysis, typical compounds of the metabolomic profile of each culture were identified. Organic acids, phenol-containing compounds and polyols prevailed in *L. caerulea*, while mono- and oligosaccharides, in *R. nigrum*. The qualitative composition of the black currant varieties 'Malen'kii Printz', 'Dobriyi Dzhinn', 'Tisel', 'Orlovskii Val's', and blue honeysuckle 'S 322-4', 'Malvina', 'Leningradsky Velikan' was optimal for food consumption; the varieties of blue honeysuckle 'Bazhovskaya' and black currant 'Aleander' had a good representation of biologically active compounds, which makes samples attractive as raw materials for the production of biologically active additives, including with the use of microorganisms' cultures.
Key words: *Ribes nigrum* L.; *Lonicera caerulea* L.; VIR collection; nonspecific metabolomic profiling; gas-liquid chromatography; mass spectrometry; fruit crops; biologically active substances.

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# Метаболомные профили *Ribes nigrum* L. и *Lonicera caerulea* L. из коллекции ВИР им. Н.И. Вавилова в условиях Северо-Запада РФ

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**Аннотация.** В последнее время актуализируются тенденция использования плодово-ягодных культур как ингредиентов для функционального и диетического питания, разработка и внедрение ароматизаторов, пигментов, новых лекарственных препаратов и биологически активных добавок. Направление применения зависит от биохимических характеристик плодов, которые обусловлены не только видовыми и сортовыми особенностями, но и условиями репродукции, поэтому исследование биохимического состава плодовых, выращенных в различных регионах мира, продолжает быть актуальным. В этой связи коллекция ВИР им. Н.И. Вавилова, обладающая широким разнообразием плодово-ягодных культур, представляет значительный интерес для изучения. Плоды *Ribes nigrum* отличаются сбалансированным набором сахаров, органических кислот, эфирных масел, микроэлементов, высоким содержанием витаминов, антоцианов, пектинов. Для плодов *Lonicera caerulea* характерны высокие значения фенолсодержащих веществ: биофлавоноидов, оксикоричных кислот, флавонолов, полифенолов, антоцианов, а также витаминов, каротиноидов, иридоидных гликозидов и других природных антиоксидантов. Исследование

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*L. caerulea* и *R. nigrum* из коллекции ВИР с применением газожидкостной хроматографии, сопряженной с масс-спектрометрией, позволяет получить новые сведения о биохимических характеристиках плодов, выделить сорта *L. caerulea* и *R. nigrum* с оптимальными хозяйственно ценными признаками, выявить специфику метаболомных спектров *L. caerulea* и *R. nigrum* в условиях Северо-Запада Российской Федерации. В результате анализа идентифицированы соединения, характерные для метаболомного профиля каждой из культур. У *L. caerulea* превалировали органические кислоты, фенолсодержащие соединения, полиолы, у *R. nigrum* – моно- и олигосахара. Качественный состав сортов черной смородины «Маленький Принц», «Добрый Джинн», «Tisel», «Орловский Вальс» и жимолости синей «С 322-4», «Мальвина», «Ленинградский Великан» оказался оптимальным для пищевого употребления. Сорта жимолости синей «Бажовская» и черной смородины «Алеандр» с хорошей представленностью биологически активных соединений привлекательны в качестве сырья для производства биологически активных добавок, в том числе с использованием культур микроорганизмов.
Ключевые слова: *Ribes nigrum* L.; *Lonicera caerulea* L.; коллекция ВИР им. Н.И. Вавилова; неспецифическое метаболомное профилирование; газожидкостная хроматография; масс-спектрометрия; плодовые культуры; биологически активные вещества.

## Introduction

Fruit crops represent a rich source of bioactive substances (BAS) with a broad range of properties beneficial for humans (Kylli, 2011). Recently, the trend of using fruit and berry crops as ingredients of functional and dietary foods, as well as for the development and introduction of flavors, pigments, new drugs and BAS has been gaining relevance (Konarev, Khoreva, 2000; Dudnik et al., 2018; Thole et al., 2019). The application depends on the biochemical characteristics of fruits, which are determined not only by characteristics of a species or variety, but also by the regeneration conditions, therefore, the study of the biochemical composition of fruits grown in different regions of the world maintains its relevance (Sochor et al., 2014; Gołba et al., 2020). In this regard, the collection of the N.I. Vavilov Institute of Plant Genetic Resources (VIR) that contains a wide range of fruit and berry crops is of interest for research.

*Ribes nigrum* is one of the most popular berry crops (Vitkovsky, 2003; Pikunova et al., 2011). To date, more than 1200 blackcurrant varieties have been bred and are cultivated (Knyazev, Ogoltsova, 2004). *Lonicera caerulea* has attracted attention relatively recently; its breeding has been actively developed since the 1940s–1950s. A great contribution to the promotion of blue honeysuckle was made by Prof. M.N. Plekhanova (VIR), the author of 24 varieties of *L. caerulea* (Plekhanova, 1992, 2000, 2007; Plekhanova, Streltsyna, 1998). The fruits of *R. nigrum* have a balanced set of sugars and organic acids, as well as a high content of vitamin C and dietary fiber (Dudnik et al., 2018; Thole et al., 2019; Tian et al., 2019). Honeysuckle is characterized by a high content of phenol-containing compounds (PCCs): bioflavonoids, hydroxycinnamic acids, flavonols, polyphenols, anthocyanins and other natural antioxidants. Also, the presence of iridoids is noted in its fruits (Senica et al., 2018; Gołba et al., 2020).

The purpose of the present study was to use gas-liquid chromatography coupled with mass spectrometry to obtain new information about the biochemical composition of fruits of *R. nigrum* and *L. caerulea* and to reveal the specifics of metabolomic profiles of fruits grown in conditions of the Leningrad Province, to identify varieties with optimal economically important characteristics, determine the prospects for the possible use of the selected accessions as raw material for expanding the range of products for functional and therapeutic nutrition, for the production of bioactive additives, as well as for breeding aimed at creating varieties that combine nutritional qualities with resistance to environmental stress factors.

## Materials and methods

The study was carried out on fruits of 20 *R. nigrum* and 10 *L. caerulea* accessions from the VIR collection grown in 2014 at the "Pushkin and Pavlovsk Laboratories of VIR" Research and Production Base located 30 km south of St. Petersburg. The blackcurrant varieties of Russian and foreign origin taken into the study included 'Azhurnaya', 'Muravushka', 'Orlovskii Val's', 'Orlovskaya Serenada', 'Malen'kii Printz', 'Charovnitsa', 'Syuita Kievskaya', 'Cherechneva', 'Krasa L'vova', 'Ukrainka', 'Aleander', 'Pamyati Potapenko', 'Zhuravushka', 'Mila', 'Dobriyi Dzhinn', 'Slavyanka', 'Biryusinka', 'Volshebnitsa', 'Margo', and 'Tisel', and those of blue honeysuckle included 'Avacha', 'Start', 'Leningradsky Velikan', 'S 322-4', 'Malvina', 'Morena', 'Bazhovskaya', 'Suvenir', 'Solovey', and '838-12'. The material was grown according to the technique of E.N. Sedov and T.P. Ogoltsova (1999). Meteorological conditions during the study were assessed as favorable for the vegetation of plants.

Each accession was represented by an average 50 g sample of fruits collected from three bushes at the stage of technical ripeness. The fruits were crushed in a Waring 800S laboratory blender (USA) in 100 mL of methanol (for HPLC, Vecton), centrifuged, and the supernatant was evaporated to dryness. The dry residue was silylated in 20 μL of bis(trimethylsilyl) trifluoroacetamide on Digi-Block (USA) for 15 min at 100 °C. The analysis was carried out in three analytical replications using an Agilent 6850A chromatograph coupled with an Agilent 5975 mass selective detector (USA) according to a protocol by Perchuk et al. (2020).

The obtained results were processed in the UniChrom and AMDIS programs using the NIST 2010 mass spectra libraries and in-house libraries of the Science Park of the St. Petersburg University and the V.L. Komarov Botanical Institute of the Russian Academy of Sciences (Puzanskiy et al., 2018; Shtark et al., 2019). The concentration was calculated in accordance with the recommendations by Worley and Powers (2013). The analytical data are presented in ppm (mg/kg) (Perchuk et al., 2020). The data were statistically processed in Statistica 7 and Excel 7.0 for Windows using factor analysis by the method of principal components and one-way analysis of variance.

## Results

The analysis of metabolomic profiles (MPs) of blackcurrant and honeysuckle has shown the presence of over 500 substances; less than 100 of them were precisely identified, and their indicators are presented in the article. In total, blackcurrant MPs were found to contain 88 and those of blue honeysuckle 75 components which belong to organic acids (39 and 29, respectively), free amino acids (2 and 3), polyols (6 and 7), free fatty acids (6 and 4), mono- and oligosaccharides (10 and 10; 4 and 5), sugar derivatives (7 and 4), and phenol-containing compounds (14 and 11, respectively). In addition to the above substances, honeysuckle MPs contain choline and a purine derivative (1,2,3,6-tetrahydropurine-2,6-dione) (Suppl. Material 1)[1].

The content of organic acids (ppm) in the studied honeysuckle fruit samples varied among varieties in the range from 78383.85 (S 322-4) to 29311.7 (Leningradsky Velikan), that of free amino acids from 705.2 (Malvina) to 32.4 (S 322-4), of polyols from 68035.7 (Bazhovskaya) to 36966.9 (Avacha), of pentoses from 8454.2 (S 322-4) to 2960.3 (Morena), of hexoses from 357246.3 (S 322-4) to 171672.8 (Avacha), of oligosaccharides from 63824.1 (Leningradsky Velikan) to 7053.9 (Solovey), of glycosides from 3111.4 (Bazhovskaya) to 449.5 (Start), of free fatty acids from 588.4 (S 322-4) to 130.7 (838-12), and of PCCs from 29353.3 (Bazhovskaya) to 11001.2 (Start).

In blackcurrant fruits, the range of variability was wider (ppm) for the following groups of compounds: from 110551.4 (Aleander) to 13743.7 (Orlovskii Val's) for organic acids, from 72586.1 (Malen'kii Printz) to 2938.8 (Ukrainka) for polyols, from 2865.9 (Orlovskii Val's) to 357.5 (Volshebnitsa) for free fatty acids, from 706650.7 (Malen'kii Printz) to 111403.2 (Aleander) for hexoses, and from 321665.0 (Tisel) to 16001.9 (Aleander) for oligosaccharides. A narrower range was recorded for free amino acids: from 439.4 (Dobriyi Dzhinn) to 95.2 (Slavyanka), from 5841.0 (Malen'kii Printz) to 1929.41 (Orlovskii Val's) for pentoses, from 7087.0 (Malen'kii Printz) to 1432.1 (Orlovskii Val's) for PCCs, and from 4082.6 (Aleander) to 1167.8 (Orlovskii Val's) for sugar derivates (Fig. 1).

The metabolomic profiles of *R. nigrum* and *L. caerulea* differed in terms of representation of different groups of compounds. Mono-, oligosaccharides, free fatty acids, and lactone forms of organic acids dominated in blackcurrant MPs, while organic acids, polyols, PCCs, and free amino acids dominated in honeysuckle (see Fig. 1, Suppl. Material 1). Sugar derivatives were present in almost equal amounts in the MPs of these berry crops (see Fig. 1). The content of organic acids was higher in honeysuckle MPs due to significant amounts of malic and quinic acids (see Fig. 1, Suppl. Material 1).

Malic and glucuronic acids dominated in blackcurrant fruits (17501.9 and 4271.6, respectively), while glucono-1,4-lactone (802.6) dominated among lactones, dulcitol and *myo*-inositol (28551.3 and 1513.2) among polyols, oleic and vaccenic acids (213.3 and 211.2) among free fatty acids, fructose, glucose, galactose, sorbose (192582.1, 151908.7, 20264.5, 2847.6) among monosaccharides, D-6-deoxy mannopyranoside-α-L galactofuranose (1526.9) among sugar derivatives, and shikimic acid and quercetin (451.4 and 278.6 ppm) among PCCs. The fruits of honeysuckle showed the dominance of malic and quinic acids (19124.6 and 12936.3, respectively), threono-1,4-lactone (265.9), dulcitol and mannitol (27526.1 and 12983.1), palmitic acid (111.0), fructose, glucose, galactose, arabinose (122033.3, 110907.9, 18046.9 and 3165.4), 2-O-glycerol-α-galactopyranoside, quinic acid and antirrinoside (2392.3, 12936.3 and 1209.0 ppm, respectively). In currant and honeysuckle MPs, hydroxyproline prevailed in the group of free amino acids (203.6 and 254.6 ppm, respectively), and sucrose dominated in the group of disaccharides (139416.6 and 39660.7) (see Suppl. Material 1).

An average degree of variability (20–33 %) in blackcurrant MPs was established for succinic and threonic acids, for ribose and gallocatechin, while in honeysuckle MPs it was established for lactic, phosphoric, succinic, erythronic, threonic, glyceric, aconitic acids, for dulcitol, erythritol, *myo*-inositol, ribose, fructose, sorbose, galactose, mannose, glucose, glycerol-3-phosphate, arbutin, and 1,2,3,6-tetrahydropurine-2,6-dione.

A high degree of variability (33–60 %) in blackcurrant MPs was determined for fumaric, malic, erythronic, ribonic, quinic, 4-hydroxycinnamic, ascorbic, gallic, palmitic acids, for erythrono-1,4-, threono-1,4-, xylonolactones, leucine, oxyproline, *myo*-inositol, galactinol, glyceroaldehyde, arabinose, fructose, galactose, mannose, glucose, melibiose, sucrose, stachyose, glycerol-3-phosphate, α-methyl glucofuranoside, methylrutinose, 6-deoxy-mannopyranoside-α-galactofuranose, catechin, epigallocatechin, and quercetin. In honeysuckle, a high degree of variability was found for fumaric, maleic, ribonic, quinic, glucuronic, 2-keto-gluconic, caffeic, oxalic, benzoic, palmitic, stearic acids, for chlorogenic acid and its isomers, glucono-1,4-lactone, arabinitol, mannitol, quercetin, glyceraldehyde, arabinose, xylose, sucrose, rutinose, turanose, and α-methyl glucofuranoside.

A very high degree of variability (above 60 %) in blackcurrant MPs was noted for lactic, nicotinic, citraconic, glyceric, aconitic, glucuronic, 2-keto-gluconic, caffeic, galactopyruronic, palmitic, vaccenic acids, for glycerol, isomers of inositol, sorbose, 2-O-glycerol-α-galactopyranoside, α-tocopherol, scopolin, while in honeysuckle MPs it was noted for pyruvic, nicotinic, citraconic, malic, protocatechuic, α-ketoglutaric, pipecolic, linoleic, oleic acids, for threono-1,4-lactone, glucono-6-phosphate, galactinol, raffinose, maltose, α-methyl glucofuranoside, 2-O-glycerol-α-galactopyranoside, catechin, and antirrhinoside.

The main part of the blackcurrant MP components had a high degree of variability, while the honeysuckle MP components split into almost equal groups with a slight margin in favor of those with a coefficient of variation (CV) above 33 % (Suppl. Material 2).

The metabolomic profiles of black currant and honeysuckle differed from each other in a number of parameters. The MPs of blackcurrant demonstrated significantly higher ($p = 0.05$) values of organic acids (pyruvic, phosphoric, nicotinic, fumaric, threonic, 4-hydroxybenzoic, maleic, arabic, ribonic, shikimic, gluconic, 4-hydroxycinnamic, ascorbic, and gallic acids), of lactone forms of arabic and xylonic acids, erythrono-1,4-lactone, threono-1,4-lactone, 1,4-3-ols (gallocatechin, epigallocatechin), flavonols (quercetin), and oxycoumarins (scopoline). In the honeysuckle MPs, significantly higher

[1] Supplementary Materials 1–4 are available in the online version of the paper: http://vavilov.elpub.ru/jour/manager/files/Suppl_Shelenga_27_7.pdf

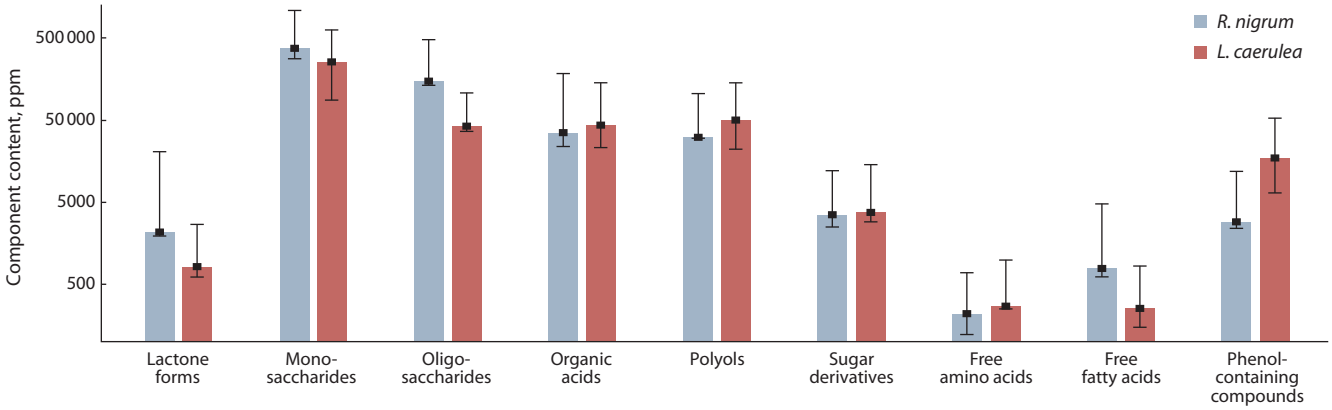


**Fig. 1.** Main groups of compounds identified in the metabolomic profiles of fruit samples of *R. nigrum* and *L. caerulea* from the VIR collection.

values were observed for succinic, erythronic, glyceric, aconitic, oxalic, protocatechuic, quinic, benzoic, α-ketoglutaric, chlorogenic stearic, and pipecolic acids, for isomers of chlorogenic acid, glucono-6-phosphate, polyols (erythritol, arabinitol, mannitol, *myo*-inositol), monosaccharides (glycerol-3 phosphate, arabinose, mannose), oligosaccharides (rutinose, maltose, and turanose), sugar derivatives (α-methyl pentafuranoside and 2-O-glycerol-α-galactopyranoside), flavonoids (catechin and kaempferol), glycosides (arbutin, antirrinoside, ammonium base of choline), and purine derivative 1,2,3,6-tetrahydropurine-2,6-dione. A lower degree of reliability ($0.1 > p > 0.05$) was demonstrated by the differences between MPs of *R. nigrum* and *L. caerulea* in terms of lactic, citraconic, galactopyranuronic acids, glyceraldehyde, sorbose, glucose, and α-tocopherol (see Suppl. Material 2).

Quantitative and qualitative differences in the MPs reflect the peculiarities of metabolism in the fruits of *R. nigrum* and *L. caerulea*. The process of accumulation of ascorbic acid, glucuronic acids, monosaccharides, especially of pentoses, fructose, mannose, galactose, as well as metabolism of free fatty acids, the Krebs cycle, glycolysis and pentose phosphate cycle are more intense in blackcurrant. The conversion of lysine along with the accumulation of pipecolic acid, the glyoxylate pathway, the exchange of phosphoric acid (phosphotransferase system) and purine bases, the synthesis of secondary metabolites (phenylpropanoids, flavonoids: flavones and flavonols) are more intense in honeysuckle. The latter is confirmed by an increase in the fraction of secondary metabolites in honeysuckle MPs up to 4.1 % compared to that in currant MPs (less than 0.5 %).

The sugars to organic acids ratio in blackcurrant and honeysuckle fruits was 15 and 7, respectively, i. e., the sugar-acid index of *R. nigrum* is optimal for food consumption. Honeysuckle is distinguished by high values of bioactive compounds, which makes the crop attractive as a raw material for the production of BAS, including the use of microorganism cultures.

Blackcurrant fruits contain more bioactive lactone forms of acids, mono- and oligosaccharides, which affect the taste quality of berries. The group of PCCs in blackcurrant has a better representation of 4-hydroxybenzoic, gallic, shikimic, and hydroxycinamic acids, of epigallocatechin, quercetin, α-tocopherol, scopolin, while in blue honeysuckle these are benzoic, protocatechuic, quinic, and chlorogenic acids, isomers of chlorogenic acid, catechin, arbutin, antirrinoside and kaempferol. Phenol-containing substances are anti-stress factors that constitute a part of the antioxidant defense system of plants. Most of the identified osmoprotective polyols are characteristic of honeysuckle MPs, while oligosaccharides with similar properties are typical of blackcurrant MPs. Free fatty acids can also be an evidence of protective mechanisms, since they indirectly reflect the activity of lipid synthesis, which are part of the membrane complex. The honeysuckle MPs were found to contain such an anti-stress factor as a non-protein pipecoline amino acid. A relatively low content of organic acids and the high content of sugars in the MPs of blackcurrant fruits, which influences the palatable attractiveness of fruits, may be associated with the breeding process aimed at improving the nutritional qualities of the created varieties.

The canonical discriminant analysis of the obtained results confirms the difference between *R. nigrum* and *L. caerulea* species at the MP level. The most 'informatively valuable' traits that confirmed the individuality of MPs of *R. nigrum* and *L. caerulea* with an accuracy up to 98 %, were indicators of phosphoric, nicotinic, succinic, 4-hydroxybenzoic, glyceric, arabic, ribonic, protocatechuic, ascorbic, gallic, caffeic, oxalic, benzoic acids and glyceraldehyde. These compounds are involved in the main reactions of primary and secondary metabolism in plant tissues, i. e. the Krebs cycle, redox reactions, glyoxylate cycle, glycolysis, and shikimate pathway of PCC biosynthesis (Fig. 2, Suppl. Material 3). The histogram of the canonical variable eigenvalues distribution shows that the value approaches –100 for *R. nigrum* accessions and 200 for *L. caerulea* (see Fig. 2, Suppl. Material 3).

The cluster analysis using the Ward method, taking into account all the identified compounds, showed that the honeysuckle accessions were divided into two clusters (Suppl. Material 4, *a*). The first one consisted of two subclusters, one of which included accessions with a predominance of polyols and oligosaccharides in the MPs ('Leningradsky Velikan' and '838-12'), while the other included those with a predominance of free amino acids ('Avacha', 'Start', 'Suvenir', 'Malvina', and 'Morena'). The next cluster was formed by honeysuckle varieties with high levels of organic acids, pentoses, hexoses, glycosides, free fatty acids, and PCCs ('Bazhovskaya', 'S 322-4', and 'Solovey'). Black currant varieties with high

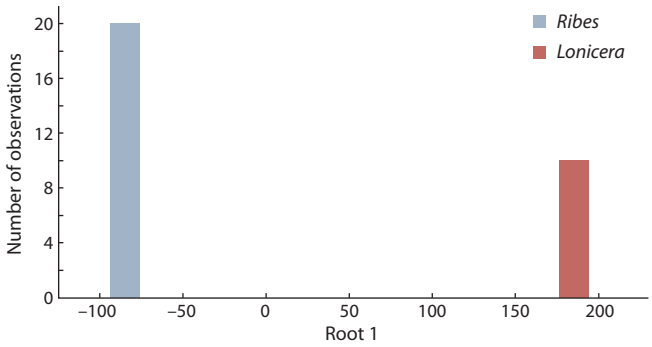


**Fig. 2.** Histogram of *R. nigrum* and *L. caerulea* fruit samples distribution according to the magnitude of the eigenvalues of the canonical variable.

levels of organic acids and sugar derivatives ('Aleander', 'Orlovskaya Serenada', and 'Charovnitsa') were included in the same cluster with blue honeysuckle accessions. The other blackcurrant varieties formed their own cluster, divided into two subclusters. The first included the bulk of the accessions with a high content of free amino acids, free fatty acids, pentoses, hexoses, polyols, and PCCs ('Mila', 'Volshebnitsa', 'Malen'kii Printz', 'Azhurnaya', 'Zhuravushka', 'Orlovskii Val's', 'Muravushka', 'Krasa L'vova', 'Cherechneva', 'Biryusinka', and 'Slavyanka'), while the second one united accessions with high values of free amino acids and oligosaccharides ('Dobriyi Dzhinn', 'Margo', 'Syuita Kievskaya', 'Pamyati Potapenko', 'Tisel', and 'Ukrainka').

A more precise separation of *R. nigrum* and *L. caerulea* accessions was achieved by taking into account the results of the PCC group (Suppl. Material 4, *b*). Blackcurrant and blue honeysuckle accessions formed two separate clusters, each of which, in turn, was divided into two subclusters. The first subcluster consisted of honeysuckle accessions with high levels of flavones and phenylpropanoids ('Leningradsky Velikan', 'Solovey', and '838-12'); while the second one included those with high levels of glycosides, flavan-3-ols, flavanones, and benzoic acid derivatives ('Avacha', 'Start', 'Suvenir', 'Malvina', 'Morena', 'Bazhovskaya', and 'S 322-4'). A separate subcluster was formed by blackcurrant varieties with high values of all the identified PCCs ('Tisel', 'Charovnitsa', 'Margo', 'Pamyati Potapenko', 'Cherechneva', and 'Malen'kii Printz').

The study has identified blue honeysuckle varieties with a high content of certain groups of compounds: 'Bazhovskaya' (PCCs), 'S 322-4' (organic acids, free fatty acids and monosaccharides), 'Leningradsky Velikan' (oligosaccharides), 'Malvina' (free amino acids), and blackcurrant varieties: 'Malen'kii Printz' (monosaccharides, PCCs, and polyols), 'Dobriyi Dzhinn' (free amino acids), 'Tisel' (oligosaccharides), 'Orlovskii Val's' (free fatty acids), and 'Aleander' (organic acids and sugar derivates).

## Discussion

We compared our data with the results of other studies. The current experiment confirmed that the total content of phenolic compounds in honeysuckle fruits is higher and their qualitative composition is different from other crops, which was previously established by VIR researchers (Streltsina et al., 2005–2007). It was also noted in the mentioned works that the high content of phenolic compounds in honeysuckle is due to its recent inclusion in the breeding process and the great similarity of the created varieties of this crop with its wild relatives. This is also confirmed by our data.

In contrast to the results obtained by Sochor et al. (2014) and Gołba et al. (2020), according to which hydroxycinnamic acids and flavonols dominate among the PCCs of *L. caerulea*, quinic acid was best represented in this group of compounds in our study, and the content of chlorogenic acid and its derivatives was significantly lower. The data on the iridoid glycoside (antirrhinoside) identified in the honeysuckle fruits studied in the present work are consistent with the results of Senica et al. (2018) and Gołba et al. (2020), but contradict those of Sochor et al. (2014). We identified only hydroxyproline and leucine in the group of free amino acids, which disagrees with the study by Sochor et al. (2014). The composition of organic acids and sugars in the honeysuckle fruit samples studied by us corresponds to the data from the works by Rop et al. (2011), Sochor et al. (2014), Senica et al. (2018), Gołba et al. (2020), and Juríková et al. (2020).

The publications of VIR researchers (Streltsina et al., 2005; Tikhonova, Streltsina, 2009, 2012; Streltsina, Tikhonova, 2010; Tikhonova et al., 2015) report on such economically important features of blackcurrant as the optimal sugar-acid index and high pectin values, which is confirmed by our results concerning the ratio of sugars and acids in the fruits of *R. nigrum* and *L. caerulea*, and the presence of uronic acids in the MPs of *R. nigrum*. According to Lee et al. (2015), and Tian et al. (2019), fructose, galactose, and glucose predominate among monosaccharides at the technical ripeness stage. Similar results were obtained in our work. According to H.J. Lee and colleagues, malonic acid dominated in the group of acids, sorbitol in the group of polyols, and quercetin and kaempferol in that of phenolic substances (Lee et al., 2015). However, this is inconsistent with our data. The paper by Tian et al. (2019) names citric and malic acids as the main organic acids in blackcurrant fruits, anthocyanins and flavanols as the main phenolic compounds, and hydroxycinnamic acids as the main phenolic acids. It was established by P.H. Mattila and colleagues that, in addition to anthocyanins, the dominant phenolic compounds in black currant are such flavonols as mirecetin and quercetin (Mattila et al., 2016). Concerning the samples studied in the present research, malic and glucuronic acids predominated in the group of organic acids, hydroxycinnamic acids and their derivatives (chlorogenic acids) in the group of phenolic acids, and shikimic acid and flavonol quercetin dominated among the PCCs. A comparative analysis of the data obtained by us with the results of other researchers revealed a number of discrepancies associated with differences in conditions for the material regeneration and methodological approaches chosen for the study. In the papers mentioned above, the authors underline the dependence of the biochemical composition of fruits on growing conditions (region), which confirms the relevance of our work (Rop et al., 2011; Sochor et al., 2014; Lee et al., 2015; Mattila et al., 2016; Senica et al., 2018; Tian et al., 2019; Gołba et al., 2020; Juríková et al., 2020).

The study of *R. nigrum* and *L. caerulea* accessions from the VIR collection within the framework of the joint international BacHBerry project confirmed the use of honeysuckle

as a donor of genes controlling the biosynthesis of secondary metabolites to be promising for the creation of microbiological producers of natural bioactive substances (Thole et al., 2019).

## Conclusion

The performed work made it possible to define features of metabolomic profiles of *R. nigrum* and *L. caerulea* berry crops grown in conditions of the Leningrad Province, to identify varieties with economically important traits, suitable for expanding the range of functional, therapeutic and prophylactic food products ('S 322-4', 'Leningradsky Velikan', 'Malvina', 'Malen'kii Printz', 'Dobriyi Dzhinn', 'Tisel', and 'Orlovskii Val's'), for producing bioactive supplements and medicines based on natural bioactive substances ('Bazhovskaya', 'Aleander'), and for breeding aimed at creating varieties that combine nutritional advantages with resistance to environmental stress factors ('Bazhovskaya', 'S 322-4', 'Leningradsky Velikan', 'Malen'kii Printz', 'Tisel', and 'Aleander').

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# Polyphenols of *Perilla frutescens* of the family Lamiaceae identified by tandem mass spectrometry

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**Abstract.** *Perilla frutescens* is mainly cultivated as an oilseed crop. Perilla seeds contain 40–53 % of oil, 28 % of protein. The growing season is 100–150 days. In Russia, perilla is grown in the Far East, where the yield is 0.8–1.2 t/ha. Perilla of different geographical origin has its own special, sharply different features that characterize two geographical groups: Japanese and Korean-Chinese. These groups differ from each other in the length of the growing season, the height of plants, the color of the stem, the surface and the size of the leaves, the shape of the bush, the shape and size of the inflorescences, the size of the cups, the size and color of the seeds. *P. frutescens* contains a large number of polyphenolic compounds that are biologically active components. The purpose of this research was a metabolomic study of extracts from leaves of *P. frutescens* obtained from the collection of Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources, grown on the fields of the Far East Experiment Station – Branch of Federal Research Center (Primorsky Krai, Russia). To identify target analytes in extracts, HPLC was used in combination with an ion trap. Preliminary results showed the presence of 23 biologically active compounds corresponding to *P. frutescens*. In addition to the reported metabolites, a number of metabolites were newly annotated in *P. frutescens*. There were hydroxycoumarin Umbelliferone; triterpene Squalene; omega-3 fatty acid Stearidonic [Moroctic] acid; higher-molecular-weight carboxylic acid: Tetracosenoic acid and Salvianic acid C; lignan Syringaresinol and cyclobutane lignan Sagerinic acid, etc. A wide range of biologically active compounds opens up rich opportunities for the creation of new drugs and dietary supplements based on extracts of perilla of the family Lamiaceae, subfamily Lamioideae, tribe Satureji and subtribe Perillinae.
Key words: *Perilla frutescens*; HPLC–MS/MS; tandem mass spectrometry; phenolic compounds; triterpene acids; lignans.

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# Полифенолы *Perilla frutescens* семейства Lamiaceae, идентифицированные с помощью тандемной масс-спектрометрии

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**Аннотация.** *Perilla frutescens* получила применение в основном как масличная культура. Семена периллы содержат 40–53 % масла, 28 % белка. Вегетационный период составляет 100–150 дней. В России периллу выращивают на Дальнем Востоке, где урожайность достигает 0.8–1.2 т/га. Это растение короткого дня, поэтому большинство форм не цветет в условиях средней полосы России. Перилла различного географического происхождения имеет свои особенные, резко отличные признаки, характеризующие две географические группы: японскую и корейско-китайскую. Эти группы различаются длиной вегетационного периода, а также по высоте растений, окраске стебля, поверхности и величине листьев, форме куста, форме и размеру соцветий, величине чашечек и цвету семян. *Perilla frutescens* содержит большое количество полифенольных соединений, которые являются биологически активными компонентами. Цель данной работы состояла в метаболомном исследовании экстрактов из листьев *P. frutescens*, полученных из коллекции Всероссийского института генетических ресурсов растений им. Н.И. Вавилова и выращенных на полях его Дальневосточной опытной станции (Приморский край, Россия). Для идентификации целевых аналитов в экстрактах использовали метод высокоэффективной жидкостной хроматографии в сочетании с ионной ловушкой. Предварительные результаты показали наличие 23 биологически активных соединений, соответствующих виду *P. frutescens*. В дополнение к упомянутым метаболитам, в экстрактах

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*P. frutescens* был впервые обнаружен ряд соединений. Это кумарин умбеллиферон; тритерпен сквален; стеаридоновая кислота; высокомолекулярные карбоновые кислоты: тетракозановая кислота и сальвиановая кислота С; лигнан сирингарезинол; циклобутановый лигнан сагериновая кислота и др. Широкий спектр биологически активных соединений открывает богатые возможности для создания новых лекарственных средств и биологически активных добавок на основе экстрактов периллы семейства Lamiaceae, подсемейства Lamioideae, трибы Satureji и подтрибы Perillinae.
Ключевые слова: *Perilla frutescens*; ВЭЖХ–МС/МС; тандемная масс-спектрометрия; фенольные соединения; тритерпеновые кислоты; лигнаны.

## Introduction

This research presents a detailed study of the metabolomic composition of *Perilla frutescens* leaves. *Perilla frutescens* L. is an annual plant belonging to the mint family Lamiaceae, subfamily Lamioideae, tribe Satureji and subtribe Perillinae (Zhou et al., 2014). Perilla is widely cultivated in Asian countries such as China, Japan, South Korea and India for its oils and leaves used in cooking. Perilla has also been cultivated in Russia in the Far East since the 1930s to obtain high quality oil.

Perilla is a heat-loving and moisture-loving plant. It requires fertile soils. Perilla is a short-day plant, so most forms do not bloom in the conditions of Central Russia or bloom only in late autumn. Perilla of different geographical origin has its own special, sharply different features that characterize two geographical groups: Japanese and Korean-Chinese. These groups differ from each other in the length of the growing season, the height of plants, the color of the stem, the surface and the size of the leaves, the shape of the bush, the shape and size of the inflorescences, the size of the cups, the size and color of the seeds. Perilla leaves are commonly used for their antioxidant, anti-allergic, antimicrobial, anti-tumor and anti-cancer effects due to the presence of phenolic compounds including rosemary acid, essential oil and vitamins (Ahmed, 2019).

The fatty acid composition of perilla oil is characterized by the presence of five main fatty acids. On average, perilla oil contains (% of the total fatty acids): palmitic acid – 5.9, stearic acid – 1.8, oleic acid – 15.3, linoleic acid – 12.4, α-linolenic acid – 61.9. The increased content of polyunsaturated fatty acids – up to 90 % – indicates a high biological activity of perilla oil. By their properties, these acids are close to vitamins (vitamin F), which are not synthesized in the human organism. In terms of the sum of these acids, perilla oil even exceeds many varieties of flax and hemp. It is important to observe the ratio of ω-3 and ω-6 fatty acids in the diet. The optimal ratio of ω-3 and ω-6 fatty acids is 1:4 (Banno et al., 2004; Gu et al., 2009; Meng et al., 2009). Since unsaturated fatty acids and α-linolenic acid are thought to have various beneficial effects on the human health, such as lowering serum cholesterol and triglyceride levels, reducing the risk of colon cancer, and preventing overgrowth of visceral adipose tissue (Longvah et al., 2000), perilla seed oil is considered to be of high quality.

Many bioactive compounds from various chemical groups have been identified from the leaves and seeds of extract of *P. frutescens*. *P. frutescens* is used as a spice as well as in medicine and consists of several chemotypes that refer to the essential oils chemical composition. A chemotype containing perillaldehyde is a major component of the essential oil that is most effective as a sedative in China's traditional medicine. Honda et al. (1986) fractionated MeOH extract of *P. frutescens* to presence of stigmasterol and perillaldehyde. Also, several studies showed the presence of other flavonoids such as apigenin and luteolin, and phenolic compounds such as caffeic acid and rosmarinic acid (Lee et al., 2013; Kauffmann et al., 2016).

Thus, we isolated and investigated the structure of phenolic compounds and triterpenic acids from *P. frutescens* leaves. A total of 23 biologically active compounds: 13 phenolic compounds, omega-3-fatty acids, lignans, sterols and triterpenic acids were identified using tandem ion trap mass spectrometry.

## Materials and methods

*Perilla frutescens* leaves served as the object of the study. The variety 'Novinka' from the collection of Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) was grown on the fields of the Far East Experiment Station – Branch of VIR, Primorsky Territory (N 43°21′34″, E 132°11′19″; yellow-brown soil). This is the only perilla oilseed variety listed in the State Register of the Russian Federation. The variety 'Novinka' is a medium-ripened variety of the Korean-Chinese ecological group with a growing season length of 106 days and an oil content of 49 %, the yield is 0.8–1.2 t/ha.

The leaves were harvested at the end of August, 2020. Weather conditions were favorable for the perilla growth and development. The average air temperature in August was 20 °C, the amount of precipitation was 250 mm. All samples morphologically corresponded to the pharmacopoeial standards of the Pharmacopoeia of the Eurasian Economic Union (2020).

**Chemicals and reagents.** HPLC-grade acetonitrile was purchased from Fisher Scientific (Southborough, UK), MS-grade formic acid was obtained from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water was prepared from a SIEMENS ULTRA clear (SIEMENS water technologies, Germany), and all other chemicals were analytical grade.

**Fractional maceration.** To obtain highly concentrated extracts, fractional maceration was applied. In this case, the total amount of the extractant (ethyl alcohol of reagent grade) was divided into 3 parts and was consistently infused on perilla with the first part, then with the second and third, correspondingly. The infusion time of each part of the extractant was 7 days. Fractional maceration technique was applied to obtain highly concentrated extracts (Azmir at al., 2013). From 300 g of the fresh sample, 50 g of leaves of *P. frutescens* were selected for maceration. The total amount of the extractant (ethyl alcohol of reagent grade) was divided into three parts and consistently infused to the leaves with the first, second and third parts. The solid–solvent ratio was 1:20. The infusion of each part of the extractant lasted 7 days at room temperature.

**Liquid chromatography.** HPLC was performed using Shimadzu LC-20 Prominence HPLC (Shimadzu, Japan)

equipped with a UV-sensor and a Shodex ODP-40 4E reverse phase column for multicomponent mixtures separation. The gradient elution program was as follows: 0.01–4 min, 100 % $C_2H_3N$; 4–60 min, 100–25 % $C_2H_3N$; 60–75 min, 25–0 % $C_2H_3N$; control washing 75–120 min 0 % $C_2H_3N$. The entire HPLC analysis was done with a UV detector at wavelengths of 230 and 330 nm; the temperature corresponded to 17 °C. The injection volume was 1 ml.

**Mass spectrometry.** MS analysis was performed on an ion trap amaZon SL (BRUKER DALTONIKS, Germany) equipped with an ESI source in negative and positive ion mode. The optimized parameters were obtained as follows: ionization source temperature: 70 °C, gas flow: 4 l/min, nebulizer gas (atomizer): 7.3 psi, capillary voltage: 4500 V, end plate bend voltage: 1500 V, fragmentary: 280 V, collision energy: 60 eV. An ion trap was used in the scan range *m/z* 100–1.700 for MS and MS/MS.

Data collection was controlled by Windows software for BRUKER DALTONIKS. All experiments were repeated three times. A four-stage ion separation mode (MS/MS mode) was implemented.

## Results and discussion

Five of the most EtOH extracts of *P. frutescens* were selected. All of them had a rich polyphenolic and triterpene composition. High accuracy mass spectrometric data were recorded on an ion trap amaZon SL BRUKER DALTONIKS equipped with an ESI source in the mode of negative/positive ions. The four-stage ion separation mode (MS/MS mode) was implemented. All the chemical profiles of the samples were obtained by the HPLC – ESI – MS/MS method. A total of 300 peaks were detected in the chromatogram (Fig. 1).

The combination of both ionization modes (positive and negative) in MS full scan mode is giving certainty to the molecular mass determination. The negative ion mode provides the highest sensitivity and results in limited fragmentation making it most suited to infer the molecular mass of the separated polyphenols especially in cases where concentration is low. A tentative identification of compounds was carried out using comparisons of the *m/z* values, the RT and the fragmentation patterns with the $MS^2$ spectral data taken from the literature (Banno et al., 2004; Vallverdu-Queralt et al., 2012; Zhou et al., 2014; Spinola et al., 2015; Cirlini et al., 2016; Pandey et al., 2016; Sharma et al., 2016; Marzouk et al., 2018; Sun L. et al., 2019; Goufo et al., 2020; etc.) or the data bases (MS2T, MassBank, HMDB). A unifying system table of the molecular masses of the target analytes isolated from the EtOH-extract of *P. frutescens* was compiled for ease of identification (see the Table). The 23 compounds are shown in the Table. Some of them belong to different polyphenolic families: anthocyanidins, flavones, hydroxycinnamic acids, hydroxybenzoic acids, lignans.

In addition to the reported metabolites, a number of metabolites were newly annotated in *P. frutescens.* The newly annotated metabolites were hydroxycoumarin Umbelliferone; triterpene Squalene; omega-3 fatty acid: Stearidonic [Moroctic] acid; higher-molecular-weight carboxylic acids: Tetracosenoic acid and Salvianic acid C; cyclobutane lignan Sagerinic acid; sterol 7-oxo-beta-sitosterol [3-Hydroxystigmast-5-en-7-one]; flavone Vicenin-2 [Apigenin-6,8-Di-*C*-Glucoside].

A total of 13 polyphenol compounds have been identified (see the Table). The flavones Chrysoeriol, Diosmetin, Apigenin 7-*O*-glucuronide, Scutellarin, Vicenin-2 have already been characterized as a component of *P. frutescens.* This identification was satisfactory according to the studied references in *P. frutescens* (Yamazaki et al., 2003; Gu et al., 2009; Meng et al., 2009; Zhou et al., 2014), *Triticum aestivum* L. (Di Loreto et al., 2018), apple (Sanchez-Rabaneda et al., 2004), rice (Chen W. et al., 2013), *Mentha* (Xu et al., 2017), *Cirsium japonicum* (Zhang et al., 2014), etc.

The CID-spectrum (collision induced dissociation spectrum) in negative ion modes of Apigenin-7-*O*-glucuronide from extracts of *P. frutescens* is shown in Figure 2. The $[M–H]^-$ ion produced three fragment ions at *m/z* 269.02, *m/z* 341.00, *m/z* 175.03 (see Fig. 2). The fragment ion with *m/z* 269.02 yields two daughter ions at *m/z* 225.04, and *m/z* 149.04. The fragment ion with *m/z* 225.04 yields three daughter ions at *m/z* 224.03, *m/z* 183.00, and *m/z* 132.08. It was identified in the bibliography in extracts from *P. frutescens* (Yamazaki et al., 2003), pear (Sun L. et al., 2019), *Hedyotis diffusa* (Chen X. et al., 2018), *Coriandrum* (Hussein et al., 2018), *Thymus vulgaris* (Justesen, 2000).

The anthocyanin Shisonin [Cyanidin 3-*O*-(6-*O*-para-coumaroyl) glucoside-5-*O*-glucoside] was found in extracts of *P. frutescens* (Fig. 3). The Shisonin CID-spectrum in negative ion mode is shown in Figure 3.

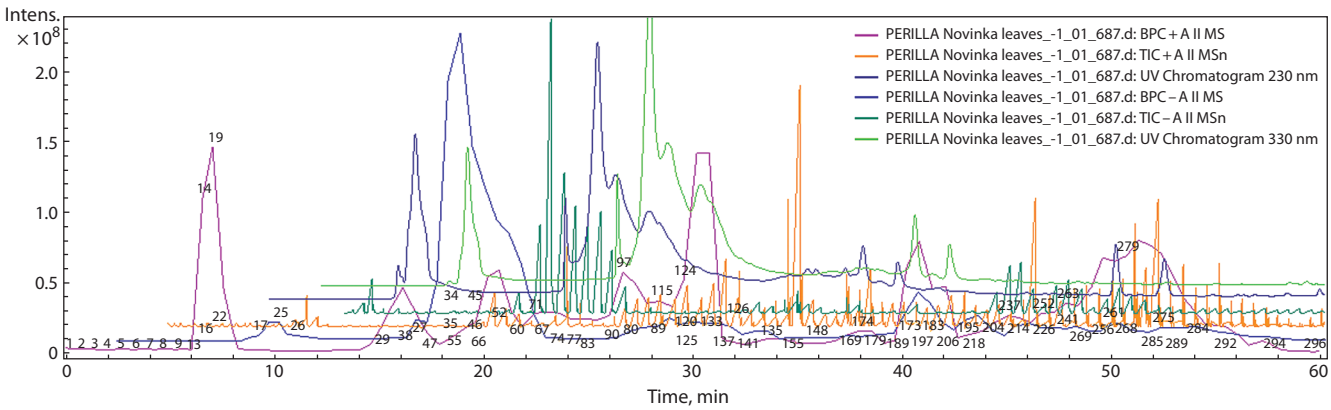


**Fig. 1.** Chemical profiles of the *P. frutescens* sample (Primorsky Territory, Russia) represented in a total ion chromatogram from EtOH-extract.

Biologically active substances identified from the EtOH-extracts of *P. frutescens*

| N | Class of compounds | Identification | Formula | Calculated mass | Observed mass | | MS/MS fragmentation | | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $[M–H]^-$ | $[M+H]^+$ | Stage 1 | Stage 2 | Stage 3 | |
| 1 | Hydroxycoumarin | **Umbelliferone** [Skimmetin; Hydragin] | $C_9H_6O_3$ | **162.1421** | | 163 | 145 | 177 | | *Vitis vinifera* (Goufo et al., 2020), *Sanguisorba officinalis* (Kim et al., 2018), *F. glaucescens* (Hamed et al., 2021) |
| 2 | Hydroxycinnamic acid | **Caffeic acid** [(2E)-3-(3,4-Dihydroxyphenyl) acrylic acid] | $C_9H_8O_4$ | **180.1574** | 179 | | 135 | | | *P. frutescens* (Zhou et al., 2014), tomato (Vallverdu-Queralt et al., 2012), *Mentha* (Chen X. et al., 2017; Marzouk et al., 2018), *Vaccinium macrocarpon* (Abeywickrama et al., 2016), *Rubus occidentalis* (Paudel et al., 2013), *Rhododendron sichotense* (Razgonova et al., 2020) |
| 3 | Trans-cinnamic acid | **Ferulic acid** | $C_{10}H_{10}O_4$ | **194.1840** | 193 | | 161; 134 | 133 | | *P. frutescens* (Peng et al., 2005), *Vaccinium macrocarpon* (Abeywickrama et al., 2016), triticum (Sharma et al., 2016) |
| 4 | Omega-3 fatty acid; octadecatetraenoic acid | **Stearidonic acid** [6,9,12,15-Octadecatetraenoic acid; Moroctic acid] | $C_{18}H_{28}O_2$ | **276.4137** | | 277 | 232; 147 | 119 | | *Salvia miltiorrhiza* (Yang et al., 2015) |
| 5 | Octadec-9-enoic acid | **Oleic acid** (*Cis*-9-Octadecenoic acid; *Cis*-Oleic acid) | $C_{18}H_{34}O_2$ | **282.4614** | 281 | | 163; 135 | 119 | | *P. frutescens* (Longvah et al., 2000), *Sanguisorba officinalis* (Kim et al., 2018) |
| 6 | Monobasic carboxylic acid | **Stearic acid** (Octadecanoic acid; Stearophanic acid) | $C_{18}H_{36}O_2$ | **284.4772** | | 285 | 253; 152 | 193 | | *P. frutescens* (Longvah et al., 2000) |
| 7 | Flavone | **Chrysoeriol** [Chryseriol] | $C_{16}H_{12}O_6$ | **300.2629** | 299 | | 284 | 227; 137 | 199 | Perilla (Gu et al., 2009), apple (Sanchez-Rabaneda et al., 2004), rice (Chen W. et al., 2013), *Mentha* (Xu et al., 2017) |
| 8 | Flavone | **Diosmetin** [Luteolin 4'-Methyl Ether; Salinigricoflavonol] | $C_{16}H_{12}O_6$ | **300.2629** | | 301 | 286; 211 | 168; 121 | 139 | *Triticum turgidum* ssp. *durum* (Di Loreto, 2018), *Cirsium japonicum* (Zhang et al., 2014), *Mentha* (Xu et al., 2017) |
| 9 | Phenylpropanoid (cinnamic acid derivative) | **Rosmarinic acid** | $C_{18}H_{16}O_8$ | **360.3148** | 359 | | 161; 197 | 133 | | *P. frutescens* (Yamazaki et al., 2003; Banno et al., 2004; Zhou et al., 2014), *Rhodiola rosea* (Wang et al., 2007), *Mentha* (Chen X. et al., 2017; Xu et al., 2017), *Salvia miltiorrhiza* (Jiang et al., 2005) |
| 10 | Higher-molecular-weight carboxylic acid | **Tetracosenoic acid** | $C_{24}H_{46}O_2$ | **366.6208** | | 367 | 349; 284; 203; 138 | 135 | | *A. cordifolia* (Hamed et al., 2021) |
| 11 | Higher-molecular-weight carboxylic acid | **Salvianic acid C** | $C_{18}H_{18}O_9$ | **378.3301** | 377 | | 341; 197; 135 | 179 | 149 | *Salvia miltiorrhiza* (Jiang et al., 2005) |

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| 12 | Benzenepropanoic acid | **Ethyl rosmarinate** | $C_{20}H_{20}O_8$ | **388.3680** | 387 | | 341; 207; 163 | 163 | 147 | *Mentha* (Chen X. et al., 2017) |
| 13 | Triterpene | **Squalene** (Trans-Squalene; Spinacene; Supraene) | $C_{30}H_{50}$ | **410.7180** | | 411 | 393; 296; 231; 175 | 175 | | Squalene (Toh et al., 2001; Sun S. et al., 2005) |
| 14 | Lignan | **Syringaresinol** | $C_{22}H_{26}O_8$ | **418.4436** | | 419 | 326 | 298; 254; 218; 174 | 279; 174; 154 | Wheat (Cukelj et al., 2011), *Punica granatum* (Bonzanini et al., 2009), *Xanthium sibiricum* (Kan et al., 2011) |
| 15 | Sterol | **7-oxo-beta-sitosterol** [3-Hydroxystigmast-5-en-7-one] | $C_{29}H_{48}O_3$ | **444.6896** | | 445 | 427; 353; 333; 245; 153 | | | *C. edulis*, *F. pottsii* (Hamed et al., 2021) |
| 16 | Flavone | **Apigenin 7-*O*-glucuronide** | $C_{21}H_{18}O_{11}$ | **446.3610** | 445 | | 269; 175 | 225; 149 | 224; 183; 132 | *P. frutescens* (Yamazaki et al., 2003), pear (Sun L. et al., 2019), *Hedyotis diffusa* (Chen X. et al., 2018), *Coriandrum* (Hussein et al., 2018), *Thymus vulgaris* (Justesen, 2000) |
| 17 | Triterpenic acid | **Oleanoic acid** | $C_{30}H_{48}O_3$ | **456.7003** | | 457 | 439; 369; 277; 203; 145 | 369; 277; 203 | 203 | Pear (Sun L. et al., 2019), *Ocimum* (Pandey, Kumar, 2016) |
| 18 | Triterpenic acid | **Ursolic acid** | $C_{30}H_{48}O_3$ | **456.7003** | | 457 | 439; 387; 277; 203 | 207 | 174 | *P. frutescens* (Banno et al., 2004]) *Ocimum* (Pandey, Kumar, 2016), *Hedyotis diffusa* (Chen X. et al., 2018), pear (Sun L. et al., 2019), *Mentha* (Xu et al., 2017) |
| 19 | Flavone | **Scutellarin** [Breviscapin; Scutellarin-7-glucuronide] | $C_{21}H_{18}O_{12}$ | **462.3604** | | 463 | 287; 445 | 269; 241; 169; 123 | 185; 119 | *P. frutescens* (Yamazaki et al., 2003; Meng et al., 2009) |
| 20 | Triterpenoid | **Corosolic acid** | $C_{30}H_{48}O_4$ | **472.6997** | | 473 | 455; 370; 259; 217; 162 | 437; 358; 323; 264; 233; 193 | 338 | *P. frutescens* (Banno et al., 2004), pear (Sun L. et al., 2019), *Folium eriobotryae* (Li at al., 2015), *Malus domestic*a (Sut et al., 2019) |
| 21 | Flavone | **Vicenin-2** [Apigenin-6,8-Di-*C*-Glucoside] | $C_{27}H_{30}O_{15}$ | **594.5181** | | 595 | 577; 541; 457; 427; 355; 271; 219; 163 | 379; 325 | 351; 308 | *Triticum aestivum* L. (Dinelli et al., 2011), lemon, passion fruits (Spinola et al., 2015), *Mentha* (Marzouk et al., 2018), *P. aculeata* (Hassan et al., 2019) |
| 22 | Cyclobutane lignan | **Sagerinic acid** | $C_{36}H_{32}O_{16}$ | **720.6297** | 719 | | 359; 555 | 161; 197; 133 | 133 | *Mentha* (Cirlini et al., 2016) |
| 23 | Anthocyanidin | **Shisonin** [Cyanidin 3-*O*-(6-*O*-para-coumaroyl) glucoside-5-*O*-glucoside | $C_{36}H_{37}O_{18}$+ | **757.6789** | 756 | | 595; 433; 397; 359; 235 | 217; 179; 135 | 205; 134 | *P. frutescens* (Yamazaki et al., 2003; He et al., 2015) |

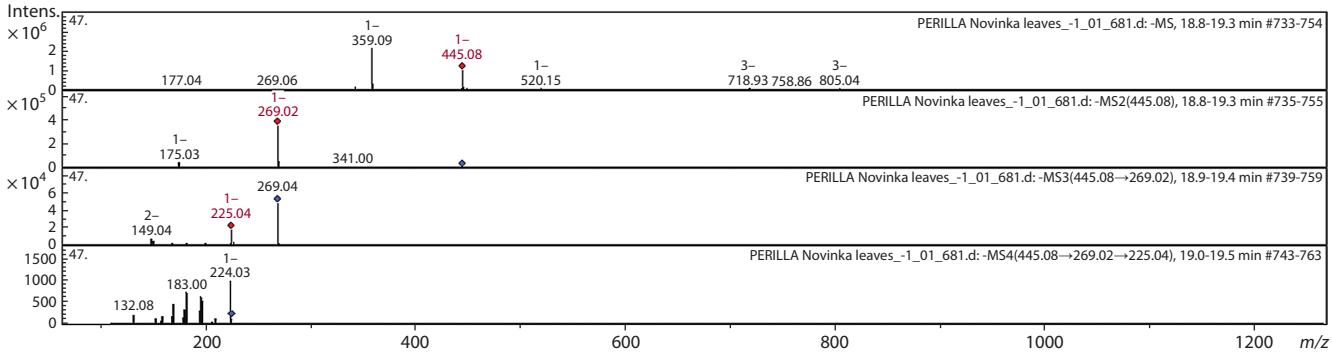


**Fig. 2.** CID-spectrum of Apigenin-7-*O*-glucuronide from extracts of *P. frutescens*, *m/z* 445.08.

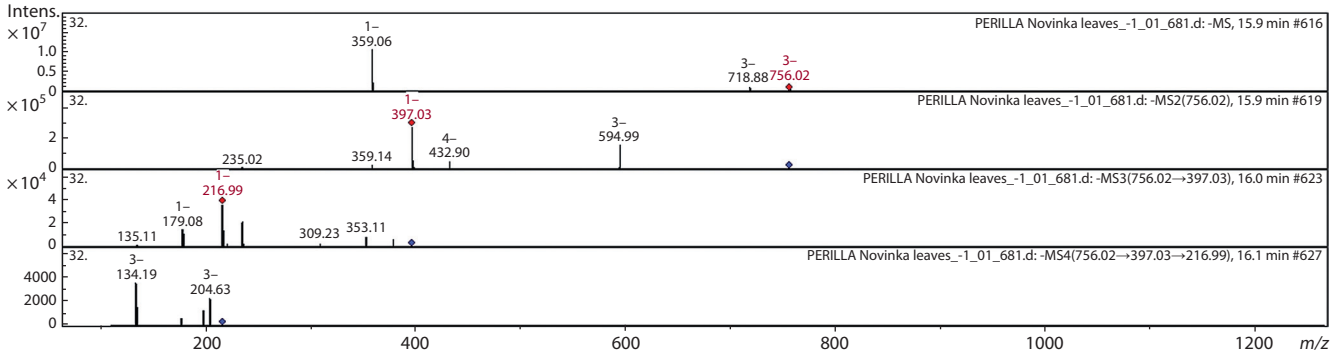


**Fig. 3.** CID-spectrum of [Cyanidin 3-*O*-(6-*O*-para-coumaroyl) glucoside-5-*O*-glucoside] from extracts of *P. frutescens*, *m/z* 756.02.

The $[M–H]^-$ ion produced five fragment ions at *m/z* 397.03, *m/z* 432.90, *m/z* 594.99, *m/z* 359.14, and *m/z* 235.02 (see Fig. 3). The fragment ion with *m/z* 397.03 yields five daughter ions at *m/z* 216.99, *m/z* 309.23, *m/z* 353.11, *m/z* 179.08, and *m/z* 135.11. The fragment ion with *m/z* 216.99 yields two daughter ions at *m/z* 204.63 and *m/z* 134.19. These results were in agreement with bibliography of *P. frutescens* (Yamazaki et al., 2003; He et al., 2015).

## Conclusions

The extracts of *P. frutescens* from the N.I. Vavilov All-Russian Institute of Plant Genetic Resources contain a large number of polyphenolic complexes, which are biologically active compounds. For the most complete and safe extraction, the method of maceration with EtOH was used. To identify target analytes in extracts, tandem mass spectrometry, HPLC and the ion trap were used. The preliminary results showed the presence of 23 bioactive compounds corresponding to *P. frutescens*. In addition to the reported metabolites, a number of metabolites were newly annotated in *P. frutescens* leaves. There were hydroxycoumarin Umbelliferone; triterpene Squalene; omega-3 fatty acid Stearidonic [Moroctic] acid; higher-molecular-weight carboxylic acids: Tetracosenoic acid and Salvianic acid C; lignan Syringaresinol and cyclobutane lignan Sagerinic acid; sterol 7-oxo-beta-sitosterol [3-Hydroxystigmast-5-en-7-one]; benzenepropanoic acid Ethyl rosmarinate; flavones Diosmetin and Vicenin-2 [Apigenin-6,8-Di-*C*-Glucoside].

The findings may support future research into the production of various pharmaceutical and dietary supplements containing *P. frutescens* extracts. A wide variety of bioactive compounds opens up rich opportunities for the creation of new drugs and bioactive additives based on extracts from mint family Lamiaceae, subfamily Lamioideae, tribe Satureji and subtribe Perillinae. In continuation of the study, we are planning to determine the quantitative content of the identified substances.

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# Identification of apple genes *Md-Exp7* and *Md-PG1* alleles in advanced selections resistant to scab

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**Abstract.** The creation of apple varieties with a high level of flesh firmness and long shelf life is one of the important goals in breeding. Among the genes controlling these traits, the role of the endogenous ethylene biosynthesis control gene, *Md-ACS1*, the expansin gene, *Md-Exp7*, and the polygalacturonase gene, *Md-PG1*, has been established. The use of DNA marker analysis to solve problems in breeding for fruit quality traits allows one not only to track several target genes simultaneously, but also to cull plants with undesirable alleles at the early stages of development. In order to select complex donors of breeding traits, molecular genetic identification of the genes that determine the quality traits of apple fruits *Md-Exp7* and *Md-PG1* was performed in 256 breeding selections carrying the scab resistance gene *Rvi6* and valuable allelic variants of the *Md-ACS-1* gene, which determines the endogenous synthesis of ethylene in fruits: 90 samples with the *Md-ACS1* allele (2/2) and 166 samples with *Md-ACS1* (1/2). As a result of the study, an allelic combination for the *Md-Exp7* and *Md-PG1* genes was established. Analysis of the parental cultivars (Renet Simirenko, Modi, Smeralda, Renoir, Fulzhion and Granny Smith) used to obtain hybrid selections revealed three alleles 198, 202, 214 bp according to the DNA marker of the *Md-Exp7* gene. The SSR marker for the *Md-PG1* gene amplified three alleles (289, 292, 298 bp) on the abovementioned cultivars. Within the 256 breeding selections samples that have the most priority for breeding alleles of the desired genes in combination with the *Rvi6* gene and/or with selection-priority allelic variants of the *Md-ACS-1* gene were identified. Of the most valuable for breeding, 46 accessions carrying the combination *Md-Exp7* (202:202) + *Md-ACS1* (2/2) were distinguished. Hybrids with alleles *Md-PG1* (292:292) + *Md-ACS1* (2/2) are also most valuable for use in breeding and as donors of selection-valuable alleles; 21 samples were identified. Accessions with a complex of breeding-valuable target alleles are valuable complex donors, as well as valuable breeding material for creating varieties with improved fruit quality characteristics and scab resistance.
Key words: apple; breeding; marker-assisted selection; fruit quality; scab resistance; *Md-Exp7*; *Md-PG1*; *Md-ACS1*; *Rvi6*; complex donors; gene pyramiding.

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# Идентификация аллелей генов *Md-Exp7* и *Md-PG1* в селекционных формах яблони, устойчивых к парше

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**Аннотация.** Создание сортов яблони, обладающих высоким уровнем плотности мякоти и лежкоспособности плодов, является одной из важных задач в селекции. Среди генов, контролирующих данные признаки, установлена роль гена контроля биосинтеза эндогенного этилена *Md-ACS1* и генов экспансина *Md-Exp7* и полигалактуроназы *Md-PG1*. Применение ДНК-маркерного анализа для решения задач в селекции на признаки качества плодов позволяет не только отслеживать одновременно несколько целевых генов, но и проводить выбраковку растений с нежелательными аллелями, не дожидаясь вступления гибридов в плодоношение, благодаря чему можно ускорить процесс отбора селекционно ценных форм. В целях отбора доноров по комплексу селекционно приоритетных аллелей с использованием мультиплексной ПЦР была выполнена молекулярно-генетическая идентификация генов *Md-Exp7* и *Md-PG1* у 256 селекционных форм, которые содержат ген устойчивости к парше *Rvi6* и аллельные варианты гена *Md-ACS-1*: 90 образцов – *Md-ACS1* (2/2) и 166 образцов – *Md-ACS1* (1/2). Анализ родительских сортов яблони (Ренет Симиренко, Моди, Смеральда, Ренуар, Фулжион и Грени Смит), использованных при получении гибридных форм, выявил три аллеля длиной 198, 202, 214 п. н. по ДНК-маркеру, разработанному для гена *Md-Exp7*. SSR-маркер для гена *Md-PG1* амплифицировал три аллеля (289, 292, 298 п. н.)

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в геноме родительских сортов. Генотипирование гибридных форм яблони позволило обнаружить образцы, содержащие сочетание приоритетных аллелей генов *Md-Exp7*, *Md-PG1* и *Md-ACS-1*. В качестве доноров ценных аллелей отобрано 46 образцов, несущих комбинацию *Md-Exp7* (202:202) + *Md-ACS1* (2/2). Среди изученных гибридов обнаружен 21 образец, содержащий аллели генов *Md-PG1* (292:292) и *Md-ACS1* (2/2). Образцы с различным сочетанием селекционно ценных аллелей генов *Md-Exp7*, *Md-PG1*, *Md-ACS-1* и *Rvi6* рекомендуются для создания сортов с высоким уровнем лежкоспособности плодов и устойчивостью к парше.
Ключевые слова: яблоня; селекция; маркер-опосредованный отбор; качество плодов; устойчивость к парше; *Md-Exp7*; *Md-PG1*; *Md-ACS1*; *Rvi6*; комплексные доноры; пирамидирование генов.

## Introduction

Some of the most important traits of fruit quality in an apple are the flesh firmness and long shelf life of fruits. These traits not only form consumer attractiveness, but also provide an increase in the economic efficiency of the apple fruits production industry by improving storability of the fruits and their transportability. In this regard, the creation of varieties that have a firm texture of the flesh and preserve it during storage is an important direction in breeding. The change in the structure of the fruit flesh during fruit ripening and storage is regulated by various physiological and biochemical processes, among which an important role belongs to the process of endogenous synthesis of ethylene, an increase in the intensity of which leads to softening of the flesh due to the activation of various enzymatic systems that affect the density of the cell wall (Ji, Wang, 2021).

Among the genes controlling the biosynthesis of endogenous ethylene, in the apple, the key role belongs to the *Md-ACS1* and *Md-ACO1* genes encoding the enzymes 1-aminocyclopropane-1-carboxylate synthase (ACC-synthase-1) and 1-aminocyclopropane-1-carboxylate oxidase (ACC-oxidase-1), which sequentially, in a chain of reactions, convert S-adenosyl-methionine to ethylene (Dong et al., 1991, 1992; Kende, 1993). These genes have been mapped, the effect of allelic variants of genes on the level of endogenous ethylene synthesis in fruits and, accordingly, on the storage quality of fruits, has been established, and effective DNA markers for identifying alleles have been developed (Sunako et al., 1999; Oraguzie et al., 2004; Costa et al., 2005). Using these markers, allelic combinations in the breeding material and collection samples of the apple tree were assessed in the world (Oraguzie et al., 2007; Zhu, Barritt, 2008; Nybom et al., 2012; Suprun, Tokmakov, 2013; Savel'ev et al., 2014b; Lyzhin, Savelyeva, 2020; Shamshin et al., 2020). The influence of the *Md-ACS3a* gene on the synthesis of endogenous ethylene in fruits was also revealed (Bai et al., 2012). However, the contribution of this gene to the formation of this trait is lower than that of *Md-ACS1* (Dougherty et al., 2016).

Along with the abovementioned genes, the expansin gene – *Md-Exp7* and the polygalacturonase gene – *Md-PG1* play an important role in the control of physiological and biochemical processes associated with the formation of the flesh structure and the preservation of its density during storage in the apple tree. Expansin is a protein involved in the enzymatic rearrangement of cell walls by breaking noncovalent bonds between the hemicellulose matrix and cellulose microfibrils, which increases the susceptibility of this structural polymer to the action of other enzymes (Cosgrove, 2000). The activity of the ethylene-dependent enzyme polygalacturonase contributes to the destruction of the structure of the cellular pectin polymer by biochemical catalysis of the hydrolytic cleavage of (1–4) galacturonan (Brummell, Harpster, 2001).

In genetic studies of the *Md-Exp7* and *Md-PG1* genes, microsatellite markers cosegregating with them were identified. For the microsatellite marker Md-Exp7SSR of the *Md-Exp7* gene, localized in the first linkage group, it was found that an increase in the size of the amplification product correlates with the level of fruit flesh softening during storage: for a fragment of 198 bp characterized by a lower level of softening, for 202 bp – medium and for 214 bp – the highest (Costa et al., 2008). H. Nybom (Nybom et al., 2012) made a preliminary conclusion about a possibly more significant effect of the allele with the size of the amplification product at the Md-Exp7SSR microsatellite locus of 202 bp in comparison with the 198 bp allele. The polygalacturonase gene – *Md-PG1*, mapped at a distance of 37 cM from the *Md-ACO1* gene in linkage group 10, has a more pronounced contribution to the phenotypic variation in the change in flesh density during storage of fruits at temperatures close to room temperature, and not in refrigerators in the temperature range 2–4 °C (Costa et al., 2010). This is of great importance for commercial attractiveness of the fruits stored during transportation without compliance of the temperature regime, in temporary warehouses of shopping malls, and in logistics centers. The studies revealed a number of DNA markers closely linked to this gene (Costa et al., 2010), including the microsatellite marker Md-PG1$_{10kd}$, which is the most informative (Longhi et al., 2013b). Analysis of allelic variants of this DNA marker showed that the presence of an allele with a fragment size of 298 bp is undesirable for breeding varieties with improved flesh density retention without special storage conditions. At the same time, the homozygous variant for the allele 298 bp is the least promising for use in breeding (Longhi et al., 2013a).

It is noteworthy that a high level of influence on the phenotypic manifestation of the trait was found at temperatures close to room temperature not only for the *Md-PG1* gene (Costa et al., 2010), but also for the *Md-ACS1* gene. When comparing data on allelic variants of the *Md-PG1*, *Md-ACS1* and *Md-ACO1* genes and the level of fruit flesh density in 108 apple varieties at the stage of harvesting maturity and after 20 days of storage (at a temperature of 20–25 °C) after harvesting, a relationship was established between the allelic variants of the *Md-PG1* and *Md-ACS1* genes and the degree of reduction in fruit flesh density (Kwon et al., 2020). Using the DNA markers of the *Md-PG1* and *Md-Exp7* genes, a number of studies were carried out to identify their alleles, including cultivars and species specimens of the genus *Malus* (Costa et al., 2008; Longhi et al., 2013a, b; Nybom et al., 2013; Savel'ev et al., 2014a; Shamshin et al., 2018; Savelyeva, Lyzhin, 2019; Dolzhikova et al., 2020), for the purposes of breeding and as

part of the study of the allelic diversity of these genes within the genus *Malus*. For the *Md-PG1* and *Md-ACS1* genes, allele-specific SNP markers were also developed and further integrated into the SNP-array for MAS-selection – International RosBREED SNP Consortium OpenArray v1.0, which allows for the total detection of alleles of 11 genes (Chagné et al., 2019).

Obviously, the presence of DNA markers for genes that determine such economically valuable traits as flesh density and preservation of its characteristics during storage makes it possible to increase the efficiency of the breeding process, as well as to conduct pre-breeding work for more efficient selection of parental pairs for crossing. Especially relevant is the issue of using DNA markers for the analysis of the allelic composition of genes that determine quality traits in connection with the polygenic control of this trait and the different contribution to the phenotypic manifestation of the trait depending on the combinations of alleles of different genes: *Md-ACS1*, *Md-ACO1*, *Md-PG1* and *Md-Exp7*. An important advantage of using marker-assisted selection is the ability to simultaneously track several genes that control not only one, but several traits, including resistance to pathogens.

As part of our previous work, using DNA marker analysis, we created a wide range of apple breeding selections carrying the *Rvi6* scab resistance gene in combination with various alleles of the *Md-ACS1* gene. Expansion of the set of priority genes, the alleles for which will be identified in the created breeding selections, will make it possible to select the most valuable material for breeding. In this regard, in the presented study, the task was to identify the alleles of the *Md-PG1* and *Md-Exp7* genes in apple samples carrying the *Rvi6* gene and selection-valuable variants of the *Md-ACS1* gene alleles (1/2, 2/2) to create apple varieties that combine a complex of economically valuable traits.

## Material and methods

The object of research was 256 apple selections obtained in six combinations of crossing: (1) Renet Simirenko/Modi (62 pcs); (2) Renet Simirenko/Smeralda (65 pcs); (3) Renet Simirenko/Renoir (33 pcs); (4) Renet Simirenko/Fujion (22 pcs); (5) Renoir/Granny Smith (9 pcs); (6) Modi/Granny Smith (65 pcs). The hybrids were obtained earlier as part of a marker-assisted breeding program aimed at the development of apple scab-resistant varieties with improved fruit quality characteristics. The presence of the scab resistance gene *Rvi6* (Suprun et al., 2018), as well as breeding-valuable alleles of the *Md-ACS1* gene, was previously determined by DNA-marker based analysis.

For DNA extraction, a CTAB-based method was used (Murray, Thompson, 1980). Molecular genetic identification of the alleles of the *Md-PG1* and *Md-Exp7* genes was performed using microsatellite markers Md-PGl$_{10kd}$ and Md-Exp7SSR, respectively (Costa et al., 2008; Longhi et al., 2013b). The analysis was carried out by two markers simultaneously in one PCR reaction, which included: 20 ng of DNA, 1.5 mM dNTPs, 10 pM of each primer, 1 u. Taq polymerase and 2.5 mM 10×standard PCR buffer. PCR program: 94 °C – 150 s, 32 cycles: 60 °C – 45 s, 72 °C – 60 s, 94 °C – 30 s; 1 cycle 72 °C – 10 min. Electrophoresis of PCR products was carried out on an automatic genetic analyzer Nanofor 05. Analysis of the results was performed using the GeneMarker V3.0.1 program.

## Results

The absence of overlapping in the size ranges of the amplified fragments by the used DNA markers (198–214 bp for the Md-Exp7SSR marker and 289–302 bp for the Md-PG1$_{10kd}$ marker) made it possible to apply multiplex identification (Fig. 1).

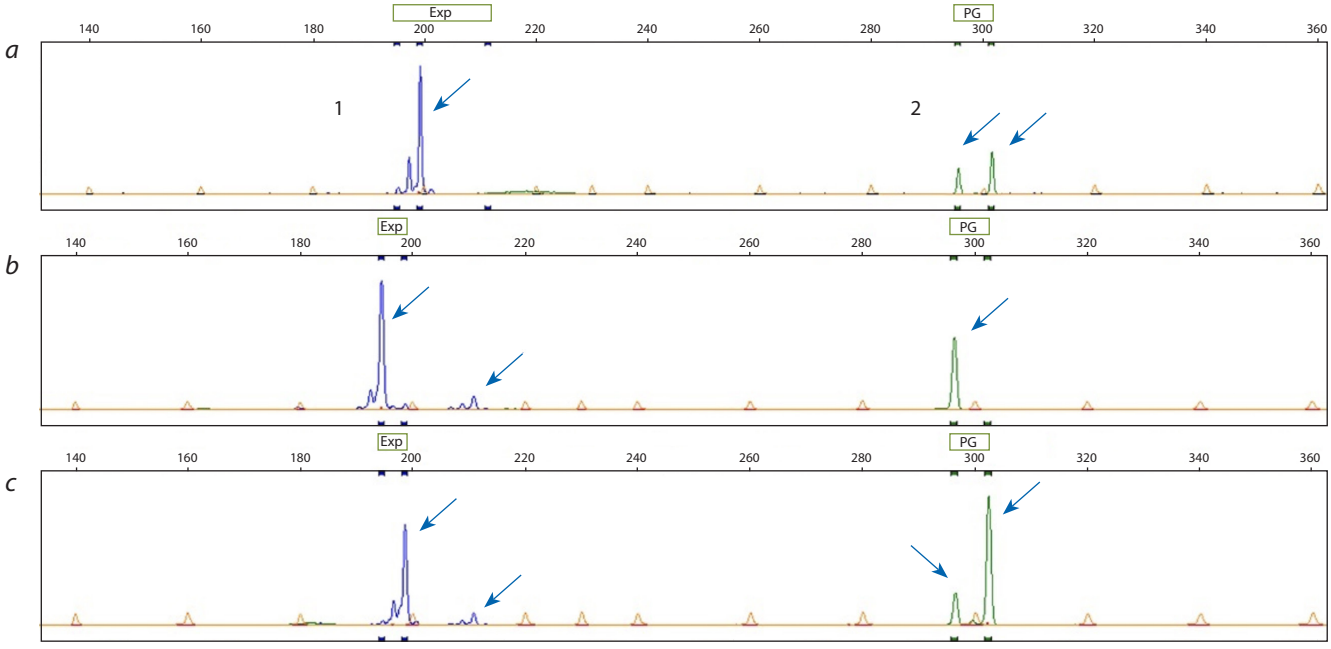


**Fig. 1.** Multiplex fragment analysis of amplification products for DNA markers of the *Md-Exp7* (1) and *Md-PG1* (2) genes.

The electropherogram shows examples of the results of the analysis of a sample homozygous for Md-Exp7SSR and heterozygous for Md-PG1$_{10kd}$ (*a*); heterozygous for Md-Exp7SSR and homozygous for Md-PG1$_{10kd}$ (*b*) and simultaneously heterozygous for two target loci (*c*).

**Table 1.** The size of DNA marker amplification fragments for the *Md-Exp7* and *Md-PG1* genes in parental apple cultivars

| Parental variety | Md-Exp7SSR | | $Md\text{-}PG1_{10kd}$ | |
|---|---|---|---|---|
| Renet Simirenko | 202 | 202 | 298 | 298 |
| Modi | 202 | 214 | 292 | 292 |
| Smeralda | 202 | 202 | 292 | 292 |
| Renoir | 202 | 202 | 289 | 298 |
| Fujion | 202 | 214 | 289 | 289 |
| Granny Smith | 198 | 202 | 292 | 292 |

Analysis of the parental cultivars used to obtain apple selections revealed three fragments, 198, 202, 214 bp in size by the marker of the *Md-Exp7* gene, while fragments of 289, 292, 298 bp in length were identified by the SSR marker of the *Md-PG1* gene (Table 1).

DNA marker-based analysis of hybrid plants revealed various combinations of alleles. Taking into account the fact that for the Md-Exp7SSR marker in the parental cultivars the allele with the size of the amplified fragment of 202 bp was most common (represented in all varieties, wherein in the varieties Renet Simirenko, Smeralda and Renoir in the homozygous state), its presence was detected in all hybrid samples, with the exception of 21 hybrids from combination No. 6 (Modi/Granny Smith), carrying the allelic combination 198:214. At the same time, the allele 202 bp in the homozygote was present in 113 samples. Allelic combinations 198:202 and 202:214 were found in seven and 115 hybrid plants, respectively. Identification of the alleles of the *Md-PG1* gene marker revealed that the most common was the allele with a product size of 292 bp, while in 65 samples it was found in the homozygote. Along with the allelic variant 292:292, allelic combinations 289:292 were identified (7 samples); 292:298 (129 samples); 289:298 (38 samples) and 298:298 (17 samples).

## Discussion

Molecular genetic analysis of parental cultivars based on DNA markers of the *Md-Exp7* and *Md-PG1* genes made it possible to identify allelic combinations for a number of cultivars for the first time, as well as confirm the already available scientific information for the Granny Smith and Modi cultivars. According to S. Longhi et al. (2013b), cultivar Granny Smith has an allele of 292 bp in homozygote for the DNA marker of the *Md-PG1* gene. A similar allelic variant was previously identified in the Modi variety (Longhi et al., 2013a). According to the DNA marker Md-Exp7SSR for the Granny Smith cultivar, the presence of an allelic variant 198:202 bp is known (Costa et al., 2008), which was also confirmed in our study.

Among the cultivars that were used as parental forms for the production of hybrid plants, the genotypes with the most breeding-valuable combinations of allelic variants of two genes simultaneously are the Smeralda and Granny Smith cultivars. According to the $Md\text{-}PG1_{10kd}$ marker, the least valuable allele is 298 bp; it was identified in the Renoir cultivar – 289:298 and in Renet Simirenko – 298:298. At the same time, according to the *Md-Exp7* gene marker, an allelic variant was identified in them, which is valuable for selection 202:202, which can probably compensate for the negative effect of allelic variants for the *Md-PG1* gene. This is supported by the fact that the Renet Simirenko variety, although inferior to the Granny Smith variety in terms of storability, however, exhibits a fairly high level of this trait. At the same time, it is characterized by a sharp decrease in the density of the fruit flesh with an increase in storage temperature, which cannot be said about the Granny Smith variety, which is the variety with the highest fruit keeping quality (Prichko, 2018; Prichko et al., 2019). It can be assumed that in this way the Renet Simirenko variety showed a negative effect of the 298:298 allelic variant by the DNA marker of the *Md-PG1* gene, because, as mentioned above, this gene has a more pronounced contribution to the phenotypic variation in the change in flesh firmness during storage of fruits at temperatures close to room temperature (Costa et al., 2010). In general, the availability of information about allelic combinations of DNA markers of target genes makes it possible to correct pairs of crosses to increase the yield of hybrids with the most valuable allelic combinations.

Considering the distribution of alleles of the DNA marker of the *Md-Exp7* gene, we can note hybrid progenies No. 2 and 3, in which all hybrid accessions are homozygous for the 202 bp allele, which corresponds to the allelic variants of the parent varieties (202:202 in all parental forms in these combinations). In hybrid combination No. 5, for which nine plants were analyzed, allelic variants 198:202 and 202:202 were identified, which corresponds to the alleles of the parental varieties. A small sample size does not allow to reliably estimate the deviation of the distribution from the expected 1:1 – (198:202) : (202:202). Specific distribution was observed in progenies No. 1, 4 and 6. Plants with the 214 bp allele predominate in these hybrid populations (allelic variants 202:214 and 198:214) (Table 2). However, taking into account the alleles for the DNA marker of this gene in parental varieties, the ratio of plants carrying the 202:214 allele variant to plants with the 202 allele in the homozygote (i. e. 202:202) in hybrid combinations No. 1 and 4 should be close to a 1:1 distribution, and in the sample of plants obtained in combination No. 6, the expected distribution is 1:1:1:1 for allelic combinations 198:202, 198:214, 202:202, 202:214. Obviously, there is a significant predominance of plants carrying the 214 bp allele.

The reason for the deviation in the distribution of allelic variants is the fact that the *Md-Exp7* gene and the *Rvi6* scab resistance gene are located on the first chromosome, while the distance between them is about 9 cM (Costa et al., 2008). In this study, mapping of the *Md-Exp7* gene was carried out using a hybrid population obtained in a combination of crossing varieties Prima (202:214), *Rvi6rvi6*/Fiesta (202:202), *rvi6/rvi6*, which made it possible to establish the distance between these genes.

In our study, in hybrid combinations No. 1, 4 and 6, the Modi variety with an allelic combination of 202:214 bp by DNA marker Md-Exp7SSR was used as a donor of the scab resistance gene. Taking into account the fact that in the presented work, the analysis of plants carrying the dominant allele of the *Rvi6* gene was carried out, we can speak about the regularity of the result obtained and the confirmation of the genetic distance between the *Md-Exp7* and *Rvi6* genes.

**Table 2.** Allelic combinations of DNA markers of the *Md-Exp7* and *Md-PG1* genes in combination with alleles of the *Md-ACS1* gene

| Md-ACS1* | Md-Exp7SSR | | Md-PG1$_{10kd}$ | | Number of samples (progenies number) |
|---|---|---|---|---|---|
| 1/2 | 198 | 202 | 289 | 292 | 4 (No. 5) |
| | | | 292 | 292 | 1 (No. 6) |
| | | | 292 | 298 | 2 (No. 5) |
| | | 214 | 292 | 292 | 14 (No. 6) |
| | 202 | 202 | 289 | 292 | 2 (No. 5) |
| | | | 289 | 298 | 13 (No. 3 – 12 samples, No. 4 – 1 sample) |
| | | | 292 | 292 | 2 (No. 6) |
| | | | 292 | 298 | 37 (No. 1 – 6 samples, No. 2 – 31 samples) |
| | | | 298 | 298 | 13 (No. 13) |
| | | 214 | 289 | 298 | 9 (No. 4) |
| | 202 | 214 | 292 | 292 | 27 (No. 6) |
| | 202 | 214 | 292 | 298 | 42 (No. 1) |
| 2/2 | 198 | 214 | 292 | 292 | 7 (No. 6) |
| | 202 | 202 | 289 | 292 | 1 (No. 5) |
| | | | 289 | 298 | 4 (No. 3) |
| | | | 292 | 298 | 37 (No. 1 – 3 samples, No. 2 – 34 samples) |
| | | | 298 | 298 | 4 (No. 3) |
| | | 214 | 289 | 298 | 12 (No. 4) |
| | | | 292 | 292 | 14 (No. 6) |
| | | | 292 | 298 | 11 (No. 1) |

* Allelic variants of the *Md-ACS1* gene are indicated, according to the numbering proposed by Sunako et al. (1999).

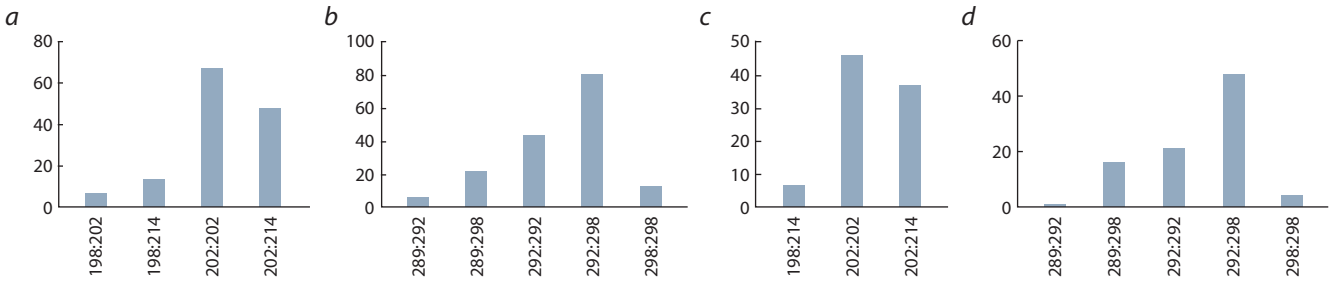


**Fig. 2.** The correlation of allelic variants of the *Md-Exp7* (*a*, *c*) and *Md-PG1* (*b*, *d*) genes in hybrid plants with different allelic variants of the *Md-ACS1* gene: 1/2 (*a*, *b*) and 2/2 (*c*, *d*).

The summation of all plants from three hybrid combinations No. 1, 4 and 6 shows that out of 149 plants, an allele of 214 bp is present in 136 plants, and the total number of plants without it is 13 (about 9 % of the total number of plants), which is consistent with the genetic distance between the *Md-Exp7* and *Rvi6* genes.

For additional verification of the absence of erroneously interpreted results, on the example of the largest hybrid family of the three for which there was a deviation of the observed distribution of alleles from the expected – hybrid family No. 1, a molecular genetic analysis was performed using the Md-Exp7SSR DNA marker for all hybrid plants, regardless from the presence of a dominant allele of the *Rvi6* gene. It was found that out of 231 hybrid plants, 113 have the 202:214 allelic variant, and 118 plants have the 202:202 allelic variant. Thus, there is no significant deviation from the 1:1 ratio ($\chi^2$ (1:1) = 0.11 at $\chi^2_{crit}$ = 3.8).

The distribution of alleles for the DNA marker of the *Md-PG1* gene corresponds to allelic variants in parental cultivars: in combinations No. 1, 2, 4 and 6, the hybrid progeny is uniform and has allelic variants 292:298, 292:298, 289:298 and 292:292, respectively. In hybrid combinations No. 3 and 5, two types of allelic combinations are present, consistent with the allelic variants of the parent varieties.

Considering combinations of allelic variants of the *Md-Exp7* and *Md-PG1* genes with alleles of the *Md-ACS1* gene present in the studied apple hybrid accessions, the predominance of the 202:202 bp allelic variant is seen by the DNA marker of the *Md-Exp7* gene and 292:298 by the DNA marker of the *Md-PG1* gene both in the sample of hybrids with the 1/2 allele variant and in the sample of homozygous for the allele 2 of the *Md-ACS1* gene hybrid samples (Fig. 2).

It is also necessary to note a rather high share of plants with an allele set of 202:214 for the DNA marker of the

*Md-Exp7* gene and 292:292 for the DNA marker of the *Md-PG1* gene.

The samples carrying the combination *Md-Exp7* (202:202) + *Md-ACS1* (2/2) have the highest value for breeding. 46 such accessions were identified. Accessions carrying combinations of alleles *Md-PG1* (292:292) + *Md-ACS1* (2/2) are also the most valuable for use in breeding and as donors of selection-valuable alleles – 21 accessions were identified.

However, given the fact that no homozygous samples for the 214 allele of the *Md-Exp7* gene marker were found, and for samples with the allele variant 298:298 (the least priority for selection) for the DNA marker of the *Md-PG1* gene, an insignificant number was detected – 17 samples out of 256 of plants included in the study sample, we can talk about the presence of a wide list of breeding forms that are valuable both for further breeding and for use as donors of scab resistance (*Rvi6* gene) and a complex of breeding-valuable alleles of several genes simultaneously that determine flesh density – *Md-Exp7*, *Md-PG1* and *Md-ACS1*. This is supported by the fact that among modern industrial cultivars that are actively used in world horticulture, allele variants that determine the average level of phenotypic expression of the target trait are quite widespread (Costa et al., 2008, 2010; Nybom et al., 2012; Longhi et al., 2013a, b), which is apparently due to the polygenic control of the trait, in which the presence of alleles "average" in terms of selection value simultaneously at the loci of several genes gives the desired phenotypic effect.

## Conclusion

Thus, the performed study made it possible to identify groups of apple breeding selections with different combinations of alleles of the *Md-Exp7* and *Md-PG1* genes among accessions carrying the *Rvi6* scab resistance gene and possessing selection-valuable allelic variants of the *Md-ACS1* gene. The information obtained made it possible to identify donors with a complex of priority alleles that are of high value for use in breeding in order to create new generation varieties that are resistant to apple scab and have a high level of fruit storability.

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# Genome variability of domestic tomato varieties: data from AFLP analysis

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**Abstract.** Tomato *Solanum lycopersicum* L. is one of the main vegetable crops, accessions and cultivars of which are characterized by a low level of genomic polymorphism. Introgressive tomato breeding uses related wild *Solanum* species to improve cultivars for stress tolerance and fruit quality traits. The aim of this work was to evaluate the genome variability of 59 cultivars and perspective breeding lines of *S. lycopersicum* and 11 wild tomato species using the AFLP method. According to the AFLP analysis, four combinations of primers E32/M59, E32/M57, E38/M57, and E41/M59, which had the highest PIC (polymorphism information content) values, were selected. In the process of genotyping a collection of 59 cultivars/lines of *S. lycopersicum* and 11 wild tomato accessions, the selected primers revealed 391 fragments ranging in size from 80 to 450 bp, of which 114 fragments turned out to be polymorphic and 25 were unique. Analysis of the amplification spectra placed wild tomato accessions into separate clades. Sister clades included cultivars of FSCV breeding resistant to drought and/or cold and, in part, to late blight, *Alternaria*, *Septoria*, tobacco mosaic virus and blossom end rot, as well as tomato accessions not characterized according to these traits, which suggests that they have resistance to stress factors. In accessions of distant clades, there was clustering on the basis of resistance to *Verticillium*, cladosporiosis, *Fusarium*, tobacco mosaic virus, gray rot, and blossom end rot. The combination of accessions according to their origin from the originating organization was shown. The primer combinations E32/M59, E32/M57, E38/M57 and E41/M59 were shown to be perspective for genotyping tomato cultivars to select donors of resistance to various stress factors. The clade-specific fragments identified in this work can become the basis for the development of AFLP markers for traits of resistance to stress factors.
Key words: *Solanum lycopersicum*; tomato cultivars; genomic polymorphism; AFLP markers; clustering.

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# Вариабельность генома отечественных сортов томата: данные AFLP-анализа

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**Аннотация.** Томат *Solanum lycopersicum* L. является одной из основных овощных культур, образцы и сорта которой характеризуются низким уровнем геномного полиморфизма. В интрогрессивной селекции томата используют родственные дикорастущие виды *Solanum* для улучшения сортов по признакам устойчивости к стрессовым факторам и качества плодов. Целью работы была оценка вариабельности генома 59 сортов и перспективных селекционных линий *S. lycopersicum* и 11 дикорастущих видов томата с помощью метода AFLP. По данным AFLP-анализа было выбрано четыре комбинации праймеров E32/M59, E32/M57, E38/M57 и E41/M59, которые отличались наиболее высокими показателями PIC (polymorphism information content). В процессе маркирования коллекции из 59 сортов/линий *S. lycopersicum* и 11 дикорастущих образцов томата отобранными праймерами выявлен 391 фрагмент размером от 80 до 450 п. н., из которых 114 фрагментов оказались полиморфными и 25 – уникальными. Анализ спектров амплификации выделил дикорастущие образцы томата в отдельные клады. Сестринские клады включали сорта селекции Федерального научного центра овощеводства, устойчивые к засухе и/или холоду и, частично, к фитофторозу, альтернариозу, септориозу, вирусу табачной мозаики и вершинной гнили плода, а также не охарактеризованные по данным признакам образцы томата, что позволяет предположить наличие у них устойчивости к стрессовым факторам. У сортовых образцов отдаленных клад присутствует кластеризация по признакам устойчивости к вертициллезу, кладоспориозу, фузариозу, вирусу табачной мозаи-

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ки, серой гнили и вершинной гнили плода. Показано объединение образцов согласно их происхождению от организации-оригинатора. Продемонстрирована перспективность праймерных комбинаций E32/M59, E32/M57, E38/M57 и E41/M59 для генотипирования сортов томата с целью отбора доноров устойчивости к различным стрессовым факторам. Выявленные в настоящей работе кладоспецифичные фрагменты могут стать основой для разработки AFLP-маркеров для признаков устойчивости к стрессовым факторам.
Ключевые слова: *Solanum lycopersicum*; сорта томата; геномный полиморфизм; AFLP-маркеры; кластеризация.

## Introduction

The assessment of genetic diversity, considering the pedigrees of crop cultivars and associations with important traits, is one of the foundations of modern breeding. Various methods of molecular genome analysis are used in the selection of parental genotypes, as well as in identifying the level of variability both within a variety and between varieties (Nurmansyah et al., 2020; Sheeja et al., 2021). Both the entire plant genome and its particular regions (gene families, specific loci, individual genes) are subjected to DNA genotyping. Polymorphism data are used, for example, to develop molecular DNA markers linked to important traits. Markers are used to search for donors of the corresponding genotypes, as well as to certify varieties and lines (Semagn et al., 2006; Swiecicka et al., 2009).

One of the commonly used methods for assessing plant genome variability is the AFLP (Amplified Fragment Length Polymorphism), which is based on the assessment of unique and moderately repetitive genome sequences, but does not require the determination of the sequences themselves (Vos et al., 1995; Karp et al., 1997; Despres et al., 2003). The evaluation is based on selective PCR amplification of restriction fragments from a total genomic DNA digest (Vos et al., 1995). The use of AFLP markers is applicable to all species, highly reproducible, and highly efficient in determining genetic distances and phylogenetic relationships in taxonomy (Kardolus et al., 1998; Mba, Tohme, 2005; Arif et al., 2010). The method has been successfully applied to study wild and endangered plant species (Zawko et al., 2001; Ronikier, 2002; van Ee et al., 2006; Manoko et al., 2007; Elameen et al., 2008; Li et al., 2008; Sánchez-Teyer et al., 2009; Tatikonda et al., 2009). In addition, AFLP is popular in modern plant breeding and is used to determine pedigrees, variability, homogeneity, and the degree of introgression and hybridity of varieties, as well as to search for molecular markers associated with economically valuable traits (Mba, Tohme, 2005; Swiecicka et al., 2009; Arif et al., 2010). Such studies have been carried out, for example, on wheat (Hassan et al., 2018), barley (El-Esawi et al., 2018a), peas (D'iachenko et al., 2014; El-Esawi et al., 2018b), pepper (Kochieva, Ryzhova, 2009) and potato (McGregor et al., 2002; Jacobs et al., 2008; Bamberg, del Rio, 2014; Bryan et al., 2017; Dyachenko et al., 2020).

The AFLP has also been used for genotyping tomato (*Solanum lycopersicum* L.). Thus, with this method, an intraspecific map of the tomato genome was obtained (Saliba-Colombani et al., 2000), the transcriptional response of tomato to nematode infection was studied (Święcicka et al., 2017), and DNA markers linked to resistance to tomato bacterial wilt (Miao et al., 2009) and cladosporiosis (Thomas et al., 1995) were identified. The use of AFLP for comparing the response of heat-tolerant and heat-sensitive tomato genotypes to moderate heat stress conditions revealed a number of differentially expressed constitutive genes, presumably determining heat tolerance and differences in genotype adaptation to elevated temperatures (Bita et al., 2011).

The phylogenetics and genogeography of crop wild relatives are effective approaches to understanding their evolutionary patterns and unlocking their potential to improve crops. AFLP genotyping against geographic and climatic indicators has contributed to the study of the spatial genetics of wild tomato species *S. lycopersicum*, *S. pimpinellifolium* (Nakazato, Housworth, 2011) and *S. peruvianum* (Nakazato et al., 2012). The *S. lycopersicum* and *S. pimpinellifolium* evolutionary patterns, including demographic history, dispersal patterns, interspecific divergence and hybridization, have been shown to be closely related to the complex geographic and ecological conditions in the Andes (Nakazato, Housworth, 2011). An AFLP study of 19 natural populations of *S. peruvianum* revealed a moderate degree of population differentiation, probably reflecting partial geographic isolation between tomato species (Nakazato et al., 2012).

In addition to solving taxonomic and phylogenetic problems, the AFLP method is used to determine the variability of tomato varieties. Various DNA marking systems showed low efficiency for studying the genetic diversity of tomato cultivars with limited genetic variability. The use of AFLP in combination with SSR markers to characterize 48 closely related Spanish tomato varieties made it possible to obtain a unique fingerprint for each analyzed accession (García-Martínez et al., 2006).

Cultivated varieties and lines of tomato belong to the species *S. lycopersicum*. Compared to wild related species (section Lycopersicon of the genus *Solanum*) (Peralta et al., 2008), their genomes are significantly less polymorphic (20 or more times) (The 100 Tomato Genome Sequencing Consortium et al., 2014). Hundreds of genes and loci of quantitative traits linked to resistance, yield, flower and fruit characteristics, and plant architecture have been mapped in the genome of wild species (Foolad, 2007). Due to the relative ease of crossing with *S. lycopersicum*, wild species are actively used in introgressive tomato breeding to improve economic traits associated with stress resistance, yield and quality (Hajjar, Hodgkin, 2007; Labate, Robertson, 2012). For example, sources of varying degrees of resistance to bacterial wilt are *L. pimpinellifolium* (= *S. pimpinellifolium*) PI127805A, *L. esculentum* var. *cerasiforme* (= *S. lycopersicum* var. *cerasiforme*) CRA66, *L. pimpinellifolium* PI129080 and *L. esculentum* AS52 (Chellemi et al., 1994). In cultivars with purple fruits, the trait of anthocyanin biosynthesis in the fruit was obtained by introgression from

the genomes of wild species *S. chilense* and *S. cheesmaniae* (Povero et al., 2011; Maligeppagol et al., 2013).

Thus, the low level of genomic polymorphism of tomato varieties is combined with introgressive genes/loci associated with economically valuable traits. Therefore, multilocus genome mapping methods can presumably separate cultivars according to useful traits.

Despite the importance of varietal certification and assessment of intervarietal genome variability, there are few studies on marking the genotypes of tomato cultivars in Russia, and these are mainly works on genotyping using already known markers (Shcherban, 2019). For example, a collection of tomato varieties and hybrids from the Michurinsky State Agrarian University was screened using the P7 molecular marker to identify donors of cladosporiosis resistance (Shamshin et al., 2019).

In this study, using the AFLP method, we assessed the genomic variability of tomato *S. lycopersicum* cultivars and lines of domestic and foreign breeding from the collection of the Federal Scientific Vegetable Center (FSVC) in comparison with wild accessions of tomato species.

## Materials and methods

For the study, 59 tomato *S. lycopersicum* cultivars and perspective breeding lines of domestic and foreign breeding from the FSVC collection were selected (Table 1). 11 wild tomato species were used as an outgroup (see Table 1). 34 varieties of the sample (~58 %) are included in the State Register of Breeding Achievements Approved for Use of the Russian Federation for 2022 (https://reestr.gossortrf.ru/). Seeds of accessions were germinated under standard greenhouse conditions (23 °C/25 °C, 16 h/8 h – day/night). Genomic DNA was isolated from freshly harvested 5–6 day old seedlings using the CTAB method (Puchooa, 2004).

Data on drought and cold resistance, resistance and susceptibility to diseases (late blight, *Fusarium*, *Verticillium*, cladosporiosis, alternariosis, *Septoria*, tobacco mosaic virus, gray rot, blossom end rot) were partially taken from the State Register of Breeding Achievements (http://reestr.gossortrf.ru/), as well as kindly provided by the originators of the varieties and Ph.D. I.A. Engalycheva.

AFLP analysis was carried out according to the standard protocol: hydrolysis of 350 ng of genomic DNA of each accession with restriction enzymes *Eco*RI and *Mse*I followed by ligation with *Eco*RI and *Mse*I adapters (Vos et al., 1995). Selective amplification was performed in two stages: (1) preamplification (denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, synthesis at 72 °C for 1 min, 24 cycles) with adapter primers *Eco*RI+1 and *Mse*I+1 (Vos et al., 1995) with one selective nucleotide (A) at the 3′ end; (2) amplification with primers *Eco*RI+3 and *Mse*I+3 with three selective nucleotides at the 3′ end. The results were visualized in a denaturing 6 % polyacrylamide gel using a LI-COR 4300 gel analyzer (LI-COR operator manual; LI-COR, USA). The experiment was carried out in one repeat for each combination of primers. The polymorphic information content (PIC) index for each primer combination was calculated according to Botstein et al. (1980) and Krishnamurthy et al. (2015).

Molecular panels of AFLP fragments were documented in the form of binary matrices (Excel program). Based on the constructed spectra and matrices, variety-specific DNA markers were identified, coefficients of pairwise genetic similarity/difference between accessions (GS) and genetic distances (GD = 1 – GS) were calculated, cluster analysis was performed (Neighbor Joining method; method of principal coordinates, PCA) and groups of genetically similar accessions were determined (PAST software package) (Hammer et al., 2001). Analysis of the genomic structure of the population of the studied accessions was carried out using the Structure v.2.3.4, which makes it possible to identify common genetic blocks and their ratio in each accession (Pritchard et al., 2000; Hubisz et al., 2009).

## Results

Since up to 80 % of the standard AFLP spectrum can serve as markers for the detection of genetic polymorphisms, and the effectiveness of AFLP depends on primer combinations (Vos et al., 1995), primer/enzyme combinations were selected and tested for multilocus AFLP analysis of tomato accessions. On a sample of five tomato accessions, seven combinations of primers *Eco*RI+3/*Mse*I+3 were tested, differing in the composition of selective nucleotides at the 3′ end: E32/M59 (E-AAC/M-CTA); E32/M57 (E-AAC/M-CGG); E38/M57 (E-ACT/M-CGG); E41/M59 (E-AGG/M-CTA); E32/M61 (E-AAC/M-CTG); E38/M47 (E-ACT/M-CAA); E38/M59 (E-ACT/M-CTA). It was shown that the use of combinations of E32/M59, E32/M57, E38/M57 and E41/M59 gives a polymorphic, well-differentiated spectrum with an optimal number of fragments.

Four selected primer combinations were used to label 59 *S. lycopersicum* cultivars/lines and 11 wild tomato accessions. As a result, 391 fragments 80–450 bp in size were detected, of which 114 (29.2 %) fragments turned out to be polymorphic (Table 2). The primer combination E41/M59 was the most effective: 47 out of 67 obtained fragments were variable. At the same time, the E32/M59 combination corresponded to the largest number of fragments unique for individual accessions (11 out of 25 found) (see Table 2). In case of the combinations E32/M61, E38/M47, and E38/M59 (the number of obtained fragments was 31, 24, and 41, respectively) no polymorphic and unique fragments were identified. The PIC value ranged from 0.367 (E32/M57) to 0.658 (E41/M59) (see Table 2) with a mean value of 0.504, indicating that a large number of polymorphisms can be detected using the E41/M59 primer pair.

Based on the results of the AFLP analysis, a dendrogram that clearly divided the tomato accessions into clusters I and II was constructed (Fig. 1).

Wild tomato accessions were grouped into two clades of cluster I: accessions 1 to 7 (including representatives of wild tomato species and a wild accession of *S. lycopersicum*) were separated into clade A; accessions 8–11, including wild accessions of cultivated species (*S. lycopersicum* var. *succentuariatum*, var. *humboldtii*, var. *cerasiforme* and var. *pyriforme*) fell into clade C. Clade C was sister to clade B, consisting of seven *S. lycopersicum* cultivars (accessions 12–15, 17, 18,

**Table 1.** Tomato accessions used for AFLP analysis and their resistance to various stresses

| Accession (species, cultivar or line; cat number TGRC (LA), VIR (k) or SBR*) | Cold / Drought | Late blight / *Fusarium* | Blossom end rot / Gray rot | Verticillosis / Cladosporiosis | *Alternaria* / *Septoria* / TMV |
|---|---|---|---|---|---|
| [1]*S. peruvianum* LA1278; [2]*S. cheesmaniae* LA0421; [3]*S. galapagense* LA1044; [4]*S. pimpinellifolium* var. *racemigerum* LA2348; [5]*S. pimpinellifolium* LA1578; [6]*S. pimpinellifolium* k-1018 | R / R | n / n | n / n | n / n | n / n / n |
| [7]*S. lycopersicum* LA1673; [8]*S. lycopersicum* var. *succenturiatum* k-732; [9]*S. lycopersicum* var. *humboldtii* k-2912; [10]*S. lycopersicum* var. *cerasiforme* k-342; [11]*S. lycopersicum* var. *pyriforme* k-2911 | S / S | n / n | n / n | n / n | n / n / n |
| [12]Osennia Rapsodia 8153507# | R / R | S / n | RR / n | n / n | n / RR / RR |
| [13]Magnat 9154078#; [14]Charovnica 9553320#; [18]Chernomor 9553287#; [19]Raduzhnaia vdova 9154081#; [24]Perst 9608141# | R / R | RR / n | RR / n | n / n | RR / RR / RR |
| [15]Dolgonosik 8456311# | R / R | RR / n | RR / n | RR / n | RR / RR / RR |
| [16]Revansh 9705233#; [23]Monah 9154082#; [28]Gurman 9900616#; [29]Shtambovyi Alpatieva 905a w/n# | R / R | S / n | RR / n | n / n | RR / RR / RR |
| [17]Yunona 9359147# | R / R | RR / RR | S / n | RR / n | RR / RR / RR |
| [20]Geia 9608133# | R / R | RR / n | RR / n | n / n | RR / RR / S |
| [21]Talisman 9705235# | R / R | RR / n | RR / n | n / n | S / S / S |
| [22]Rosinka 9359149# | R / R | RR / R | RR / n | R / n | RR / RR / RR |
| [25]Argo 8901902# | R / S | S / n | S / n | n / n | RR / RR / S |
| [26]Gruntovyi Gribovskii 4500237#; [31]Otradnyi 8006741# | R / R | S / n | RR / n | n / n | RR / RR / S |
| [27]Kameia 9900640# | R / R | R / n | RR / n | n / n | RR / RR / RR |
| [30]Dubrava 9401288# | R / R | MR / MR | RR / n | n / n | RR / RR / S |
| [32]Bychie Serdce 9810228## | S / S | S / n | S / n | n / n | RR / RR / S |
| [33]Hohloma 9609982### | S / S | RR / RR | S / n | n / n | n / n / n |
| [34]Rosovyi Buton 8355731#### | S / S | S / n | S / n | n / n | n / n / n |
| [35]Medovaia Kaplia 8262258#### | n / n | RR / n | RR / RR | n / S | n / n / RR |
| [36]Altaiskii Oranzhevyi 9463931& | R / R | n / n | RR / RR | n / RR | n / n / n |
| [37]Bokari 8262335# | R / R | n / n | RR / S | n / RR | n / n / n |
| [38]Rozovyi nash w/n# | R / R | n / n | RR / S | n / S | n / n / n |
| [39]Sodruzhestvo 8456314# | R / R | RR / n | RR / S | n / n | RR / RR / RR |
| [40]Organza 9359003&&; [51]L-270-20 (*RIN/rin*) w/n# | R / R | n / RR | RR / S | n / RR | n / n / RR |
| [41]Korneevskii 8262334# | R / R | n / n | RR / S | n / S | n / n / RR |
| [42]Malinovyi Silach 8653837&&&; [43]Garmoshka 8556947&&&&; [44]Kopilka Zheltaia w/n# | R / R | n / RR | S / S | n / S | n / n / RR |
| [45]LM-298-19 w/n# | R / R | n / RR | n / RR | RR / RR | n / n / RR |
| [46]LP-296-19 w/n#; [47]G-67-19 $F_1$ w/n#; [48]G-68-19 $F_1$ w/n#; [49]G-69-19 $F_1$ w/n# | R / R | n / RR | n / S | RR / RR | n / n / RR |
| [50]Viking 9253767# | R / R | S / n | RR / n | n / n | RR / n / n |
| [52]Cherry Ukrainskie w/n# | R / R | n / RR | RR / RR | n / RR | n / n / RR |
| [53]Cherry Zhelto-oranzhevye w/n#; [54]Red Cherry w/n#; [55]Black Cherry LA4451 | R / R | n / RR | RR / RR | RR / RR | n / n / n |
| [56]Cherry Rose w/n#### | R / R | n / RR | RR / S | n / RR | n / n / n |

**Table 1 (end)**

| Accession (species, cultivar or line; cat number TGRC (LA), VIR (k) or SBR*) | Cold / Drought | Late blight / *Fusarium* | Blossom end rot / Gray rot | Verticillosis / Cladosporiosis | *Alternaria* / *Septoria* / TMV |
|---|---|---|---|---|---|
| [57]Indigo Rose w/n[####] | R / R | n / RR | RR / RR | n / RR | n / n / n |
| [58]Troia[####] | RR / RR | S / S | S / RR | S / RR | n / n / n |
| [59]OP-EF-1-6 w/n[####] | R / R | n / n | RR / n | n / n | n / n / n |
| [60]Zemba 8262336[#] | R / R | n / RR | RR / RR | n / RR | S / n / RR |
| [61]DeBarao Oranzhevyi 9803327[#####] | R / R | MR / n | S / S | n / S | RR / n / RR |
| [62]L-271-20 w/n[#] | n / n | n / n | RR / RR | n / RR | RR / n / RR |
| [63]Moneymaker LA2706 | n / n | RR / n | RR / RR | n / RR | n / n / RR |
| [64]A11round LA2463 | n / n | n / n | RR / n | n / RR | n / n / RR |
| [65]Mazero-6111 $F_2$ w/n[####] | n / n | n / n | RR / RR | n / RR | n / n / n |
| [66]White Beauty LA2464A | n / n | n / n | RR / n | n / RR | n / n / n |
| [67]Heinz 1706-BG LA4345 | R / R | n / n | RR / n | n / n | RR / n / n |
| [68]Black-Jack 8457464[###]; [69]Paul Robeson LA4450; [70]Christmas Blueberry w/n[#] | n / n | n / RR | RR / RR | n / RR | RR / n / n |

Note. Abbreviations: w/n – without number; n – no data; R – resistant (<0.5 score), RR – relatively resistant (0.5–1.0), MR – moderately resistant (1.1–2.0); S – sensitive (>2.0). Late blight (*Phytophthora infestans* de Bary A); *Fusarium* (*Fusarium oxysporum* (Schlecht.) f. sp. *lycopersici* (Sacc.)); Verticillosis (*Verticillium alboatrum* and *V. dahliae*); Cladosporiosis (*Cladosporium fulvum* Cooke); Gray rot (*Botrytis cinerea* Pers); *Alternaria* (*Alternaria solani* Sorauer); *Septoria* (*Septoria lycopersici* Speg); TMV – Tobacco mosaic virus.

* According to SBR (State Register of Breeding Achievements; http://reestr.gossortrf.ru/), TGRC – Tomato Genetic Resource Center (https://tgrc.ucdavis.edu/) or VIR (The N.I. Vavilov All-Russian Institute of Plant Genetic Resources).

[1–70] Numbering of accessions (used in Fig. 1–3).

[#] FSVC; [##] LLC 'Agrofirm Poisk'; [###] LLC 'Research Institute of Vegetable Breeding', LLC 'Agrofirma GAVRISH'; [####] LLC 'Breeding company GAVRISH'; [#####] LLC 'Breeding and seed-growing company 'Gisok'; [&] LLC Agrofirma 'Demetra-Sibir'; [&&] MONSANTO HOLLAND B. V.; [&&&] LLC 'Agrofirma Aelita'; [&&&&] LLC 'Premium seeds'.

**Table 2.** Results of AFLP analysis of tomato species, cultivars, hybrids and lines

| Primer combination | PIC | Number of fragments | | |
|---|---|---|---|---|
| | | Total | Polymorphic, pcs (%) | Unique |
| E32/M59 | 0.481 | 109 | 20 (18.0) | 11 |
| E32/M57 | 0.367 | 142 | 16 (11.2) | 9 |
| E38/M57 | 0.511 | 73 | 31 (42.5) | 2 |
| E41/M59 | 0.658 | 67 | 47 (70.1) | 3 |
| Total | – | 391 | 114 (35.5) | 25 |

and 29; see Table 1, Fig. 1). Clade D (intermediate position between A and B+C) combined 14 tomato varieties/lines. The two clades of cluster II, in turn, were divided into two subclades each (see Fig. 1).

On the graph constructed by the method of principal components, the analyzed cultivars formed three diffuse pools of genotypes, where, as in the dendrogram, a group of wild accessions stood out, and tomato varieties/lines were clustered in a similar way (Fig. 2). There was a clear division between clusters I and II (according to the dendrogram). Wild accession 11 (*S. lycopersicum* var. *pyriforme*) was the closest to subclade B varieties/lines.

It was interesting to analyze the possible relationship between the clustering of cultivars and accessions obtained from AFLP data and resistance to various biotic and abiotic stresses.

Varieties/lines of tomato included in cluster I (clades B, D) are the result of breeding by the FSVC (except accession 34). All of them are resistant to cold and/or drought, while accession 34 is susceptible. A similar situation is observed in the case of resistance to blossom end rot, *Septoria* and *Alternaria*. All clade B accessions are resistant to tobacco mosaic virus, as are half of clade D accessions (the other half are susceptible). Six accessions of clade D and five accessions of clade B are

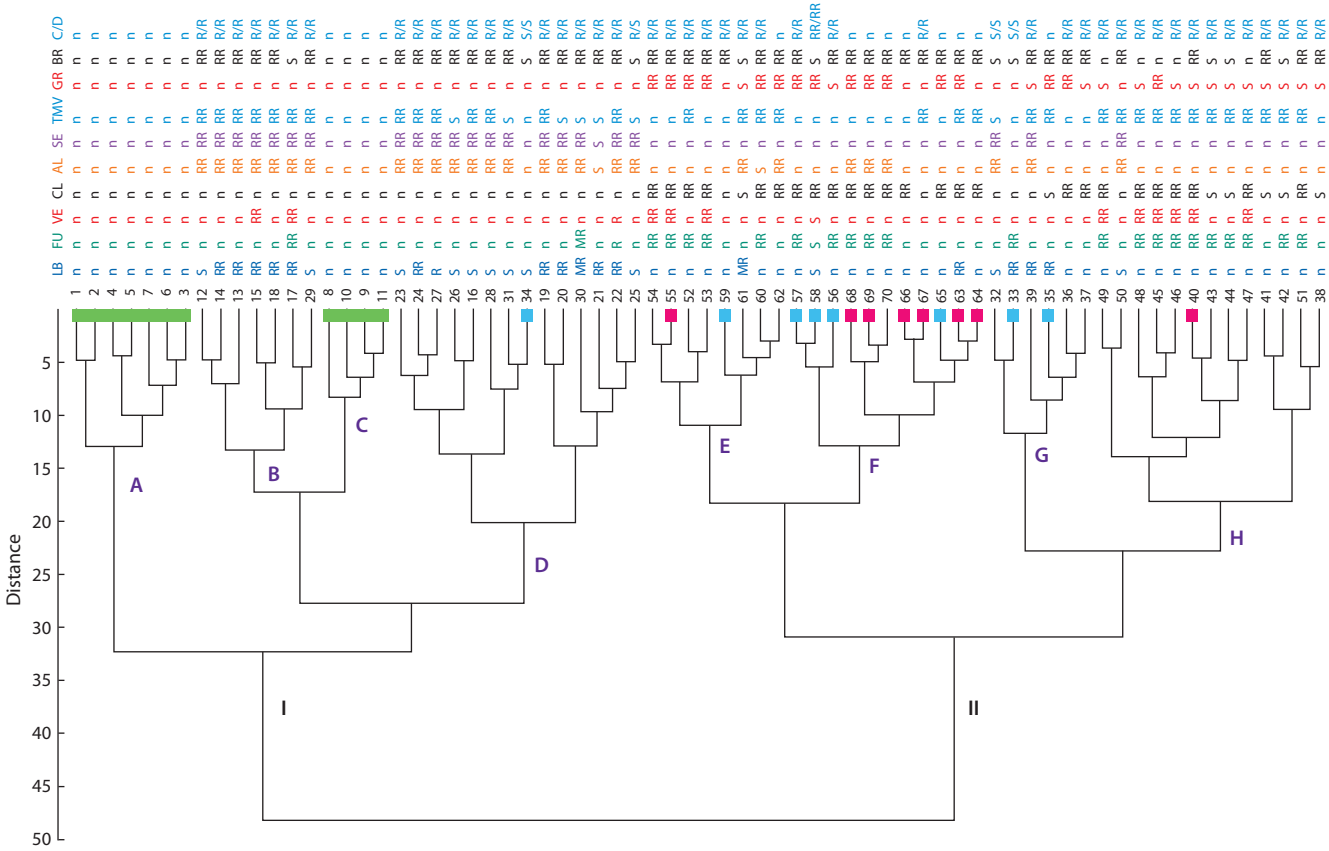


**Fig. 1.** Dendrogram based on AFLP data for cultivated and wild tomato accessions.

According to Table 1, the accessions are numbered (1–70), and resistance to late blight (LB), *Fusarium* (FU), *Verticillium* (VE), cladosporiosis (CL), alternariosis (AL), *Septoria* (SE), tobacco mosaic virus (TMV), gray rot (GR), blossom end rot (BR), cold (C) and drought (D) is indicated. The degree of resistance of the accessions is given according to Table 1: n – no data, S – susceptible, R – resistant, RR – relatively resistant, MR – moderately resistant. Boxes mark accessions: wild (green), foreign breeding (pink), breeding of LLC 'Breeding company GAVRISH' (blue); the rest are breeding of the FSVC.

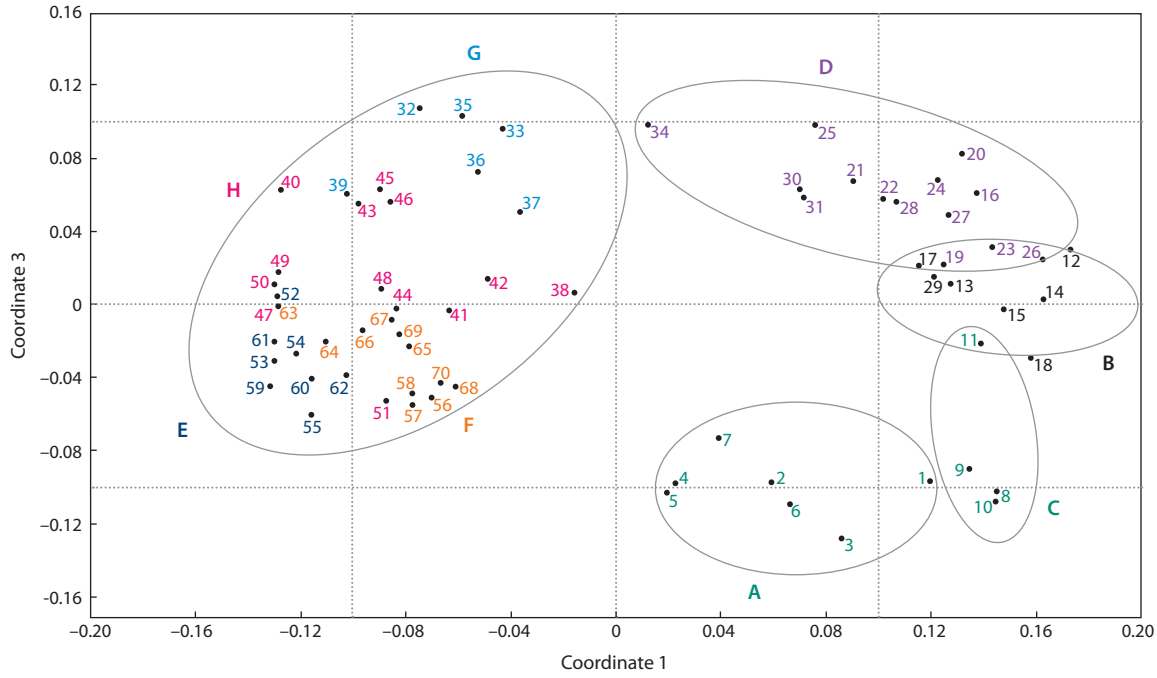


**Fig. 2.** PCA plot of AFLP data for 70 cultivated and wild tomato accessions.

The numbers correspond to the numbering of accessions in Table 1. The distribution of accessions by clades is shown in accordance with the dendrogram in Fig. 1: clades A and C are highlighted in green, B in black, D in lilac, E in dark blue, F in orange, G in blue, H in pink.

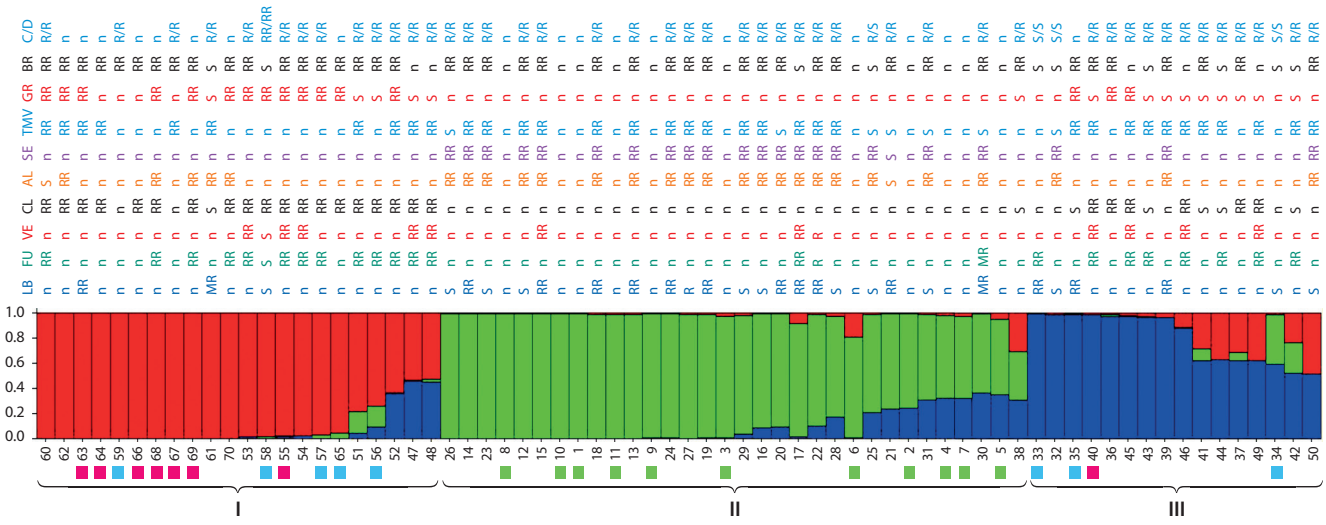


**Fig. 3.** Genomic structure of 59 cultivated and 11 wild tomato accessions according to AFLP analysis ($k$ = 3).

According to Table 1, the accessions are numbered (1–70), and the resistance to late blight (LB), *Fusarium* (FU), *Verticillium* (VE), cladosporiosis (CL), alternariosis (AL), *Septoria* (SE), tobacco mosaic virus (TMV), gray rot (GR), blossom end rot (BR), cold (C) and drought (D) is indicated. The degree of resistance of the accessions is given according to Table 1: n – no data, S – susceptible, R – resistant, RR – relatively resistant, MR – moderately resistant. Boxes mark accessions: wild (green), foreign breeding (pink), breeding of LLC 'Breeding company GAVRISH' (blue); the rest are breeding of the FSVC.

resistant to late blight; the remaining accessions of these clades are susceptible to this disease.

Accessions of subclades E and H, with the exception of one uncharacterized accession (62), are characterized by resistance to cold and drought; in subclades F and G, four and three accessions are resistant, respectively. Subclades E and F are distinguished by resistance to blossom end rot, gray mold and cladosporiosis (except for single susceptible or uncharacterized varieties). About half of subclade E accessions are resistant to *Verticillium* and *Fusarium*. Most of subclade H accessions, as well as two groups of the subclade F, are resistant to *Fusarium*. Subclade G accessions have resistance to late blight (see Fig. 1). Almost all subclade H accessions originated from the FSVC. Accessions of foreign breeding (except for 55 and 40) stand out in subclade F, clustering together with accessions of breeding of LLC 'Breeding company GAVRISH'.

The study also included an analysis of the population structure of 70 tomato accessions, which revealed common genetic blocks and their ratio in each accession. This distributed the analyzed accessions into clusters. In total, 16 options for the number of subgroups ($k$) from 3 to 18 were analyzed. The best result (LnLike = –12363.6) was obtained for $k$ = 3.

On the graph, the genomic structure of the studied 70 tomato accessions is presented in the form of various ratios of three blocks (Fig. 3). All accessions of wild species, including accessions of *S. lycopersicum*, fell into cluster II. An analysis of the correlations between the distribution of accessions by clusters and the traits under consideration (see Table 1) showed a tendency to combine accessions in terms of resistance to gray rot, blossom end rot, *Fusarium*, cladosporiosis, and *Septoria* (cluster I). Cold and drought resistant accessions are presented in large numbers in all three clusters. Resistance to *Alternaria*, *Septoria*, and TMV proved to be the most typical for cluster II (see Fig. 3). Also, half of the varieties in cluster II are resistant to blossom end rot, and a third of the accessions are resistant to late blight. Cluster III accessions were characterized by different variants of resistance; we can assume clustering on the basis of resistance to TMV (11 out of 16 accessions), as well as susceptibility to gray rot. Except for accession 40 (cluster III), all tomato accessions of foreign breeding were identified in cluster I. The accessions of the LLC 'Breeding company GAVRISH' were distributed similarly (four accessions – cluster I, three accessions – cluster III) (see Fig. 3).

## Discussion

In this study, using the AFLP method, we analyzed 11 wild and 59 cultivated (*S. lycopersicum*) tomato accessions, mainly of domestic breeding (see Table 1). It should be noted that data on resistance to various diseases (Gossortreestr, originators) are unknown for some analyzed cultivated and wild accessions studied. The species *S. lycopersicum* (wild accessions 7–11 in Table 1) comes from the humid tropics of South America and is a classic example of a cold-sensitive crop (Rick, 1976). The remaining wild species used (accessions 1–6 in Table 1) grow in different climatic zones of South America, from the tropics of the Amazon basin to deserts along the coast and the cold high mountains of the Andes (Nakazato et al., 2010). This suggests that accessions 1–6 are resistant to cold and drought, and accessions 7–11 are sensitive to these stresses.

Each of the 70 accessions was characterized by a specific range of fragments obtained using a combination of four primer pairs (see Table 2). The efficiency obtained (391 fragments, including 114 polymorphic fragments) was comparable with the results of other studies. For example, an AFLP analysis of 21 tomato varieties with four primer combinations revealed 298 fragments, including 159 polymorphs (Suliman-Pollatschek et al., 2002). The percentage of polymorphic fragments obtained by us (29.16 %) also fit into the known data on different crops – in a number of studies it varies from 17.4 to 78.3 % (Kim et al., 1998; Vetelainen et al., 2005).

Analysis of the obtained AFLP data using various bioinformatic methods distributed the studied tomato accessions in a similar way (see Fig. 1–3). Wild tomato accessions isolated themselves into a separate group (see Fig. 2, 3) or divided into clades within cluster I (see Fig. 1). In the dendrogram, accessions 1–6 (tomato species except *S. lycopersicum*) constituted a separate clade A, and 8–11 (various wild *S. lycopersicum* accessions) constituted clade C (see Fig. 1). At the same time, accession 7 (*S. lycopersicum* LA1673) did not combine with 8–11, but entered the subclade with red-fruited accessions 3–6 (*S. pimpinellifolium*, *S. galapagense*), which may indicate a probable interspecific introgression. Sister clades B and D consisted of *S. lycopersicum* cultivars, for which resistance to drought and/or cold was shown (see Fig. 1). This, on the one hand, confirms our assumptions about the possible resistance of wild accessions 1–6 taken for analysis to drought/cold, and also suggests this trait in accessions 7–11. Cold/drought resistance in more than half of the samples of clusters I and II (see Fig. 1) allows us to assume the presence of such resistance in varieties for which there are no data. In addition, the results may indicate the presence of traits of resistance to abiotic stresses introgressed from wild tomato species in the genome of varieties of both clusters.

A fairly clear grouping of accessions by origin shows the effectiveness of the analysis and, at the same time, helps to trace possible links in the pedigree of varieties both from one originator and between breeding centers.

## Conclusion

Thus, using AFLP genotyping of selectively neutral regions of the genome of *S. lycopersicum* cultivars/lines and wild tomato species, clustering of accessions was shown according to resistance to biotic and abiotic stress factors, as well as according to origin from different breeding centers. The prospects of AFLP with the set of primer combinations chosen in this study for genotyping tomato varieties in order to select cultivars with resistance to various stresses were demonstrated. The obtained clade-specific fragments can become the basis for the development of specific molecular markers associated with economically important traits. Sequencing polymorphic AFLP fragments that underlie differences between accession clusters, mapping them on the genome, and assessing the variability of such regions among the analyzed varieties may be promising for obtaining STS markers.

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# Earliness and morphotypes of common wheat cultivars of Western and Eastern Siberia

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**Abstract.** The global and local climate changes determine the producing of highly-adaptive common (bread) wheat commercial cultivars of a new generation whose optimal earliness matches the climatic features of the territory where the cultivars are farmed. Principal component analysis involving our own and published data has been applied to investigate 98 commercial common wheat cultivars from Western and Eastern Siberia comparing their morphotypes; cultivar zoning time; length of the vegetation period; 1000-grain weight, and inheritance of spring growth habit. It demonstrated that the dominant *Vrn* gene polymorphism determining the spring growth habit of the Siberian cultivars was minimally polymorphic. In 75 % of the tested cultivars, the spring growth habit was controlled by digenic, namely dominant *Vrn-A1* and *Vrn-B1* genes. In 25 % of them (24 cultivars), spring growth habit is controlled by a single gene. In 19 and 5 of these cultivars spring growth habit is controlled by only one dominant gene, *Vrn-B1* or *Vrn-A1*, respectively. In cv. Tulun 15, a trigenic control was identified. A conclusion about the optimality of the digenic control for the climatic conditions of both Western and Eastern Siberia has been confirmed. However, since none of the tested cultivars had the dominant *Vrn-D1* gene typical of the regions of China and Central Asia bordering Siberia, it can be considered as an additional argument in favor of the European origin of Siberian common wheat cultivars. The revealed high frequency of the *Vrn-B1c* allele in the Western Siberian cultivars and the *Vrn-B1a* allele in the Eastern Siberian cultivars suggests their selectivity. The analysis also confirmed the dominance of red glume (*ferrugineum*, *milturum*) and awned spike (*ferrugineum*, *erythrospermum*) varieties in the Eastern Siberian cultivars, and white glume and awnedless spike (*lutescens* and *albidum*) ones in the Western Siberian cultivars. Small grain size cultivars are more typical of Eastern than Western Siberia. The retrospective analysis based on the cultivars' zoning time included in the "State Register for Selection Achievements Admitted for Usage" brought us to the conclusion that the earliness/lateness of modern Siberian commercial cultivars was not regionally but rather zonally-associated (taiga, subtaiga, forest-steppe and steppe zones).
Key words: common wheat; *Vrn* genes; commercial and local cultivars; earliness; morphotype; breeding.

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# Скороспелость и морфотип сортов мягкой пшеницы Западной и Восточной Сибири

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**Аннотация.** Глобальное и локальное изменение климата обусловливает создание высокоадаптивных сортов яровой мягкой пшеницы нового типа с оптимальной скороспелостью, отражающей региональные особенности климатических условий территорий ее возделывания. Методом главных компонент с использованием собствен-

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ных данных и результатов, опубликованных другими исследователями, проведено сравнение 98 селекционных коммерческих сортов яровой мягкой пшеницы Западной и Восточной Сибири по морфотипу, времени районирования, длине вегетационного периода, массе 1000 зерен и характеру наследования ярового типа развития. Полиморфизм по доминантным генам *Vrn*, контролирующим яровость у селекционных сортов Сибири, минимален. У 75 % из них он контролируется дигенно доминантными *Vrn-A1* и *Vrn-B1*; у 25 % – моногенно доминантными генами (у 24 сортов, из них у 19 и 5 соответственно только одним доминантным геном – *Vrn-A1* или *Vrn-B1*). У одного сорта, Тулун 15, описан тригенный контроль. Подтвержден вывод об оптимальности для климатических условий как Западной, так и Восточной Сибири контроля яровости двумя доминантными генами *Vrn*. Ни у одного из сортов не обнаружено характерного для приграничных с Сибирью областей Китая и Центральной Азии доминантного гена *Vrn-D1*. Данное наблюдение может служить дополнительным аргументом в пользу гипотезы европейского происхождения сортимента Сибири. Выявлена повышенная частота встречаемости аллеля *Vrn-B1c* у сортов Западной Сибири и аллеля *Vrn-B1a* у сортов Восточной Сибири, что позволяет говорить об их селективности. Подтверждено преобладание красноколосых (*ferrugineum*, *milturum*) и остистых (*ferrugineum*, *erythrospermum*) разновидностей в Восточной Сибири, безостых и белоколосых (*lutescens* и *albidum*) – в Западной Сибири. Для Восточной Сибири характерны более мелкозерные сорта. Выполненная ретроспектива по годам районирования (включения в «Государственный реестр селекционных достижений, допущенных к использованию» РФ) позволяет отметить, что скороспелость/позднеспелость современных сортов Сибири не имеет ярко выраженной региональной компоненты, а является зональной (тайга, подтайга, лесостепь, степь).
Ключевые слова: мягкая пшеница; гены *Vrn*; селекционные и местные сорта; скороспелость; морфотип; селекция.

## Introduction

Wheat is one of the three most wide-spread crops in the world, but, unlike rice and corn, both its winter and spring cultivars are widely cultivated. Spring common (bread) wheat, in this respect, mains a crucial crop for the regions of South and North America, Australia, Central and South-East Asia as well as for those of Northern Asia which have harsh continental climate (Morgounov et al., 2018; Garcia et al., 2019; Rivelli et al., 2021).

In the Russian Federation, these are mainly the winter and spring cultivars of common wheat (*Triticum aestivum* L.) and the spring cultivars of macaroni ones (*T. durum* Desf.) that are farmed. Only insignificant areas are taken to grow winter macaroni (Fomenko, Grabovets, 2016) and Indian dwarf wheat (*T. sphaerococcum* Perciv.) (Bespalova et al., 2015) cultivars as well as emmer (*T. dicoccum* Schrank. ex Schübler) (Temirbekova et al., 2020). The "State Register for Selection Achievements Admitted for Usage (National List)" also includes two cultivars of Rivet wheat (*T. turgidum* L.) and one of spelt (*T. spelta* L.) (State Register…, 2021)[1]. Among the mentioned ones, the common wheat species the backbone of Russia's crop farming, dominating not only over other species of genus *Triticum* L. but overall crops cultivated in the country.

Along with Southern Urals and the Volga region, Eastern and Western Siberia are main territories of spring common wheat farming in Russia. The global and local climate changes determine the producing of highly-adaptive common wheat cultivars of a new generation with optimal earliness to match the climatic features of the territory where they are farmed, including the harsh continental areas of Western and Eastern Siberia.

The duration of vegetation period (earliness) in wheat, as the most important adaptive trait (Lozada et al., 2021), determines not only plant productivity (yield) but also affects its resistivity to such external environmental stress factors as drought, low temperature, insects, diseases, etc. (Zotova et al., 2019). Moreover, farming spring cultivars of different earliness enables one to control harvesting times to reduce peak loads on agricultural machinery and yield losses due to overmature (Belan et al., 2021). Duration of vegetation period in wheat is a complex trait whose extent is mainly determined by the allele diversity of the *Vrn* genes controlling the type growth habit (spring *vs*. winter) and response to vernalization and by that of the *Ppd* genes controlling response to photoperiod (Kiseleva, Salina, 2018). Currently, a set of VRN loci (*Vrn-A1*, *Vrn-B1*, *Vrn-D1*, *Vrn-B3*, and *Vrn-D4* genes) (Goncharov, 2003; Yan et al., 2003, 2004, 2006; Kippes et al., 2014) and at least two PPD loci (*Ppd-B1*, *Ppd-D1* genes) (Welsh et al., 1973; Beales et al., 2007; Diaz et al., 2012) have been identified in hexaploid wheat species.

In wheat, the molecular basis of genetic control of earliness is being intensively investigated (Royo et al., 2020), but there is still considerable uncertainty when it comes to its phenotypical manifestations determined by the interaction of *Vrn* and *Ppd* gene alleles. Some experts claim *Vrn* genes control up to 75 % of the variability related to the duration of the vegetation period (earliness), while *Ppd* genes account for only 20 % of them (Stelmakh, 1981). However, in the spring cultivars and when winter and spring wheat are cultivated northward of 55° N and southward of 55° S, the influence of these genes on the trait manifestation changes significantly. The results of the correlation analysis of the vegetation period duration in the wheat cultivars with yield have been contradictory (Vedrov, 2006; Meng et al., 2016; Piskarev et al., 2018; Rigin et al., 2018; Sidorov, 2018; Kuz'min et al., 2019; among others) and, due to their importance, call for comprehensive study.

The current diagnostic molecular markers have been developed to identify the alleles of the *Vrn* and *Ppd* genes. They have made it possible to detect the presence/absence of

[1] For the last two species, the "State Register…" indicates no type of growth habit (spring *vs*. winter). Moreover, the State Commission for Breeding Test System and Protection uses the wheat taxon names nonrelated to the Russian scientific tradition (Dorofeev et al., 1979; Goncharov, 2011).

dominant *Vrn* and *Ppd* genes in both local and commercial (cultivated) wheat cultivars from the countries of Europe, Asia, North and South America, Africa and Australia (Zheng et al., 2013; Gomez et al., 2014; Cho et al., 2015; Shcherban et al., 2015; Whittal et al., 2018; Royo et al., 2020). It has been shown that the most early-maturing cultivars possess at least three dominant *Vrn* genes (Zhang et al., 2008; Rigin et al., 2019, 2021a, b), among which some authors include the rare dominant *Vrn-B3* allele (Zhang et al., 2008). It is noteworthy that this allele has been detected in the only one Russian cv. Tulun 15 being most early maturing among those permitted for cultivate in Siberia (Lysenko et al., 2014). A new dominant *Vrn-A3* gene controlling the early maturity in the accession TN26 of *T. dicoccum* has been described (Nishimura et al., 2018). It is assumed that the early maturity is caused by the GATA-box element found in this gene, while this locus as well as the VRN-2 one described in *T. monococcum* L. (Tan, Yan, 2016) is not functional in hexaploid wheat cultivars.

Genogeographic studies have been performed in our country to investigate the *Vrn* genes in spring wheat landraces (Genotypes…, 1985; Goncharov, Shitova, 1999; Moiseeva, Goncharov, 2007) as well as in the Russian commercial cultivars (Genotypes…, 1985; Catalogue…, 1987; Shcherban et al., 2012b; Lysenko et al., 2014; etc.). The other investigated cultivars were spring common wheats from Siberia (Fait, Stelmakh, 1993; Dzhalpakova et al., 1996; Likhenko et al., 2014; etc.); the local cultivars of seven hexaploid wheat species from different regions of Eurasia (Dragovich et al., 2021); macaroni wheat cultivars (Dzhalpakova et al., 1995; Konopatskaia et al., 2016), and emmer landraces (Rigin et al., 1994). As an earliness donor, *Aegilops squarrosa* L. (syn. *Aegilops tauschii* Coss.) the D genome donor of polyploid wheats has been suggested (Goncharov, Chikida, 1995).

The search for *Vrn* gene polymorphisms and studying its influence to the earliness expression has been one of the key directions of the Russian wheat breeding programs, including Eastern and Western Siberia since dominant *Ppd* genes are not that widespread in Siberian cultivars (Likhenko et al., 2014; Balashova, Fait, 2021).

The purpose of the present study was to compare the bred (commercial) cultivars of spring common wheat from Western and Eastern Siberia for their dominant *Vrn* alleles and morphotypes to investigate their effect on the earliness, yield and cultivar zoning time.

## Materials and methods

**Biological material.** Only cultivars of common wheat included in the "State Register for Selection Achievements Admitted for Usage (National List)" (State Register…, 2021) were studied in our investigation. Information on inheritance of them spring growth habit included in the was either obtained in this investigation or taken from publications (Dzhalpakova et al., 1996; Likhenko et al., 2014; Lysenko et al., 2014, etc.) (Supplementary Material)[2]. The data about morphotypes, duration of vegetation period and 1000-grain weight were taken from the official publications of The State Commission for Breeding Test System and Protection (Guidelines…, 1928, 1937; among others)[3], "Catalog…" (2009), since they provided information on trait fluctuations for all state agricultural facilities in a region, and these data were necessary for obtaining integral estimations. In total, the information about 98 commercial spring common wheat cultivars from Eastern and Western Siberia (zoning time from 1929 through 2021) was collected as well as the information concerning four our breeding lines was used (see Supplementary Material).

In case of incomplete data, a cultivar was removed from analysis, such as the cvs Soanovskaya 4 and Khludovka. In addition, the cultivars produced in Siberian agricultural institutions but zoned in other regions such as Perm (cv. Tayezhnaya) or the Far East (cv. Priobskaya) were removed. Since the local cultivar data were mainly represented by East Siberian cultivars (Goncharov, Shitova, 1999) and Tuva landraces (Moiseeva, Goncharov, 2007), they were used only in discussion.

**PCR amplification conditions and total DNA isolation protocol.** The total DNA isolation and PCR amplification conditions were carried out as described in (Konopatskaia et al., 2016). For PCR amplification were used the primers specific for the *Vrn-A1*, *Vrn-B1* and *Vrn-D1* genes (Konopatskaia et al., 2016).

**Data.** The information about the genotypes and phenotypes of on such biological traits as botanical varieties, the duration of vegetation period (earliness), 1000-grain weight and *Vrn* gene alleles presented in Supplementary Material. Note that the cultivar botanical varieties were first studied as a whole, e. g., *ferrugineum* and *lutescens*, and then subset into their element traits such as spike color, awnedness/awnedlessness, etc. according their spike and grain traits (Table 1).

**Statistical analysis.** For statistical processing, quantitative and qualitative characteristics of cultivars were used. Both quantitative (mean duration of vegetation period, 1000-grain weight) and binary traits (awnedness/awnedlessness, spike and grain color, *Vrn-A1* gene alleles) were aligned and normalized in a way the sum of squares for each of them was equal to one. Every qualitative character, whose gradation exceeded three (*Vrn-B1* gene alleles, morphotypes), was coded using a binary number in which one marked belonging to this particular gradation, and zero – to all other graduations used. Since this population still represented just a single trait, it was aligned and normalized for its sum of squares to be equal to one as well. In such a way all the traits were equally weighted. To estimate the principal components for all the investigated biological traits, a Euclidean distance matrix was built and the principal coordinate method was applied (Gower, 1966).

[2] Supplementary Material is available in the online version of the paper: http://vavilov.elpub.ru/jour/manager/files/Suppl_Smolenskaya_27_7.pdf

[3] As it has been mentioned earlier, the vegetation period grades of the different cultivars studied in different sites almost never change (Goncharov, Efimov, 1990; Smiryaev et al., 1992). For that reason, in our study we followed E.S. Kuznetsova's (1929) approach who considered that studying a cultivar core collection set enabled one to obtain proper information about the whole species polymorphism.

**Table 1.** Reduced classification of the most important common wheat botanical varieties (from: Plotnikov et al., 1937)

| Grain color | Spike glume | |
|---|---|---|
| | Naked glumes | Hairy glumes |
| | Awnedless spike | |
| | White glumes | |
| White | *albidum* Al. | *anglicum* Mazz.(=syn. *leucospermum* Körn.) |
| Red | *lutescens* Al. | *velutinum* Schübl. |
| | Red glumes | |
| White | *alborubrum* Körn. | *Delfi* Körn. |
| Red | *milturum* Körn. | *pyrothrix* Al. |
| | Awned spike | |
| | White glumes and white awns | |
| White | *graecum* Körn. | *meridionale* Körn. |
| Red | *erythrospermum* Körn. | *hostianum* Clem. |
| | Red glumes and red awns | |
| White | *erythroleucon* Körn. | *turcicum* Körn. |
| Red | *ferrugineum* Al. | *barbarossa* Al. |
| | Gray-blue or black-blue glumes and gray-blue awns | |
| Red | *caesium* Al. | *coeruleovelutinum* Al. |

**Table 2.** Principal component dispersions (λ) and their accumulated sums (Sum)

| PC | λ | λ, % | Sum | Sum, % |
|---|---|---|---|---|
| PC1 | 3.17 | 39.6 | 3.17 | 39.6 |
| PC2 | 1.27 | 15.9 | 4.44 | 55.5 |
| PC3 | 1.11 | 13.9 | 5.55 | 69.3 |
| PC4 | 0.77 | 9.6 | 6.32 | 79.0 |
| PC5 | 0.52 | 6.5 | 6.84 | 85.5 |
| PC6 | 0.37 | 4.6 | 7.21 | 90.1 |
| PC7 | 0.32 | 4.0 | 7.53 | 94.1 |
| PC8 | 0.28 | 3.5 | 7.81 | 97.6 |
| PC9 | 0.10 | 1.3 | 7.91 | 98.9 |
| PC10 | 0.04 | 0.5 | 7.96 | 99.5 |
| PC11 | 0.04 | 0.5 | 8.00 | 100.0 |

Note. PC – the principal components.

## Results

The results of the statistical data processing for the cultivars biological traits and their agronomical characteristics (see Materials and methods) can be seen in Tables 2 and 3, Fig. 1–3. The contributions of the first three principal components gave 69.3 % in total (see Table 2) that comprised around 70 % of the total dispersion.

A cultivar agronomical characteristics are included its farming region and the zoning time, i. e., the year it was included in "State Register for Selection Achievements Admitted for Usage (National List)". In our study, these characteristics were considered as external ones and for that reason were excluded from the principal component estimation. For biological interpretation of the obtained com-

**Table 3.** Principal component correlation matrix (×1000) between the biological traits and agronomical characteristics of the studied cultivars

| Parameter | Region01 | West | East | Year | PC1 | PC2 | PC3 | Awn | Spike color | Grain color | DVP | 1000-weigh grain | *Vrn-A1* | *Vrn-B1a* | *Vrn-B1c* | *Vrn-B1null* | *albidum* | *caesium* | *erythro-spermum* | *ferrugineum* | *lutescens* | *milturum* | *pyrothrix* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Region01 | ***1000*** | ***–926*** | ***940*** | –203 | ***392*** | 7 | –81 | 363 | 363 | 66 | –47 | –339 | 190 | 280 | –316 | 20 | –35 | –76 | 3 | ***460*** | –260 | 3 | –76 |
| West | ***–926*** | ***1000*** | ***–741*** | 253 | ***–439*** | 22 | 16 | ***–413*** | ***–413*** | –31 | 33 | 377 | –150 | –345 | 329 | 40 | 5 | 60 | –29 | ***–497*** | 331 | –29 | 60 |
| East | ***940*** | ***–741*** | ***1000*** | –132 | 299 | 32 | –129 | 273 | 273 | 89 | –53 | –262 | 202 | 186 | –265 | 70 | –59 | –80 | –20 | 369 | –164 | –20 | –80 |
| Year | –203 | 253 | –132 | ***1000*** | ***–585*** | 26 | –244 | ***–485*** | ***–582*** | 55 | 95 | ***640*** | 10 | –166 | 36 | 145 | –83 | –270 | –81 | ***–431*** | ***537*** | –266 | 42 |
| PC1 | ***392*** | ***–439*** | 299 | ***–585*** | ***1000*** | 0 | 0 | ***859*** | ***893*** | –25 | ***–550*** | ***–863*** | 207 | ***398*** | –295 | –133 | 21 | 236 | 120 | ***867*** | ***–790*** | 184 | 12 |
| PC2 | 7 | 22 | 32 | 26 | 0 | ***1000*** | 0 | 127 | 111 | ***949*** | –147 | –1 | –312 | –308 | 231 | 100 | ***–857*** | 44 | 16 | 126 | ***420*** | –14 | ***–390*** |
| PC3 | –81 | 16 | –129 | –244 | 0 | 0 | ***1000*** | 103 | 132 | –234 | 377 | –147 | ***–861*** | –202 | ***415*** | –208 | 234 | 52 | 22 | 90 | –244 | 78 | 50 |
| PC4 | 232 | –239 | 196 | –123 | 0 | 0 | 0 | 244 | 153 | 150 | ***680*** | 105 | 252 | –155 | –277 | ***459*** | –104 | 59 | 334 | 61 | –185 | 161 | –124 |
| PC5 | –152 | 185 | –103 | 146 | 0 | 0 | 0 | 297 | –152 | –129 | –244 | 218 | –40 | ***–614*** | 125 | ***548*** | 98 | 54 | ***529*** | 3 | –173 | –326 | 91 |
| PC6 | –144 | 170 | –103 | –120 | 0 | 0 | 0 | –119 | 212 | –13 | 15 | 35 | 203 | ***–425*** | ***697*** | –255 | 39 | 125 | –199 | –63 | –95 | ***431*** | –47 |
| PC7 | –8 | –47 | –57 | 101 | 0 | 0 | 0 | 236 | –65 | 45 | 8 | 274 | 19 | 337 | 191 | ***–571*** | –76 | 90 | ***469*** | –45 | –122 | –106 | 49 |
| PC8 | –45 | 26 | –57 | 125 | 0 | 0 | 0 | –124 | 269 | –7 | –105 | 299 | –110 | 106 | –268 | 161 | –60 | 22 | –139 | –69 | –163 | ***611*** | 134 |
| PC9 | –176 | 165 | –163 | –212 | 0 | 0 | 0 | –32 | –61 | 47 | –16 | –92 | –7 | 6 | –18 | 12 | –24 | 93 | ***535*** | ***–409*** | –153 | ***479*** | –57 |
| PC10 | –36 | 37 | –30 | 12 | 0 | 0 | 0 | –5 | –8 | –27 | 6 | –12 | 3 | –8 | 9 | 0 | ***–421*** | –62 | 8 | 12 | –12 | 3 | ***890*** |
| PC11 | –143 | 143 | –126 | –103 | 0 | 0 | 0 | 28 | 29 | –12 | 1 | 0 | –1 | 4 | –10 | 7 | –21 | ***947*** | –169 | –207 | 42 | –168 | 67 |
| Awn | 363 | ***–413*** | 273 | ***–485*** | ***859*** | 127 | 103 | ***1000*** | ***716*** | 85 | –345 | ***–640*** | 93 | 179 | –216 | 27 | –75 | 272 | ***476*** | ***799*** | ***–739*** | –65 | –37 |
| Spike color | 363 | ***–413*** | 273 | ***–582*** | ***893*** | 111 | 132 | ***716*** | ***1000*** | 85 | –340 | ***–732*** | 93 | 280 | –180 | –122 | –75 | 272 | –65 | ***799*** | ***–739*** | ***476*** | –37 |
| Grain color | 66 | –31 | 89 | 55 | –25 | ***949*** | –234 | 85 | 85 | ***1000*** | –80 | 48 | –58 | –178 | 72 | 122 | ***–890*** | 23 | 40 | 68 | ***459*** | 40 | ***–438*** |
| DVP | –47 | 33 | –53 | 95 | ***–550*** | –147 | 377 | –345 | –340 | –80 | ***1000*** | ***410*** | –196 | –220 | 107 | 132 | 112 | –90 | 44 | ***–408*** | 214 | 52 | –45 |
| 1000-weigh grain | –339 | 377 | –262 | ***640*** | ***–863*** | –1 | –147 | ***–640*** | ***–732*** | 48 | ***410*** | ***1000*** | –54 | –354 | 190 | 195 | –72 | –166 | 74 | ***–752*** | ***589*** | –100 | 36 |
| *Vrn-A1* | 190 | –150 | 202 | 10 | 207 | –312 | ***–861*** | 93 | 93 | –58 | –196 | –54 | ***1000*** | 246 | ***–390*** | 134 | 52 | 25 | 44 | 75 | –126 | 44 | 25 |
| *Vrn-B1a* | 280 | –345 | 186 | –166 | ***398*** | –308 | –202 | 179 | 280 | –178 | –220 | –354 | 246 | ***1000*** | ***–566*** | ***–516*** | 136 | 12 | –107 | 277 | –279 | 85 | 122 |
| *Vrn-B1c* | –316 | 329 | –265 | 36 | –295 | 231 | ***415*** | –216 | –180 | 72 | 107 | 190 | ***–390*** | ***–566*** | ***1000*** | ***–413*** | –40 | 39 | –69 | –230 | 215 | –1 | –78 |
| *Vrn-B1null* | 20 | 40 | 70 | 145 | –133 | 100 | –208 | 27 | –122 | 122 | 132 | 195 | 134 | ***–516*** | ***–413*** | ***1000*** | –108 | –53 | 190 | –68 | 84 | –93 | –53 |
| *albidum* | –35 | 5 | –59 | –83 | 21 | ***–857*** | 234 | –75 | –75 | ***–890*** | 112 | –72 | 52 | 136 | –40 | –108 | ***1000*** | –21 | –36 | –60 | ***–408*** | –36 | –21 |
| *caesium* | –76 | 60 | –80 | –270 | 236 | 44 | 52 | 272 | 272 | 23 | –90 | –166 | 25 | 12 | 39 | –53 | –21 | ***1000*** | –18 | –30 | –201 | –18 | –10 |
| *erythrospermum* | 3 | –29 | –20 | –81 | 120 | 16 | 22 | ***476*** | –65 | 40 | 44 | 74 | 44 | –107 | –69 | 190 | –36 | –18 | ***1000*** | –52 | –352 | –31 | –18 |
| *ferrugineum* | ***460*** | ***–497*** | 369 | ***–431*** | ***867*** | 126 | 90 | ***799*** | ***799*** | 68 | ***–408*** | ***–752*** | 75 | 277 | –230 | –68 | –60 | –30 | –52 | ***1000*** | ***–590*** | –52 | –30 |
| *lutescens* | –260 | 331 | –164 | ***537*** | ***–790*** | ***420*** | –244 | ***–739*** | ***–739*** | ***459*** | 214 | ***589*** | –126 | –279 | 215 | 84 | ***–408*** | –201 | –352 | ***–590*** | ***1000*** | –352 | –201 |
| *milturum* | 3 | –29 | –20 | –266 | 184 | –14 | 78 | –65 | ***476*** | 40 | 52 | –100 | 44 | 85 | –1 | –93 | –36 | –18 | –31 | –52 | –352 | ***1000*** | –18 |
| *pyrothrix* | –76 | 60 | –80 | 42 | 12 | ***–390*** | 50 | –37 | –37 | ***–438*** | –45 | 36 | 25 | 122 | –78 | –53 | –21 | –10 | –18 | –30 | –201 | –18 | ***1000*** |

Note. The light-red and light-green colors mark $p < 0.001$, and red and green – $p < 10^{-4}$; West – Western Siberia, East – Eastern Siberia; PC – the principal component; DVP – duration of vegetation period.

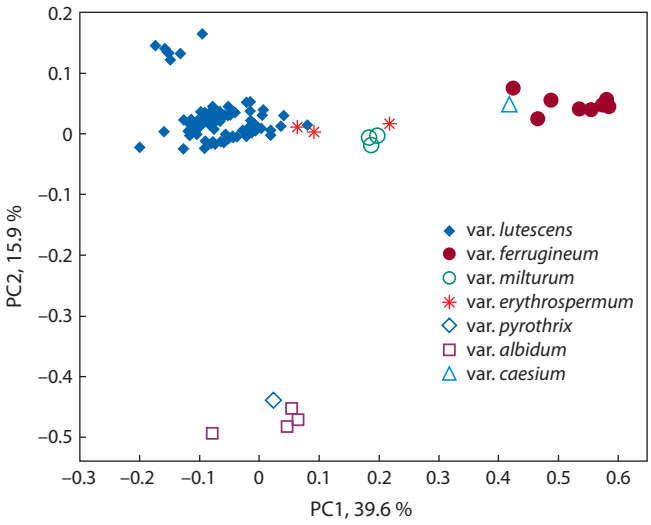


**Fig. 1.** Disposition of cultivar varieties with respect to the first two principal components.

ponents, it was enough to calculate their correlations with any qualitative traits including biological and agronomical ones (see Table 3).

Cultivar position determined, first-hand, by the their morphotypes, namely, *lutescens*, *erythrospermum* and *milturum* varieties take the upper left-hand (LEM group), morphotypes *ferrugineum* and *caesium* (FC group) – the upper right-hand, and *albidum* and *pyrothrix* (AP group) – lower left-hand corner. Thus, the primary component is determined by the differences between groups LEM and FC, and the secondary – between groups LEM and AP (see Fig. 1).

The agronomical characteristics farming region and cultivar zoning time correlated only with the PC1, giving $r = 0.392$ and $r = –0.585$, respectively. The biological traits correlating with the PC1 included the awnedness, spike color, duration of vegetation period, 1000-grain weight, *Vrn-B1a* allele presence, var. *ferrugineum* and *lutescens*. A direct correlation was discovered between *Vrn-B1a* and

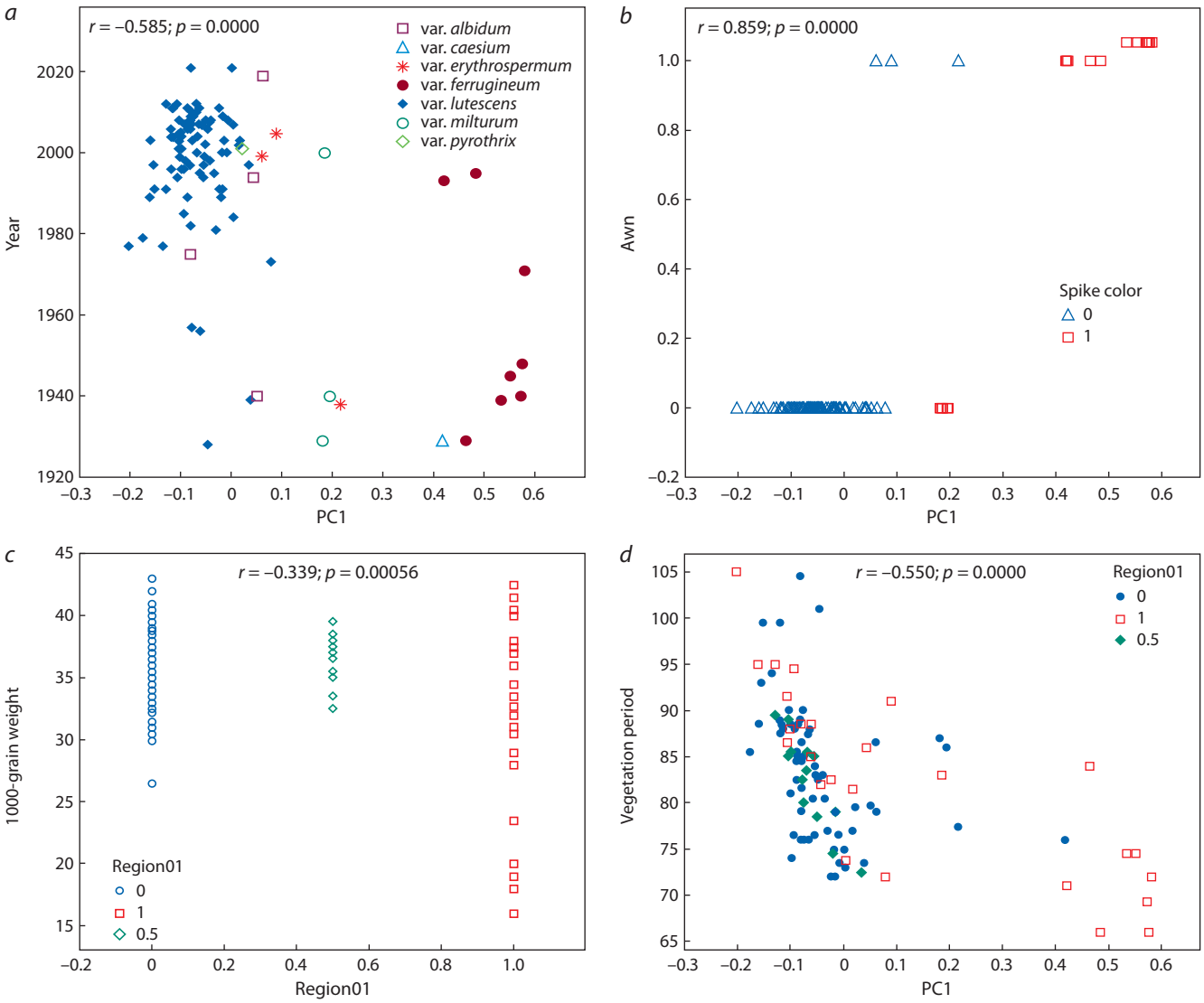


**Fig. 2.** Trait-pair scattering diagram.

*a* – morphotype-based cultivar zoning time against PC1; *b* – spike color and awnedness against PC1; *c* – region-based against 1000-grain weight; *d* – region-based duration of vegetation period against PC1.

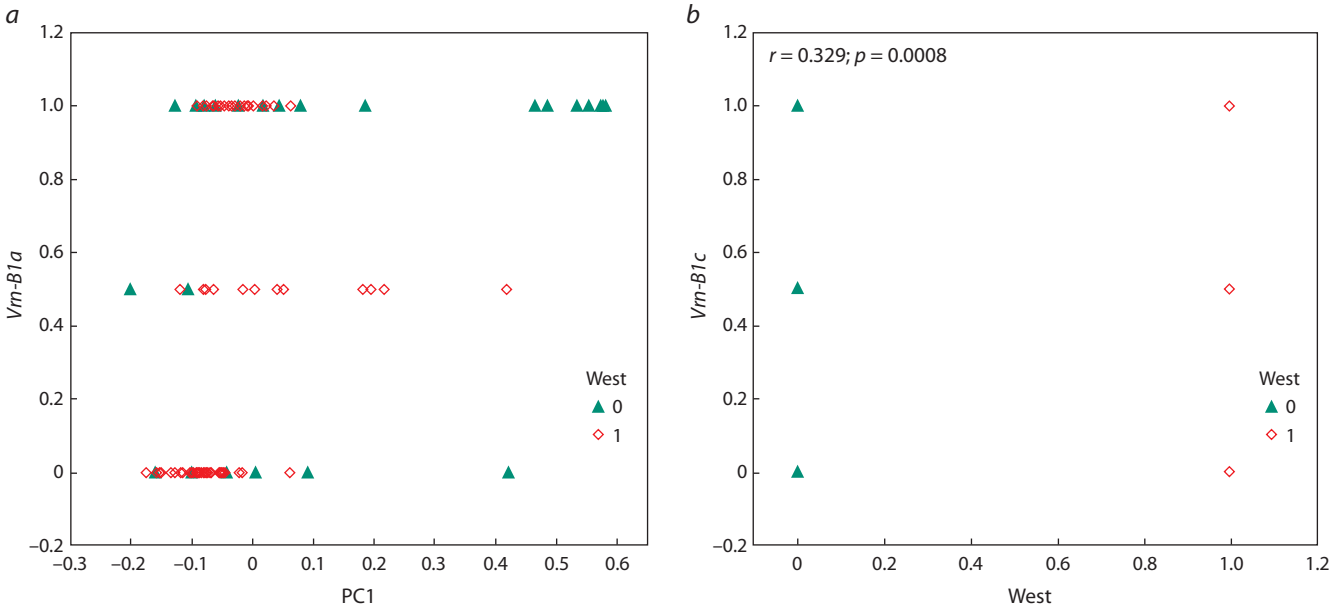


**Fig. 3.** Scatterplot of *Vrn-B1a* allele and *Vrn-B1c* allele against: (*a*) – principal component (PC1); (*b*) – zoning.

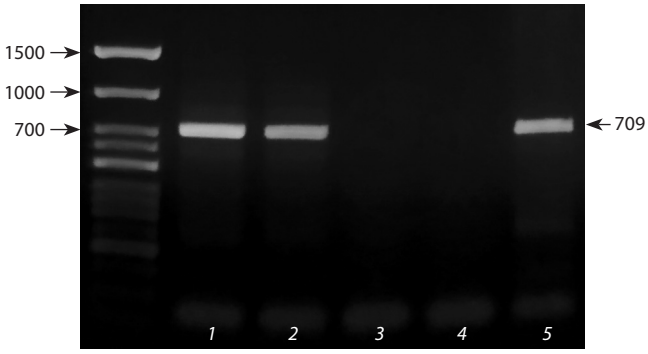


**Fig. 4.** The gel electrophoresis of the PCR fragments contained the first intron of the *Vrn-B1* gene in the tested cultivars.

*1* – Baganochka; *2* – ANK; *3* – k-39218 *T. aestivum* var. *lutinflatum* Zhuk.; *4* – k-30234 *T. araraticum* Jakubz.; *5* – Barnaulskaya 32. The arrow marks the resulted fragments of 709 bps in size.

1000-grain weight ($r$ = –0.354), i. e., the presence of this dominant allele resulted in 1000-grain weight reduction. At the same time, the presence of any dominant *Vrn* gene alleles did not affect the other biological traits. In other words, they correlated only against one another, forming a separated group of principal components from PC3 to PC6.

Hence, the biological meaning of the PC1 (see Fig. 2 and 3) is the early cultivar zoning time, spike awnedness and color of the cultivars from Eastern Siberia as well as their reduced duration of vegetation period, 1000-grain weight and var. *lutescens*, and increased *Vrn-B1a* allele frequency and var. *ferrugineum*.

At the same time, small-grained cultivars are typical for Eastern Siberia than for Western Siberia ($r$ = –0.262), while large-grained cultivars are typical for Western Siberia ($r$ = 0.377) (see Table 3, Fig. 2, *c*).

**Cultivar zoning time.** As Fig. 2, *a* demonstrates, before the 1990th, breeding in Eastern Siberia mainly produced awned varieties, but this tendency has changed since then. Hard to say why.

***Vrn* genes.** An important consideration of our study was the presence/absence in the tested cultivars of VRN-1 locus multiple alleles that control the duration of vegetation period. An important in the study was the identification of alleles of the VRN-1 locus and the assessment of their influence on duration of vegetation period. The polymorphism of the dominant *Vrn* genes controlling cultivar spring growth habit in the 98 investigated cultivars was minimally expressed (see Supplementary Material). It demonstrated that the dominant *Vrn* gene polymorphism determining the spring growth habit of the Siberian cultivars was minimally polymorphic. In 75 % of the tested cultivars, the spring growth habit was controlled by digenic, namely dominant *Vrn-A1* and *Vrn-B1* genes. In 25 % of them (24 cultivars), the spring growth habit of control is monogenic. In 19 and 5 of these cultivars spring growth habit is controlled by only one dominant gene *Vrn-A1*, *Vrn-B1*, respectively. In cv. Tulun 15, a trigenic control was identified. A conclusion about the optimality of the digenic control the climatic conditions of both Western and Eastern Siberia has been confirmed. However, since none of the tested cultivars had the dominant *Vrn-D1* gene typical for the regions of China and Central Asia bordering Siberia, it can be considered as an additional argument in favor of the European origin of Siberian common wheat cultivars. Two alleles of the dominant *Vrn-A1* gene were detected. While the frequency of the *Vrn-A1b* allele comprised less than 2 %, the *Vrn-A1a* allele presented in the most of the tested cultivars. The exception is a number of cultivars of Omsk breeding, such as cvs Omskaya 9, Omskaya 12, etc., the spring growth habit of which is de-

termined by the monogenic by dominant gene *Vrn-B1*, but their number does not exceed 5 % of the assortment (see Supplementary Material).

The *Vrn-B1* gene has three alleles, namely the dominant *Vrn-B1a*, *Vrn-B1c* alleles and recessive *vrn-B1* allele, i. e. neither *Vrn-B1a* nor *Vrn-B1c*. An increased frequency of occurrence of the *Vrn-B1c* allele was revealed for Western Siberia (see Table 3, Fig. 3, *b*) and *Vrn-B1a* (see Fig. 3, *a*) for Eastern Siberia.

Fig. 4 presents gel electrophoresis of the PCR fragments contained the first intron of the *Vrn-B1* gene in the tested cultivars. The amplification fragment of 709 bps marks the presence of the *Vrn-B1a* allele controlling a cultivar spring growth habit.

## Discussion

**Morphotype.** Plant breeders are commonly interested in complex analysis of the phenotypes of produced cultivars. For that reason, it was important for us to consider the setting significant volume accessions from 98 cultivars released in terms of morphotype difference between the cultivars from Western and Eastern Siberia and their dynamics during the last 100 years of scientific breeding during 1929–2021[4]. For the purposes of the present study, a morphotype, i. e., the approbation (classification) traits of a common wheat cultivar, had been divided into two groups such as 1) awnedness/awnedlessness and spike color that are known to affect the earliness (Pisarev, 1925); 2) grain color as a neutral parameter for there are no mentions about its effect in the publications. The results of data analysis presented at Table 3 and Fig. 2, *b* allow to conclude that the maximum contribution in the PC1 came from the region (both Eastern and Western Siberia), awnedness ($r = 0.859$) and spike color ($r = 0.893$). Another conclusion is that red spike (*ferrugineum*, *milturum*) and awned (*ferrugineum*, *erythrospermum*) varieties prevailed in Eastern Siberia ($r = 0.863$).

While other main morphotypes were distributed more or less equally with a little prevalence of white spike and awnedless varieties (*lutescens*) in Western Siberia ($r = 0.321$). The main contribution in the PC1 came from the spike color and its awnedness. Our study has confirmed the multiple conclusions made about the prevalence of red spike (*ferrugineum*, *milturum*) and awned (*ferrugineum*, *erythrospermum*) varieties in Eastern Siberia, and of white-spike awnedless varieties (*lutescens*) in Western Siberia (see Fig. 2, *a*, *b*).

**1000-grain weight.** This trait is correlated with yield (Melnikova et al., 2020) and milling quality parameters (Pototskaya et al., 2019). Our study demonstrated that the cultivars of Eastern Siberia were more small-grained than those from Western Siberia (see Table 3, Fig. 2, *c*). Earlier it had been demonstrated that the cultivars grown in the North Kazakhstan were also more small-grain than those farmed in Western Siberia (Moskalenko, 2007), so a conclusion can be made that more continental climatic conditions determine the small size of the grain. The trait "1000-grain weight" is correlated with the duration of vegetation period ($r = 0.410$).

Estimating the correlation between the region and the two the most important traits such as earliness and 1000-grain weight using the methods of multivariate statistics brought us to the conclusion that the first was related to the regional component and the second – to that of polymorphism of trait "1000-grain weight".

***Vrn* genes.** The results obtained in our study make it possible to say that spring growth habit-related polymorphism in the Siberian cultivars of common wheat is supported only by the alleles of two dominant *Vrn* genes: *Vrn-A1* and *Vrn-B1* (see Supplementary Material). Furthermore, in 95 % of studied local cultivars and landraces of Siberia (Goncharov, Shitova, 1999) and Tuva (Moiseeva, Goncharov, 2007) this polymorphism is determined by two dominant *Vrn* genes.

For the dominant *Vrn-A1* gene, the presence of two alleles, *Vrn-A1a* and *Vrn-Ab*, was shown (Lysenko et al., 2014; Efremova et al., 2016; etc.). The last allele is rather rare in Siberian cultivars and in our study was found only in 2 % of all tested ones (see Supplementary Material). It is possible that another allele of this gene can be found in the cultivars of North Kazakhstan, a territory that borders Western Siberia (Koval, Goncharov, 1998).

At the same time, allelism at the VRN-B1 locus is widespread in the Siberian cultivars (Shcherban et al., 2012a). Herewith, the *Vrn-B1c* allele prevails in the cultivars with monogenic spring growth habit control from Western Siberia and Northern Kazakhstan (Shcherban et al., 2012b). The same authors consider that in absence of the epistatic effects of the dominant *Vrn-A1* gene, this allele provokes earlier earing if compared to *Vrn-B1a* allele, which enables these plants to evade first autumn frosts. However, the cases of monogenic control in Western Siberian cultivars are quite rare: in the past 70 years only two such spring cultivars have been registered that comprises 2 % of the spring cultivar sets in Siberia. At what, both cultivars were produced in today's Omsk Agricultural Research Center.

Increasing the number of tested commercial cultivars and applying the principal component method for processing the genogeographic data has enabled us to demonstrate the non-random distribution of the dominant *Vrn-B1* alleles in the cultivars of Western and Eastern Siberia (see Fig. 3, *a* and *b*). And, if earlier it was concluded that it is selective only for late-ripening varieties of Western Siberia and Northern Kazakhstan with monogenic control by *Vrn-B1* (Shcherban et al., 2012a, b), our study detected the digenic control of highly-frequent *Vrn-B1c* (Western Siberia) and *Vrn-B1a* (Eastern Siberia) alleles (see Supplementary Material, Fig. 3, *a*, *b*). These findings make it possible to conclude that such digenic control is an optimal combination for the climatic conditions of Western and Eastern Siberia and confirms a possible breedability of multiple *Vrn-B1* alleles in the cultivars with digenic control.

[4] Preliminary zoning of agricultural crops in the State Breeding Test System and Protection was carried out in 1924 (Guidelines..., 1928).

Using published data also allowed us to compare the frequencies of the genotypes with different dominant *Vrn-1* alleles in the cultivars of Siberia and its neighboring regions (Moiseeva, Goncharov, 2007; Efremova et al., 2016). The analysis demonstrated that none of the considered cultivars had the dominant *Vrn-D1* gene typical for the neighboring regions of China and Central Asia. This observation may be an additional argument in favor of the European origin of the modern Siberian cultivars.

The only cultivar to have trigenic spring growth habit control was cv. Tulun 15 (Lysenko et al., 2014), which is probably too many for a Siberian cultivar. Indirectly, this has been confirmed by the results of investigation into ultra-early-ripening common wheat lines Rico, Rimax, Fori, Rifor from the north-west of Russia, whose spring growth habit was controlled by *Vrn-A1*, *Vrn-B1*, *Vrn-D1* and *Ppd-D1a* genes (Rigin et al., 2019, 2021a). At the same time, Tulun 15 has a different dominant *Vrn* gene than theirs, namely, *Vrn-B3* typical of Chinese wheat (Zhang et al., 2008). Its other dominant gene is *Ppd-D1a* (Berezhnaya et al., 2021) that is alien not only for Siberia but for Russia as well (Balashova, Fait, 2021). Note the Prilenskaya cultivar zoned in Siberia also has a dominant *Ppd-D1a* gene (Lysenko et al., 2014). However, the photoperiodic sensitivity of Siberian cultivars was beyond the scope of our investigation.

We believe that increasing the spring growth habit-related polymorphism of common wheats requires either introgressing the dominant *Vrn* alleles from their wild relatives (Goncharov, Chikida, 1995; Goncharov, 1998) or using the rare alleles available in the gene pool of commercial cultivars (Stelmakh, Avsenin, 1996; Koval, Goncharov, 1998) that are understudied and have rarely been used in breeding. Whether the dominant *Vrn-B3* gene can be used in breeding remains an open issue that requires further investigation. For the time being, only the *Vrn-A1* and *Vrn-B1* have shown the multiple alleles affecting the earing period. It is noteworthy that the donor of the $A^u$ *T. urartu* Thum. ex Gandil. genome provides no new mutations for spring common wheat (Golovnina et al., 2010) as well as using *T. monococcum* L. (Gonchrov et al., 2007) will never prove worthy.

Fig. 4 presents gel electrophoresis of the PCR fragments contained the first intron of the *Vrn1B* gene in the tested cultivars demonstrates the diagnostic product of the *Vrn-B1* gene. It is 709 bps in size and was obtained from the Barnaulskaya 32 (Ozimka) spring cultivar. It is said to be produced by transformation of a winter cultivar into a spring one (Catalog…, 2009). In the sequence presents the standard deletion characteristic for other Siberian cultivars. Probably Barnaulskaya 32 is not a result of transformation, so its mutation cannot be used to extend *Vrn* polymorphism.

**Cultivar zoning time.** Changes of cultivar morphotypes in dynamic is another interesting topic and a subject of vivid discussions among breeders (Goncharov N.P., Goncharov P.L., 2018). Until the 1990s, breeding for awned and red-spike cultivars was clearly maintained in Eastern Siberia (see Fig. 2, *a*) (Catalog..., 2009). Since 1990 this tendency has changed due to disbanding a number of scientific and research facilities that carried out planned breeding and provided scientific supervision (Goncharov, Kosolapov, 2021).

It is a well-known fact that before starting to breed a new cultivar, a breeder should set strategic goals and find the ways of their achievement taking into account that in 15–20 years the requirements for the cultivar can change drastically due to changes in the economic situation, farming and processing techniques. However, within a properly organized breeding process, producing a new cultivar should not be a problem for it is what happens on regular basis and new cultivars being the products of the breeding programs started earlier are regularly sent to the State Commission for Breeding Test System and Protection. The only problem with this approach is to carefully preserve succession that refers both to the people and plants. In this respect, it would be interesting to investigate what germplasm materials had entered Siberian fields since the interregional program of the Kazakhstan-Siberian network for wheat improvement (KASIB) was launched (Kuz'min et al., 2019).

It goes without saying that cultivar replacement is crucial for crop farming in the Siberian Federal District. However, a cultivar must be cultivated as long as it can provide a sufficient yield of high quality.

**Duration of vegetation period.** Comparing the obtained data against the results for local cultivars highlighted the absence of sufficient changes in the frequencies of *Vrn* genes in the commercial cultivars of Siberian common wheat at least during the last 100 years (see Supplementary Material). The first preliminary zoning of wheat cultivars in our country was performed by V.V. Talanov in 1924 (Guidelines…, 1928), while the first Soviet Unione State Cultivar Zoning Register was produced only in 1929.

Duration of vegetation period is one of the main breeding parameters to characterize a cultivar or a sample in terms of their ripeness (from early- to late-ripening thorough middle-early, middle-ripening, middle-late, etc.) (Goncharov N.P., Goncharov P.L., 2018). This scale varies is for different species, but earliness and lateness remain the most expressed characteristics of any agricultural species. The results obtained in our study and presented in Table 3 do not permit to make firm conclusions since the contributions of traits into their own vectors and dispersions included in the corresponding principal components were too small. Moreover, such traits as red spikes ($r = 0.893$) and awnedness ($r = 0.859$) were those that correlated with the primary component, i. e., they had smaller joint duration of vegetation period.

Another important thing was that the earliness trait did not include a regional component (see Table 3). So, despite the fact that a number of cultivars were zoned as in Eastern as in Western Siberia, their percentage was rather small even in the recent years when one started to zone wheat cultivars not by regions but by bigger federal districts.

When producing new cultivar, breeders proceed from the concept of matching the duration of vegetation period to the conditions of the proposed farming area. The retrospective

analysis of the most perspective trends of common wheat breeding demonstrated that the earliness/lateness of modern cultivars had no longer been related to a region but rather to an ecological zone (taiga, subtaiga, forest-steppe and steppe), which raises a question of its latitudinal/longitudinal components that have never been previously studied (Goncharov, Rechkin, 1993; Rechkin, Goncharov, 1993), for geographic sowing defiantly showed the non-latitudinal character of the trait expression (Goncharov, Rechkin, 1993). At the same time, N.I. Vavilov (1928) and E.S. Kuznetsova (1929) insisted on having two groups of plants for geographical sowing: the first is to limit the seedling – ripening period from the South to North, and the second – to extend this period. Today, only two spring common wheat cultivars from Siberia have a *Ppd-D1a* gene (Lysenko et al., 2014; Berezhnaya et al., 2021). The absence of a close relationship (correlation) between the duration of vegetation period in spring common wheat and them yield has been repeatedly shown (Vedrov, Chalipsky, 2009; Piskarev et al., 2018).

It is also noteworthy that the accumulated perennial data has enabled us to see the retrospective that is crucial considering the reduced level of scientific supervision of crop research both in Siberia and Russia.

## Conclusion

Breeding for earliness is one of the important directions of spring wheat breeding in Siberia. The accumulated perennial data has made it possible to apply the methods of multivariate statistics to extract the meaningful insights they contain. The simplicity and representability of the approach make it a useful tool for decision taking when it comes to including a new cultivar into the "State Register for Selection Achievements Admitted for Usage (National List)".

The present study investigating the geographical distribution of dominant *Vrn* genes has allowed us to estimate the advantages of the cultivars with certain alleles of those genes for specific territories of Siberia. It has been found that the digenic spring growth habit control is an optimum solution for the harsh climatic conditions of both Western and Eastern Siberia. The performed retrospective analysis has made it possible to indicate the most perspective breeding trands and revealed that the earliness/lateness trait of many modern spring common wheat cultivars no longer regionally related to either Western or Eastern Siberia. Nevertheless, the Eastern cultivars mainly have the *Vrn-B1a* allele, and the Western one – *Vrn-B1c* allele.

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# Localization of the quantitative trait loci related to lodging resistance in spring bread wheat (*Triticum aestivum* L.)

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**Abstract.** The yield and grain quality of spring and winter wheat significantly depends on varieties' resistance to lodging, the genetic basis of this trait being quantitative and controlled by a large number of loci. Therefore, the study of the genetic architecture of the trait becomes necessary for the creation and improvement of modern wheat varieties. Here we present the results of localization of the genomic regions associated with resistance to lodging, plant height, and upper internode diameter in Russian bread wheat varieties. Phenotypic screening of 97 spring varieties and breeding lines was carried out in the field conditions of the West Siberian region during 2017–2019. It was found that 54 % of the varieties could be characterized as medium and highly resistant to lodging. At the same time, it was noted that the trait varied over the years. Twelve varieties showed a low level of resistance in all years of evaluation. Plant height-based grouping of the varieties showed that 19 samples belonged to semi-dwarfs (60–84 cm), and the rest were included in the group of standard-height plants (85–100 cm). Quantitative trait loci (QTL) mapping was performed by means of genome-wide association study (GWAS) using 9285 SNP markers. For lodging resistance, plant height, and upper internode diameter, 26 significant associations ($-\log p > 3$) were found in chromosomes 1B, 2A, 3A, 3D, 4A, 5A, 5B, 5D, 6A, and 7B. The results obtained suggest that the regions of 700–711 and 597–618 Mb in chromosomes 3A and 6A, respectively, may contain clusters of genes that affect lodging resistance and plant height. No chromosome regions colocalized with the QTLs associated with lodging resistance or upper internode diameter were found. The present GWAS results may be important for the development of approaches for creating lodging-resistant varieties through marker-assisted and genomic selection.
Key words: spring wheat; lodging; plant height; upper internode diameter; GWAS; QTL.

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# Картирование локусов, ассоциированных с устойчивостью к полеганию у яровой мягкой пшеницы (*Triticum aestivum* L.)

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**Аннотация.** Урожайность и качество зерна яровой и озимой пшеницы в значительной степени зависят от устойчивости сортов к полеганию. Генетический контроль устойчивости к полеганию носит количественный характер и контролируется большим числом генных локусов, поэтому изучение генетической архитектуры данного признака необходимо для создания и усовершенствования современных сортов. В работе представлены результаты по выявлению геномных районов, ассоциированных с устойчивостью к полеганию и сопряженными с ним признаками «высота растения» и «диаметр верхнего междоузлия» у российских сортов яровой мягкой пшеницы. Фенотипический скрининг 97 яровых сортов и селекционных линий мягкой пшеницы был проведен в полевых условиях Западно-Сибирского региона в 2017–2019 гг. Установлено, что 54 % сортообразцов можно охарактеризовать как средне- и высокоустойчивые к полеганию, при этом отмечено варьирование признака по годам. Двенадцать сортов проявляли низкий уровень устойчивости во все годы проведения испытаний. Группировка растений по высоте показала, что 19 образцов относятся к полукарликам (60–84 см), остальные вошли в группу низкорослых растений (85–100 см). Картирование локусов было проведено с помощью полногеномного ассоциативного (GWA) анализа с использованием 9285 маркеров SNP. Для признаков «устойчивость к полеганию», «высота растения» и «диаметр верхнего междоузлия» найдено 26 значимых ассоциаций ($-\log p > 3$) в хромосомах 1B, 2A, 3A, 3D, 4A, 5A, 5B, 5D, 6A и 7B. Полученные результаты позволяют предположить, что районы 700–711 и 597–618 Mb хромосом 3A

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и 6A соответственно могут содержать кластеры генов, влияющих на устойчивость к полеганию и высоту растения. Не обнаружено районов хромосом с колокализацией локусов, ассоциированных с устойчивостью к полеганию и диаметром верхнего междоузлия. Данные GWA анализа могут иметь значение для разработки методов создания устойчивых к полеганию сортов с помощью маркер-ориентированной и геномной селекции.
Ключевые слова: яровая пшеница; полегание; высота растения; диаметр верхнего междоузлия; GWAS; QTL.

## Introduction

Spring bread wheat (*Triticum aestivum* L.) is one of the main food crops grown in Western Siberia taking about 40 % of all agricultural lands (5.5 mln ha). According to the Russian Statistics Agency, the wheat yield has been growing recently in the region, comprising in different years from 21 to 28 cwt/ha[1]. Lodging is one of the important factors resulting in a serious yield loss and reducing the technological quality of the grain. In lean years, early lodging in spring soft wheat can lead to a yield loss of up to 20–50 % in the milky ripeness phase, and up to 15 % – in the wax ripeness phase (Stapper, Fischer, 1990; Zhuchenko, 2004; Khobra et al., 2019). Lodging complicates mechanized harvesting, which results in additional yield loss. The weather conditions monitoring carried out in Western Siberia from 1976 to 2016 has shown that the regional climate has become more extreme, so its increased frequency of gales, showers and thunderstorms significantly reduces the yield (Kharyutkina et al., 2019). Considering this unfavorable weather conditions, it becomes essential to create the wheat varieties that are resistant to lodging.

Lodging resistance is a trait that depends on a number of features, the most important of them being the stem's anatomical and morphological properties. So far, it has been found that the plant height is crucially important for the trait in question. Discovering the genes of reduced height (*Rht*) as well as introduction of the most effective genes and their alleles (*Rht-B1b*, *Rht-D1b*, *Rht8*) into the wheat genome have resulted in creation of the varieties resistant to lodging (Khobra et al., 2019; Liu et al., 2022). Meanwhile, a number of studies have demonstrated that reducing the plant's height below a certain value leads to reduced grain size, 1000-grain weight and a worse yield in general (Miralles, Slafer, 1995; Flintham et al., 1997; Li et al., 2006). In unfavorable weather conditions, the alleles of the *Rht-B1b* and *Rht-D1b* can have a negative effect on the plant's coleoptile length and root size preventing proper rooting and reducing drought resistance (Rebetzke et al., 1999; Ellis et al., 2004; Yan, Zhang, 2017). The undesirable effects of the *Rht* gene alleles also include reduced nitrogen content in grain and a longer heading time, resulting in worse yield and grain quality (Casebow et al., 2016; Sukhikh et al., 2021). Apart from the stem's height, its other parameters are of crucial importance, e. g., it has been found that the culm's diameter, wall thickness and weight, number of vascular bundles and mechanical tissue sizes may determine wheat resistance to lodging (Berry et al., 2003; Zakharov et al., 2014).

Being of quantitative character, lodging is controlled by a large number of genes that complicates the creation and selection of resistant genetic lines using the methods of classical breeding and phenotyping. Many researchers believe phenotypic assessment of lodging resistance may be controversial since lodging occurs at different stages of plant development and its degree is affected by certain external factors (Atkins, 1938; Hai et al., 2005). On the other hand, marker-based analysis and identification of the genome regions associated with lodging may be used for indirect selection of the varieties unsusceptible to lodging.

The modern technologies for mapping of genes and quantitative trait loci (QTL) enable one to determine the chromosomal and genomic localization of target loci and the architecture of their quantitative traits. For the time being, genome-wide associated studies (GWAS) have become one of the most commonly applied approaches for mapping the QTLs of agronomically important traits. The effectiveness of the technique has been confirmed to detect and localize the loci responsible for wheat resistance to biotic (Aoun et al., 2021; Kokhmetova et al., 2021) and abiotic (Wang N. et al., 2019; Pshenichnikova et al., 2021) stress factors and their effect on the yield capacity (Luján Basile et al., 2019; Gahlaut et al., 2021), grain protein content, and baking quality (Battenfield et al., 2018; Leonova et al., 2022).

Currently, there have been just a few studies using GWAS for mapping the loci correlating with lodging resistance or responsible for related stem characteristics in the wheat (Cericola et al., 2017; Malik et al., 2019; Akram et al., 2021), so the objective of the present study was (1) to perform comparative screening of spring soft wheat varieties for lodging resistance, plant height, and upper internode diameter; (2) to detect the potential genome regions associated with lodging or its related stem characteristics using the association mapping technique.

## Materials and methods

**Plant material and phenotyping.** A collection of 97 varieties and breeding lines of spring soft wheat (*T. aestivum* L.) from different breeding centers of the Russian Federation that have been recommended for cultivation in Western Siberia was used in this study. Detailed information on the plant material can be found in Suppl. Material 1[2].

The plant material was grown in the field of Siberian Research Institute of Plant Production and Breeding, a Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk Region, 54.9191° N, 82.9903° E) for three seasons (2017–2019). The samples were sown manually following the systematic method in two replications in plots of 1 m wide, 60 seeds in a row, and 25 cm between rows. The plants' lodging was estimated during the wax ripeness phase according to the grading scale (Shamanin, Truschenko, 2006): 1 = very strong lodging, mechanized harvesting impossible; 2 = strong lodging; 3 = medium lodging, the stems are at 45° to the soil surface; 4 = weak lodging, the stems are barely inclined; 5 = no lodging. Height-based grouping was carried out as indicated in the methodological

[1] Agriculture in Russia. https://rosstat.gov.ru/folder/210/document/13226

[2] Supplementary Materials 1–4 are available in the online version of the paper: http://vavilov.elpub.ru/jour/manager/files/Suppl_Leonova_27_7.pdf

recommendations of the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) (Guidelines for Studying…, 1987). To measure the upper internode diameter, stem cross-sections were fixed in ethanol (96 %) and dyed in 1 % safranin solution (Safranin O, LLC 'Dia-M') to be photographed using a stereoscopic microscope Altami CM0655 (LLC 'Altami') equipped with a camera Altami UCOS5100KPA. For statistical processing of the results, at least 10 plants of every sample were used.

The weather conditions within the years of investigation were, in general, favorable to yield formation (Suppl. Material 2). In the summer of 2017, 278 mm of precipitation was registered, in 2018 – 380.3 mm and in 2019 – 194.7 mm, the long-run annual average being 220 mm. According to the data of the Ogurtsovo agrometeorological station, the 2017 vegetation period was characterized by temperature fluctuations and often rains. In May and June of 2017, the temperature regime exceeded its long-run annual average, and there were not enough rains in the third decade of May and the second decade of June (10.5 mm, 65 % of the normal rate). The precipitation rate in July was 101 mm, the first decade being most rainy (49 mm). Selyaninov's Hydrothermic Coefficient (HTC) comprised 0.9. The meteorological conditions in August remained within the normal rates, the third decade being characterized by insufficient precipitation (9.3 mm, 40 % of the normal rate). The average temperature during the summer months was 18.2 °C, which exceeded the long-run annual average by 0.6 °C.

The 2018 vegetation period was marked by lower temperatures in May (averagely, 5 °C below the normal range) and excessive precipitation in May–June if compared to the other seasons. During these two months, the precipitation rate comprised 211.4 mm being 80 % of the seasonal precipitation rate. May's HTC was 10.2 and it reduced to 2.8 in June, whose temperature regime and precipitation level matched the long-run annual average. In August, precipitation deficiency was observed (–33.3 mm, HTC = 0.4).

The 2019 vegetation period was marked by unstable weather conditions due to uneven precipitation fallout, and temperature fluctuations in the second half of the period. The weather in May and July was rainy (HTC = 2.3 and 1.4, respectively). In June and August, a small drought was observed (HTC = 0.7 and 0.5, respectively).

**DNA extraction, genotyping and GWAS.** DNA was extracted from 5–7 day-old seedlings following a modified protocol as per Kiseleva et al. (2016). For the purposes of genotyping, the obtained DNA samples were purified in BioSilica microcolumns as per the manufacturer's instructions. DNA concentrations were detected using a NanoDrop M2000 spectrometer (Thermo Scientific). Genotyping was carried out using a *Triticum aestivum* (wheat) genotyping Illumina Infinium 15K chip comprised of 13 006 SNP markers by the TraitGenetics company (Germany, www.traitgenetics.com).

The number of the polymorphic markers included in GWAS comprised 9235. Before the study, the markers were filtered and those with allele frequency of less than 5 % or not amplified in 20 % or more samples were excluded from the analysis, which was performed using a mixed linear model (MLM) in the TASSEL v. 5.2.70 software (Bradbury et al., 2007). The analysis considered population structure (Q-matrix) and genetic kinship (K-matrix), the first calculated using a Bayesian algorithm implemented in the STRUCTURE 2.3.4 software (Pritchard et al., 2000). The probable subscluster number was estimated using the Delta K (ΔK) statistics (Evanno et al., 2005) in the Structure Harvester web program (Earl, vonHoldt, 2012). The K-matrix was calculated using TASSEL v. 5.2.70. To find statistically reliable associations, the Benjamini–Hochberg method (1995) and FDR control at $p < 0.001$ were applied. The chromosomal localization of the SNP markers was determined as per The IWGSC RefSeq v1.0 annotation (https://triticeaetoolbox.org) and the consensus maps of wheat chromosomes (Wang S. et al., 2014).

**Statistical analysis** of the obtained results was carried out in the STATISTICA v. 10 software (http://statsoft.ru/). To estimate the statistical reliability between the averaged values of two sampled populations, Student's *t*-test was applied. The relation between lodging resistance, plant height and upper internode diameter was determined using Spearman's correlation. The contributions of genotype and environment to trait manifestation were estimated using the ANOVA, whose statistical reliability was assessed through F-test. The heritability ($H^2$) was calculated based on the following formula:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_{G\times E}^2}{n_E} + \frac{\sigma_e^2}{n_E}},$$

where $\sigma_G^2$, $\sigma_{G\times E}^2$, $\sigma_e^2$ are the mean square deviations (SDs) of the genotype, genotype/environment interaction and residual variance, respectively, and $n_E$ is the number of vegetation seasons.

## Results

### Phenotyping

Estimating the varieties' resistance to lodging within a 3-year period demonstrated that 53 out of 97 varieties could be related either to moderate or resistant kinds (>3.5 out of 5 points), and the trait varied from year to year (Table 1, Fig. 1, *a*). The highest degree of lodging was observed in 2018, which was related to the high precipitation level in the summer period, so the year did not produce a single variety with the highest level of resistance (5 out 5). Eight varieties (Novosibirskaya 29, Novosibirskaya 67, Novosibirskaya 91, Krasnoyarskaya 90, Vesnyanka 8, Mariinka, Salimovka, and Aleshina) demonstrated a high level of lodging resistance (4–5 points) in every year of the experiment. Unlike the above mentioned, twelve varieties (Saratovskaya 29, Saratovskaya 42, Lutescens 62, Altayskii prostor, Rosinka 2, Tulaikovskaya stepnaya, Lutescens 85, Surenta 6, Lutescens 840, Kinelskaya 40, Latona, and Volgouralskaya) had low lodging resistance (1–3 points) within the years of experiment.

The plants' height and upper internode diameter varied from 54 to 105 cm and from 1.26 to 2.46 mm, respectively, and depended on a vegetation season (see Table 1, Fig. 1, *b*, *c*). In the years 2018/19, the height varied less than in 2017, which means the characteristic depended on the soil and climate conditions. Grouping the plants by their height as per VIR Methodological Recommendations showed 19 varieties were semi-dwarfs (60–84 см) while the others comprised a group of dwarf plants (85–100 cm).

**Table 1.** Characteristics of spring soft wheat varieties in relation to lodging resistance, plant height and upper internode diameter based on trait assessment in years 2017–2019

| Trait | 2017 | | 2018 | | 2019 | |
|---|---|---|---|---|---|---|
| | Mean ± SD* | Range | Mean ± SD* | Range | Mean ± SD* | Range |
| Lodging resistance, points | 4.7 ± 0.75 | 1–5 | 2.6 ± 1.0 | 1–4 | 4.1 ± 0.86 | 2–5 |
| Plant height, cm | 82.5 ± 9.4 | 54.0–101.4 | 81.8 ± 7.91 | 62.0–97.3 | 91.0 ± 6.85 | 71.9–104.6 |
| Upper internode diameter, mm | 1.68 ± 0.17 | 1.26–2.32 | 1.71 ± 0.17 | 1.37–2.46 | 1.79 ± 0.12 | 1.48–2.17 |

SD – standard deviation.

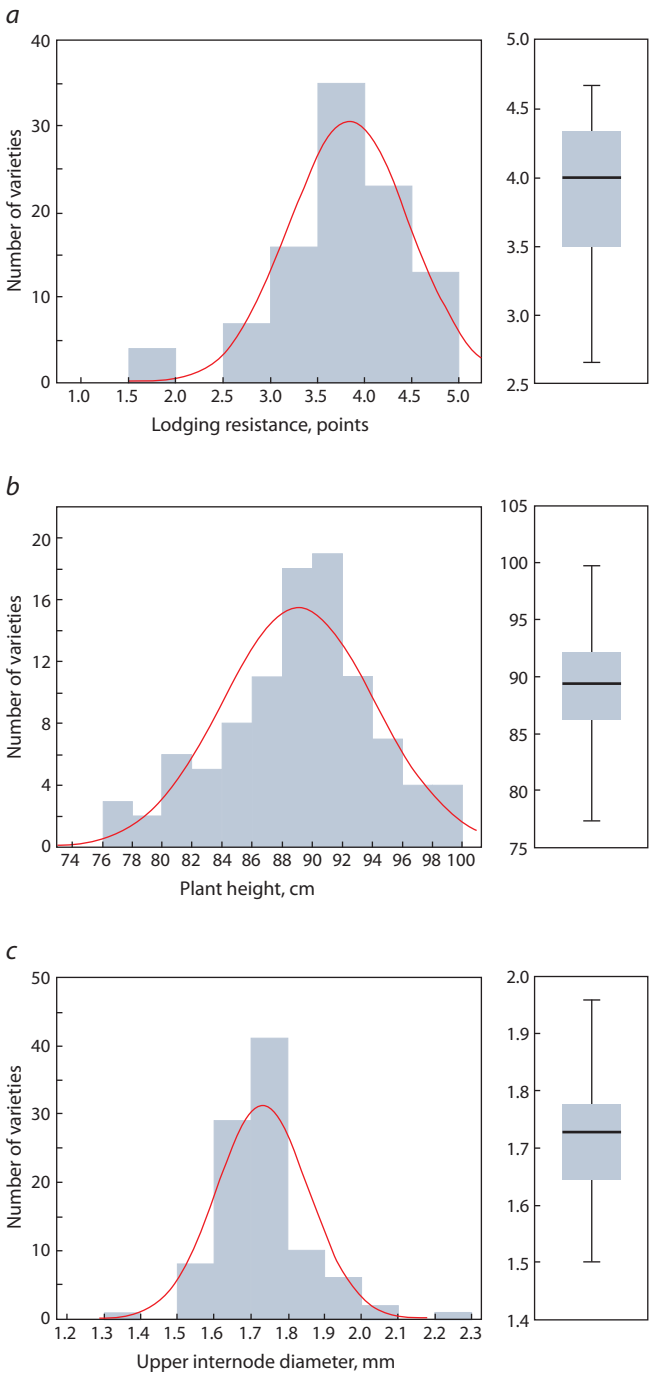


**Fig. 1.** Histograms and boxplots to illustrate the distribution of spring soft wheat varieties relative to their (*a*) lodging resistance, points; (*b*) plant height, cm; (*c*) upper internode diameter, mm.

The ANOVA demonstrated that it was the genotype (G), environmental factors (E) and their interaction (G × E) that statistically contributed to the phenotypical manifestation of the said traits (Suppl. Material 3). The heritability was high for the plant height (78 %), while for the lodging resistance and upper internode diameter it comprised 51 and 59 %, respectively, which confirms the significant effect of the environmental factors on the phenotypical manifestation of the traits. Since none of the traits had normal distribution, their correlations were analyzed using Spearman's rank correlations that showed statistically significant negative correlations between the lodging and height ($r$ = –0.48***) and positive – between the lodging and upper internode diameter ($r$ = 0.35***). The correlations between the height and the diameter were found to be weak ($r$ = 0.20**).

## GWAS

The data analysis performed in the STRUCTURE software enabled us to subdivide the investigated varieties into five subclusters including 22, 7, 20, 25 and 23 genotypes, respectively (Fig. 2). It is noteworthy that this clustering did not match the plants' origins as described by their originators (see Suppl. Material 1).

To detect genetic-marker associations with the considered characteristics, 9235 polymorphic SNP markers were used. The numbers of the markers mapped in the chromosomes of genomes A, B and D differed significantly, the smallest one registered for the chromosomes localized in homoeological group 4 (Suppl. Material 4). For 607 markers, data on their localization on the genetic and physical maps of wheat chromosomes were absent. The GWAS based on the estimation results of three vegetation seasons found 26 SNP markers that were significantly ($p$ < 0.001) associated with lodging resistance, plant height and upper internode diameter (Table 2). Eleven markers (GENE-3066_157, BS00076772_51, RAC875_c103443_475, BS00011514_51, Tdurum_contig97342_274, BS00068710_51, Excalibur_c96921_206, Ex_c69054_723, Ra_c6429_1217, BobWhite_c12261_130, Excalibur_c8931_432) sustained the association for several seasons (see Table 2).

For the lodging-resistance trait, eight true SNP markers were detected in the five loci located in chromosomes 1B, 2A, 3A, 3D and 6A as per the physical mapping of reference bread wheat variety sequence IWGSC RefSeq v1.0. Highly significant ($p$ < 0.00002) associations were observed for loci *QLdg.icg-1B* and *QLdg.icg-2A* in chromosomes 1B and 2A, respectively (see Table 2). The presence of favorable alleles, on average, increased the lodging resistance by

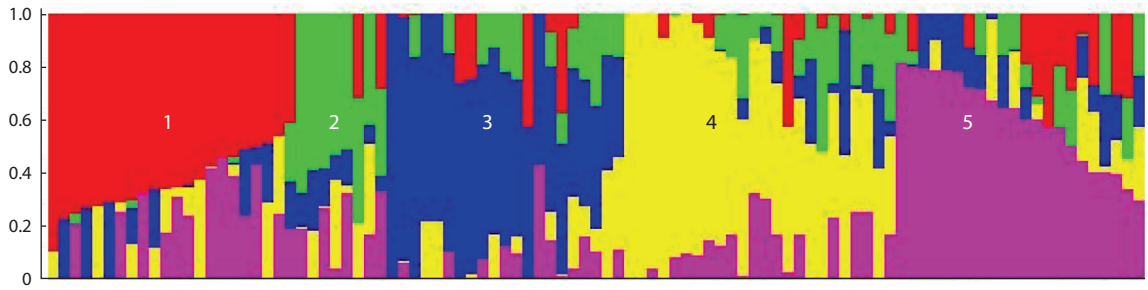


**Fig. 2.** Population structure of the spring soft wheat varieties based on their SNP-marker genotyping results.
The vertical axis marks the coefficients assigning a sample to a certain subcluster. Cluster compositions can be found in Suppl. Material 1.

**Table 2.** List of the SNP markers associated with lodging resistance, upper internode diameter and plant height in spring soft wheat varieties

| Trait | Marker* | Chromosome | Position, Mb | Allele** | Locus | *p* | $R^2$ (%) |
|---|---|---|---|---|---|---|---|
| Lodging | wsnp_JD_rep_c63201_40318622 | 1B | 613.413276 | **T**/C | *QLdg.icg-1B* | 1.69E–06 | 14.8 |
| | GENE-3066_157* | 2A | 726.025003 | **C**/T | *QLdg.icg-2A* | 1.87E–06 | 22.8 |
| | GENE-0638_1119 | 2A | 728.071406 | **A**/C | | 5.07E–05 | 20.6 |
| | BS00076772_51* | 3A | 711.202928 | **C**/A | *QLdg.icg-3A* | 8.68E–05 | 15.8 |
| | Excalibur_c19658_127 | 3D | 1.253733 | **A**/G | *QLdg.icg-3D* | 2.30E–04 | 18.7 |
| | Kukri_c24488_1603 | 3D | 1.270574 | **G**/A | | 3.91E–04 | 15.8 |
| | Tdurum_contig75700_411 | 6A | 598.635440 | **G**/A | *QLdg.icg-6A* | 2.41E–04 | 13.2 |
| | RAC875_c103443_475* | 6A | 596.903227 | **A**/G | | 1.32E–04 | 14.2 |
| Upper internode diameter | BS00011514_51* | 5B | 572.547199 | **T**/C | *QSd.icg-5B* | 3.01E–06 | 21.4 |
| | Tdurum_contig97342_274* | 5B | 558.118788 | **T**/C | | 1.49E–05 | 22.3 |
| | BS00068710_51* | 5B | 558.120029 | **T**/C | | 2.30E–04 | 16.1 |
| | D_GDS7LZN02I3554_251 | 5D | 548.942211 | **A**/G | *QSd.icg-5D* | 8.01E–05 | 15.1 |
| | BS00022267_51 | 5D | 550.511075 | **C**/T | | 9.67E–05 | 14.0 |
| | BS00025017_51 | 5D | 551.059358 | **T**/C | | 1.57E–04 | 10.8 |
| | Kukri_c15823_196 | 4A | 615.446891 | **T**/C | *QSd.icg-4A* | 2.80E–04 | 12.5 |
| | Excalibur_c30378_344 | 4A | 615.437250 | **G**/T | | 5.01E–04 | 12.1 |
| | RAC875_c21489_908 | 7B | 634.387709 | **T**/C | *QSd.icg-7B* | 1.72E–04 | 17.6 |
| Plant height | TA001128-1276 | 3A | 577.576777 | **C**/T | *QHt.icg-3A-1* | 3.25E–04 | 20.2 |
| | Excalibur_c5977_440 | 3A | 577.576266 | **G**/T | | 2.67E–04 | 16.7 |
| | Excalibur_c96921_206* | 3A | 700.946020 | **A**/G | *QHt.icg-3A-2* | 2.02E–04 | 12.8 |
| | BS00022299_51 | 5A | 679.740028 | **T**/G | *QHt.icg-5A* | 4.36E–04 | 16.7 |
| | Ex_c69054_723* | 6A | 609.452924 | **C**/T | *QHt.icg-6A* | 4.91E–04 | 16.2 |
| | Ra_c6429_1217* | 6A | 614.164297 | **C**/T | | 5.37E–04 | 16.1 |
| | BobWhite_c12261_130* | 6A | 617.482504 | **C**/T | | 5.37E–04 | 16.1 |
| | Excalibur_c8931_432* | 7B | 638.710248 | **C**/T | *QHt.icg-7B* | 6.68E–04 | 17.5 |
| | wsnp_Ex_c45195_51056617 | 7B | 645.131391 | **G**/A | | 5.37E–04 | 17.3 |

* Markers are indicated for which associations have been established from the data at least two growing seasons.
** The favorable allele is highlighted in bold.

**Table 3.** The lodging resistance, upper internode diameter and plant height traits in spring wheat varieties and their dependance on the locus/allele status

| Locus | SNP | Allele | Lodging resistance, points | Plant height, cm | Upper internode diameter, mm |
|---|---|---|---|---|---|
| *QLdg.icg-1B* | wsnp_JD_rep_c63201_40318622 | T | 4.4 ± 0.28** | 89.7 ± 4.8 | 1.73 ± 0.13 |
| | | C | 3.7 ± 0.65 | 85.6 ± 5.3 | 1.75 ± 0.12 |
| *QLdg.icg-2A* | GENE-3066_157 | C | 4.1 ± 0.44** | 89.2 ± 5.1 | 1.75 ± 0.10 |
| | | T | 3.7 ± 0.73 | 89.5 ± 5.2 | 1.73 ± 0.13 |
| *QLdg.icg-3A* | BS00076772_51 | C | 3.9 ± 0.53** | 87.2 ± 3.6* | 1.74 ± 0.13** |
| | | A | 3.1 ± 0.78 | 90.4 ± 4.1 | 1.63 ± 0.07 |
| *QLdg.icg-3D* | Excalibur_c19658_127 | A | 3.8 ± 0.56*** | 89.8 ± 4.8 | 1.74 ± 0.13** |
| | | G | 3.1 ± 0.93 | 88.4 ± 4.8 | 1.64 ± 0.10 |
| *QLdg.icg-6A* | Tdurum_contig75700_411 | G | 3.8 ± 0.67** | 87.1 ± 4.5* | 1.72 ± 0.12 |
| | | A | 3.3 ± 0.71 | 90.2 ± 3.8 | 1.73 ± 0.13 |

Note. Means ± standard deviation (M ± SD) calculated on trait evaluation in 2017–2019. An asterisk indicates the significance of differences between trait parameter for different alleles, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

0.4–0.8 points depending on a locus (Table 3). For the loci in chromosome 3A (711.20 Mb region) and in chromosome 6A (596.90–598.63 Mb), it was found that the increased resistance to lodging of the varieties containing favorable alleles led to their reduction in height by 3 cm. Locus *QLdg.icg-3D* was mapped in the region of 1.25–1.27 Mb of chromosome 3D, and 8 varieties (Saratovskaya 29, Saratovskaya 42, Lutescens 62, Tulaikovskaya belozernaya, Volgouralskaya, Lutescens 80, Albidum 73, Ilinskaya) carried the unfavorable alleles of markers Excalibur_c19658_127 and Kukri_c24488_1603, whose presence led to statistically significant reduction of the lodging resistance and upper internode diameter (see Table 3).

Nine SNPs significantly associated with the upper internode diameter were found in the four loci of chromosomes 5B, 5D, 4A and 3A (see Table 2). The GWAS performed demonstrated that the favorable alleles of positive loci were detected in no more than 10 % of studied varieties. For the plant height the most valuable associations were identified in chromosomes 3A, 5A, 6A and 7B (see Table 2). In chromosome 3A, two loci were found, one of which, *QHt.icg-3A-1*, was localized in the 577.58 Mb region and the other – in the 700.94 Mb region. The *QHt.icg-3A-2* locus was of particular interest because it did not only significantly reduce the plant height (by 7.3 cm on average) but also increased the lodging resistance. The favorable alleles of the loci in chromosomes 5A, 6A and 7B were found in the genomes of 11, 90 and 12 % of varieties, respectively and led to plant height reduction by 4 to 5 cm on average.

## Discussion

In the present study, we searched for the genetic factors determining resistance to lodging in a collection of the spring soft wheat varieties selected in Russia. Currently there have been limited number of publications covering the detection of the genetic determinants of this trait due its multigenic character and excessive dependance on environmental factors and plant development stages. Nevertheless, the last two decades have seen the QTLs identified as for lodging resistance as for the stem's morphological and anatomical parameters that can affect the trait in question.

The classical genetic mapping have enabled one to detect both major and minor loci associated with lodging resistance in the most of the chromosomes of soft wheat such as 1B, 2A, 2D, 3A, 4A, 4B, 4D, 5A, 5B, 6A, 6B, 6D, 7B, 7D (Keller et al., 1999; Hai et al., 2005; Berry P.M., Berry S.T., 2015; Dreccer et al., 2022) as well as the markers located in the vicinity of the target locus. At the same time, it is noteworthy that the detected regions are quite extended due to the limited number of markers that were used while mapping (Börner et al., 2002; Verma et al., 2005).

Genome-wide association mapping has proved to be a more effective method for searching target loci since it requires samples of higher genetic diversity than biparental mapping populations. Moreover, its higher SNP marker coverage enable for more accurate locus mapping and narrower localization regions. In our study, GWAS made it possible to detect five loci in chromosomes 1B, 2A, 3A, 3D and 6A associated with lodging resistance. For the time being, there have been only a few publications, whose authors pursued a similar approach for investigating the genetic factors associated with the trait and with the stem's anatomical parameters. GWAS has enabled to detect the determinants of lodging resistance in chromosomes 1B, 2A, 3A, 3D, 4B, 5B, 6D and 7A (Cericola et al., 2017; Singh et al., 2019; Akram et al., 2021). According to P.L. Malik et al. (2019) the manifestations of QTLs and their localization in a chromosome also depends on a stage of plant development, so mush so that in early stages (earing), marker–trait associations have been found in chromosomes 1B, 4B, 5B and 7A; and in late stages (maturing) – in chromosomes 1B, 2A, 3D, 4B, 5B and 6D.

Summarizing the published results of genetic and association mapping, a conclusion can be made that the most significant associations for resistance to lodging have been found in chromosomes 3A, 2A and 1B, which matches the

data obtained in our study, the only difference being QTL positioning in the chromosomes that depend on the genetic background of the variety material used in the studies. Based on our results, an assumption can be made that the regions of 700–711 and 597–618 Mb of the physical maps of chromosomes 3A and 6A, respectively, can contain clusters of the genes responsible for the plant's height and their resistance to lodging (see Table 2).

The fact that the loci associated with lodging resistance can have the same localization as those associated with the anatomic parameters has been observed by other authors. According to P.M. Berry and S.T. Berry (2015) the region of 53–82 cM of the genetic map of chromosome 3A contains a genetic cluster associated with lodging resistance, plant height, internode length/diameter and stem thickness. In other publications a colocalization of the loci associated with lodging resistance and plant height has been noted (Keller et al., 1999; Verma et al., 2005; Malik et al., 2019). Currently, the Catalogue of Gene Symbols for Wheat includes the 25 *Rht* genes determining plant height (https://shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp). Genes *Rht-B1*, *Rht-D1*, *Rht-8* and their alleles resulting in significant plant height reduction have been mapped in chromosomes 4BS, 4DS, 2DS, respectively (Gale et al., 1975; Korzun et al., 1998; Peng et al., 1999; Chernook et al., 2019). In the present study, the most significant association for the plant-height trait have been found in chromosomes 3A, 5A, 6A and 7B, making it possible to assume that the genomes of the investigated varieties lack highly effective dwarfing genes.

The fact that chromosomes 3A, 5A and 7B contain the loci associated with plant height has been confirmed by different authors through genetic mapping and GWAS (Ain et al., 2015; Gao et al., 2015; Akram et al., 2021; Muhammad et al., 2021). Several such genes have been identified in chromosome 6A, some of them (*Rht14*, *Rht16*, *Rht18*, *Rht25*) found in the short arm, and gene *Rht24* – in the long arm (Vikhe et al., 2017; Würschum et al., 2017; Ford et al., 2018; Mo et al., 2018). Genome-wide mapping of the *Rht* loci in chromosome 6A found genes *Rht18* (Ford et al., 2018) and *Rht24* (Würschum et al., 2017) in the region of 416–550 Mb of the physical map of the pseudomolecule, which corresponds to the approximate localization of the *Qht.icg-6A* locus in our study. Unfortunately, there have been no detailed data on the allele composition of dwarfing genes in Russian spring varieties. To verify a correlation between some alleles of the *Rht* genes and lodging resistance, additional investigations have to be carried out, including those to detect the presence of the *Rht* genes in the considered variety collection using specific molecular markers.

The found relation between upper internode diameter and lodging resistance is ambiguous. Some authors claim both the length and diameter of both upper and lower internodes in wheat matters for the plant's resistance to lodging (Berry P.M., Berry S.T., 2015; Packa et al., 2015; Demina, 2019). Others insists this correlation is only valid for the lower internode or absent completely (Zakharov et al., 2014; Zaytseva, Shchennikova, 2020). In the present study, no colocalizations of the loci associated with upper internode diameter and lodging resistance have been detected. It is noteworthy that the correlations between the two traits have been weak, which is probably due to the fact that for the investigated varieties the upper internode diameter plays no significant role for their resistance to lodging.

## Conclusion

Hence, the present study has demonstrated that GWAS is an effective tool for investigating the genetic architecture of a complex trait. Using this method, we have been able to identify several markers associated with lodging resistance, plant height and upper internode diameter in a collection of Russian spring wheat varieties. The obtained results, on the one hand, confirm the conclusions made by other authors about the most critical chromosomes containing the loci responsible for lodging resistance. On the other hand, these results may be important for detecting the samples combining the alleles favorable for several traits for their inclusion into breeding programs.

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# Plant genome modification: from induced mutagenesis to genome editing

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**Abstract.** The snowballing growth of scientific data obtained using modern techniques of genome editing (GE) calls for their critical evaluation and comparison against previously applied methods such as induced mutagenesis, which was a leading method of genome modification for many decades of the past century, and its application has resulted in a huge diversity of cultivars. However, this method was relatively long and included a number of stages from inducing multiple mutations using different mutagenic factors to crossing and selecting the most valuable cultivars for several generations. A new technology of genetic engineering and transgenesis enabled us to radically reduce the time required to obtain a new genetically-modified cultivar to one generation and make the modification process more effective and targeted. The main drawback of this approach was that an introduced transgene might uncontrollably affect the other genes of a recipient plant, which led to the limitations imposed on transgenesis application in many countries. These limitations have been effectively surmounted thanks to the development of GE techniques allowing for a precise modification within a single gene that in many characteristics make it similar to a natural allele (especially when it comes to ribonucleoprotein complexes), which has paved the way for wide application of GE in routine breeding. The paper reviews the main stages of GE development in its application in plants. It provides short descriptions of different GE techniques, including those using protein editors such as zinc-finger and transcription activator-like effector nucleases (TALEN) , and the CRISPR/Cas9 technology. It lists a number of achievements in using GE to produce new cultivars of higher yield that are resistant to unfavorable factors and have good nutritional properties. The review also considers the *de novo* domestication approach, which allows for faster obtaining of new cultivars from natural varieties. In the conclusion, the future ways of GE development are discussed.
Key words: induced mutagenesis; transgenesis; genome editing; nucleases; CRISPR/Cas9; pathogen; resistance; yield.

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# Модификация геномов растений: от индуцированного мутагенеза до геномного редактирования

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**Аннотация.** Лавинообразный рост научных данных, полученных с помощью современных методов геномного редактирования (ГР), обуславливает актуальность их критического осмысления и сопоставления с предыдущими методами модификации генома. В обзоре дана характеристика основных этапов развития методов модификации генома применительно к растительным объектам. Технология индуцированного мутагенеза лидировала в течение многих десятилетий прошлого века, с ее помощью получено огромное разнообразие сортов культурных растений. Однако этот процесс был довольно длительным и включал целый ряд стадий: от индукции множественных мутаций с помощью мутагенных факторов до этапов скрещивания и отбора наиболее ценных форм на протяжении ряда поколений. Пришедшая на смену технология генной инженерии (трансгенеза) позволила радикально сократить время получения новых генетически модифицированных форм до одного поколения, сделать процесс модификации более эффективным и целенаправленным. Но наряду с этим она имела главным недостатком возможность неконтролируемого влияния вводимого трансгена на другие гены растения-реципиента, что привело к существенным ограничениям применения трансгенеза во многих странах. Эти ограничения в настоящее время успешно преодолеваются с развитием методов ГР, позволяющих очень точно, в пределах одного гена, осуществлять модификацию, которая по своим свойствам практически не отличается от природного аллеля гена (особенно в случае использования рибонуклеопротеиновых комплек-

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сов), что дает возможность избежать ограничений на применение этой технологии в практической селекции. Приведена краткая характеристика различных методов ГР, включая использование белковых редакторов, ZF- и TALEN-нуклеаз, а также наиболее перспективный метод – CRISPR/Cas9. Перечислен ряд научных результатов по созданию с помощью этих методов новых форм растений: устойчивых к неблагоприятным факторам, с повышенной урожайностью и ценными питательными свойствами. В рамках обзора рассматривается новый подход «доместикация *de novo*» с целью ускоренного получения культурных растений из природных форм. Обсуждаются дальнейшие пути развития методологии ГР.
Ключевые слова: индуцированный мутагенез; трансгенез; геномное редактирование; нуклеазы; CRISPR/Cas9; патоген; устойчивость; урожайность.

## Introduction

Continuous accumulation of spontaneous mutations is the foundation of evolution in living organisms. Mutation frequency depends on the features of a creature's genetic apparatus and varies from $10^{-9}$ to $10^{-12}$ nucleotides/cell generations. Mutations commonly occur due to disrupted key biological processes such as DNA replication, reparation and recombination (Jonczyk et al., 1988; Banerjee et al., 1990), and only their insignificant part becomes involved in the evolutionary process while others are eliminated during selection. The mutations induced by chemical agents, radiation and other factors are random but of high frequency that provokes a huge number of mutation events in a genome (Sakuraba et al., 2005). However, selecting useful alleles and their combinations is a long-term process that involves crossing with wild genotypes and cultivating necessary ones for several generations. Nevertheless, significant number of modern cultivars have resulted from the breeding programs using induced mutagenesis that were launched in the beginning and middle of the 20th century, in other words, they are partially a subproduct of nuclear technology development.

The second method for obtaining new versions of genes lies with genetic engineering and transgenesis. The main advantage of this approach, if compared to induced mutagenesis, is that is allows for fast and dedicated effect on a certain trait through an induced alien transgene, which significantly reduces the time required to obtain a genetically modified organism (GMO) (Khush, 2012). However, along with the advantages, the method has certain drawbacks that will be discussed in a separate section below.

The further advancement of genome modification technologies is related to improved dedicated delivery of vector molecules so they could directly affect certain genetic loci, which has been implemented in the gene targeting strategy (Hall et al., 2009). The strategy allows one to overcome the main disadvantage of transgenesis that is a possibility for a transgene to introgress into different genomic regions, makes the expected effect more targeted and prevents off-target editing of other genes. Its foundation was initially based on the phenomenon of homologous recombination between a vector's DNA sequence and a genomic DNA sequence homologous to it (Smithies et al., 1985; Capecchi, 1989). The process results in either deletion of a gene or its part so the gene loses its functionality (gene knockout); or insertion of additional sequence; or modification of certain base pairs (point mutation). Genetic targeting is widely used in human and animals. In particular, it is applied to study the genetic diseases in cell lines for which a knockout or a modification of a potentially pathogenic gene can be performed *in vitro* (Sur et al., 2009). Together with homologous recombination, the genomes of eukaryotic organisms employ non-homologous end joining (NHEJ) that may generate unpredictable frequent mutations during DNA repair (Guirouilh-Barbat et al., 2004).

Another big advancement that has significantly increased the efficacy of genetic targeting has become the development of artificial endonucleases such as meganucleases, zinc-finger (ZF), transcription activator-like effector (TALEN) and Cas9 site-specific nucleases. It is the use of those nucleases that has given birth to a new specific term "genome editing (GE)" although today it refers to any methods of gene modification (Bak et al., 2018).

ZF and TALEN nucleases are used in combination with targeting proteins such as ZF domains and the proteins similar to TAL effectors, respectively. In case of Cas9 nucleases, it is CRISPR RNA that gave birth to the CRISPR/Cas9 technology that has revolutionized GE being the least laborious, relatively inexpensive and most precise and effective technology to the date. For the time passed since its introduction in 2012, it has been applied for editing of a huge number of living organisms from humans to yeast (Khlestkina, Shumny, 2016).

In what follows, the results obtained in plants with different genome modification techniques will be considered.

## Induced mutagenesis

The effect radiation has on heredity was first demonstrated by Russian botanist Georgy Nadson (Nadson, Philippov, 1925) and American genetic scientist Hermann J. Muller (Muller, 1927). Their discovery fostered multiple genetic studies that went in parallel with the development of wave and nuclear physics. Among such studies were those carried out by prominent Russian scientists including A. Sapegin who studied radiation-induced mutagenesis in common wheat (Sapegin, 1930), and N. Timofeev-Resovsky who started a new direction in radiation genetics (Timofeeff-Ressovsky, 1929). At the same time, chemical mutagenesis was studied by N. Koltsov and his disciple I. Rapoport whose achievements became crucial for applying the method in plant selection (Rapoport, 1946).

Since the 1930th, both radiation and chemical mutagenesis techniques have been used all over the world to produce more than 3200 cultivars of 200 species (https://mvd.iaea.org).

In this respect, Russia takes the fourth place (6.7 % of mutagenic cultivars) after China, India and Japan (Ahloowalia et al., 2004). In our country, the mutant plants have been used to obtain the cultivars of winter/spring wheat, barley, soybeans,

lupin, oat, beans, etc. For instance, common wheat cultivar Novosibirskaya 67 was created using the radiation technique and became the fruit of the joint efforts of the breeders of Novosibirsk Experimental Station and Institute of Cytology and Genetics of Siberian Branch of the USSR Academy of Sciences (Cherny, 1982). For a long time, the cultivar had remained the leading crop of Western Siberia in terms of planted areas for it combined high productivity, excellent baking properties and was resistant to a number of diseases. The scientists of Research Institute of Oil Crops (Krasnodar) used chemical mutagenesis to produce Pervenets, a new sunflower cultivar whose oil quality was comparable to that of olive trees (Russian Solar Flower, 2007).

The dwarfism mutation was used by N. Borlaug to breed the cultivars of non-lodging high-yielding common wheat that paved the way for the so-called green revolution in the middle of the last century (Gaud, 1968). E. Sears and F. Elliot used experimental mutagenesis in combination with long-term hybridization to transfer the loci of resistance to rust and smut from the wild varieties of goat and wheat grass to common wheat (Kilian et al., 2011). G. Stubbe (DRG) applied 5-time X-ray irradiation and selection in several generations of small-fruited wild tomato to increase its fruit to the size commonly observed in cultivated tomato (Stubbe, 1957).

## Transgenesis

The next technology to obtain new gene versions that came onto stage was genetic engineering or artificial transgenesis. In its essence the technology is introduction of an alien gene (transgene) into a living organism that facilitates the last to obtain predictable and inheritable traits. In plants, transgenes are delivered using the specialized vectors created using the tumor-inducing (Ti) plasmids of agrobacteria (Weising et al., 1988). Since all plant species have similar genetic code, it means a transgenic organism is able to express alien genes.

This approach had multiple advantages if compared to induced mutagenesis. First, it significantly widened the possibility for dedicated modification of living organisms because transgenes could have the traits untypical for a recipient, so they could not be obtained using mutagens (e. g., synthesis of pharmaceutical, insecticide and other agents in plants). Second, the technology significantly reduced the scale and duration of selection especially after such markers as antibiotic resistance and reporter genes were introduced into vector DNA and allowed for fast and effective identification of genetically modified organisms (GMO). In terms of fundamental science, transgenic organisms became a convenient model for studying the functions of a particular gene and their phenotypical manifestations.

Genetic engineering has been used to obtain multiple genetically modified cultivars of corn, rice, soybean, cotton, rape, potato and others whose farming areas take hundreds of millions of hectares all over the world (Genetically Engineered Crops…, 2016). One of the examples of transgenic plants is Golden Rice that has high content of β-carotene, a precursor to vitamin A whose deficiency leads to xerophthalmia, a widespread eye condition in South-East Asia. To obtain this cultivar, a gene of phytoene synthase (Narcissus) was introduced into a local variety using the bioballistics technique (Burkhardt et al., 1997). Another example of successful transgenesis in agriculture is transgenic soybean.

Its cultivars are widely represented on the market and are known for their resistance to different herbicides such as Roundup (glyphosate), glufosinate, Dicamba. Others contain the gene of *Bacillus thuringiensis* (BT), whose toxin make them resistant to insects (https://www.isaaa.org/gmapproval database).

An analogous transgene was introduced to cotton and made it resistant to the cotton budworm, a common pest for this species (Wu et al., 2008). Genetic engineering has also produced the transgenic varieties of cotton, maize and rape resistant to herbicides (Tan et al., 2005; Karthik et al., 2020), and that of maize resistant to insects (Lundmark, 2007) and many others.

All these examples prove the technology has been successfully applied in the agricultural sector of such countries as the USA, China, India, Argentina, Canada and others where industrial agriculture and transgenic plants were permitted unlike the majority of countries where the using and growing of GMOs was prohibited or unlike Russia that only allowed for import of GMOs as food products, forage, and research objects (Dudin, 2020). Although, most of GMO-related concerns have been due to prejudices or the rivalry of agrochemical companies, it is still cannot be stated that all such concerns have been completely ungrounded. GMOs present a certain danger for ecosystems, e. g., if we have produced herbicide-resistant plants, how can we be certain that these genes will not be transferred to weeds by pollen while cross hybridization (Schütte et al., 2017).

There is also a risk that a transgenic plant can affect non-target organisms such as plants possessing BT-toxin genes can kill non-hazardous insects (Marvier et al., 2007). The long-term consequences of transgenesis remain unclear since a transgene can enter different regions of a genome and ruin other genes' expression. As for their direct harm to human health, multiple scientific research has shown that GMOs and their products are of no more harm than traditional crops (König et al., 2004).

## Genome editing

The basis of GE is dedicated changing of a limited gene region that may be achieved in different ways. Considering the early days, the first experiments were applying oligonucleotides for DNA editing, e. g., two genes (defective green fluorescent protein and acetolactate synthase) of tobacco and corn were edited using chimeric RNA/DNA oligonucleotides in 1999 (Beetham et al., 1999; Zhu et al., 1999). In the last case, the editing resulted in a low-frequent resistance to imidazoline and sulfonylurea. This study was followed by analogous works to alter these and other species of plants (Zhu et al., 2000; Kochevenko, Willmitzer, 2003; Okuzaki, Toriyama, 2004), but the effectiveness of the techniques remained comparable to that of spontaneous mutagenesis (Ruiter et al., 2003).

Single-stranded DNA oligonucleotides proved to be of a bit higher efficacy (Dong et al., 2006), but it still was not high enough. Moreover, selecting edited plants became a problem

that could not be resolved without using vectors. For that reason, the perspectives of this direction remain questionable.

Another direction of GE is related to using endonucleases, special enzymes provoking double-stranded ruptures in a DNA molecule. Repairing the ruptures may occur either through recombination with a homologous DNA fragment that has been placed into a vector and transformed into a cell nucleus. The first endonucleases used for this purpose were homing endonucleases recognizing DNA regions of 12–45 nucleotides. The specificity of these regions varied and depended on a type of nuclease, e. g., using the I-*Ceu*I homing endonuclease and the 35S promoter, the *bar* gene was precisely inserted into a site of a corn genome to make the plant resistant to phosphinothricin (D'Halluin et al., 2008).

Analogous site-specific insertion was carried out in a cotton genome (genus *Gossypium*) to provide the last with genes *hppd* and *epsps* making the plant resistant to glyphosate (D'Halluin et al., 2013). The I-*Sce*I homing endonuclease was used to replace a region in a barley genome to a homologous one delivered in a vector with a functional gene of resistance to hygromycin (Watanabe et al., 2015).

## Protein editors: ZF and TALEN nucleases

In the GE techniques based on protein editing, one uses chimeric nucleases. These are complex proteins containing two structural components, one of which binds specifically with certain nucleotide sequencies of genome DNA, directing at them the second component, a nuclease catalyzing DNA splitting. These proteins are delivered into a plant's genome using expression vectors.

The first such vectors were ZF nucleases that typically contained three "zinc fingers" as a directing structure. The fingers are protein domains binded with one or two ions of zinc and capable of recognizing and specifically binding with a certain nucleotide triplet in DNA sequence. In some case, the number of these domains were increased to 6, so their specificity level raised to 18 DNA nucleotides (Liu et al., 1997).

For the first time, ZF nucleases were applied for genome editing in plants in 2005 when a corresponding vector was inserted in Arabidopsis so indels of different length, mostly deletions (78 %), were found (Lloyd et al., 2005). Since then, a lot of analogous projects have been performed in tabaco, soybean, corn, tomato, apple and fig trees (Shukla et al., 2009; Townsend et al., 2009; Curtin et al., 2011; Peer et al., 2015; Hilioti et al., 2016). However, the technique has turned out to be quite laborious and expensive for it requires a unique protein structure of ZF nuclease to be created for each individual sequence of target DNA. Additionally, the technique is not precise in recognizing nucleotide triplets, which results in a large number of DNA splits in off-target regions. For these reasons, the technique is quite rarely applied these days.

TALEN chimeric nucleases have proved to be more effective. The protein domains serving as their directing structures are the prototypes of the natural TAL effectors of certain bacteria, and each of them recognizes only one nucleotide. In this case, the DNA recognition mechanism is more unambiguous than that of ZF nucleases and allows for relatively easy creation of a structure that specifically recognizes a required DNA sequence. The last is binded with an enzyme splitting the DNA (commonly, *Fok* I endonuclease) and enables for a theoretically very precise double-stranded rupture within any genome region.

In 2011, the technique was recognized as the most perspective GE approach. By 2017, it had been used to edit 12 plant genomes including those of such domestic plants as rice, wheat, corn, tobacco, barley, potato, sugar cane, soybean, tomato, and of model plants such as Arabidopsis and Brachypodium. In total, in these plants, more than 50 genes have been edited (mostly knocked out) (Malzahn et al., 2017), e. g., to increase bioethanol output in the sugar cane, TALEN nucleases were used to knock out its genes responsible for high lignin content (Jung, Alpeter, 2016). To exclude potato sweetening while storing in cold, vacuolar invertase catalyzing the sucrose splitting into fructose and glucose was knocked out (Clasen et al., 2016). Using the TALEN and CRISPR/Cas9 approaches it became possible to knock out the alleles of powdery mildew resistant loci in every three subgenomes of allohexaploid common wheat *Triticum aestivum* L. (genome BAD; $2n = 42$) (Wang et al., 2014). To improve the quality of soybean oil, the genes of desaturase enzymes were mutated (Haun et al., 2014).

To facilitate the TALEN technique, a number of software solutions have been developed to search for edited sites, create vector structures and detect off-target sites such as TALEN-designer (http://talen-design.de).

## CRISPR/Cas9: leading GE technique

Unlike the chimeric nucleases, in the CRISPR/Cas9 technology, DNA-recognizing structures are not proteins but short RNAs that, first, are far more precise due to their complementarity and, second, are much easier and chipper to synthesize. The theoretical foundation of the technology was laid while studying the mechanism bacteria use to get protected from pathogenic viruses (bacteriophages) (Savitskaya et al., 2016). There have been published many reviews devoted to CRISPR/Cas9 (Khlestkina, Shumny, 2016; Zlobin et al., 2017; Strygina, Khlestkina, 2020). In plants, the technology was first applied in 2013 (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013).

The simplified vector included the genes of the Cas9 protein, a guide RNA (gRNA) analogous to bacterial CRISPR RNA and an additional sequence coding a nuclear localization signal (NLS). The vector was introduced in plant cells using either agrobacterial transformation or bioballistics. As a result, cellular DNA were transcripted by the intercellular RNA polymerase III. From the RNA template encoding Cas9, a protein is translated on ribosomes, which then enters the nucleus via NLS. In the nucleus the gRNA and Cas9 got united to bind with its target site following the principle of complimentary interaction.

An important element that, in many ways, determined the specificity of the binding was a protospacer adjacent motif (PAM), a nucleotide triplet (commonly NGG) placed near the 3′-end of the target site. The catalytic domains of the nuclease provoked single-stranded breaks near the PAM to activate a repair mechanism that could act in two ways: non-

homological end joining (NHEJ) being prone to the errors producing the indels of one or several nucleotides that shift the reading frame of the coded protein and disrupting its functionality to the degree of a knockout. The second way is homology-dependent repair (HDR) that edits the target site or introduces a new sequence that can be undesirable for an experiment, but the last is only possible if such a fragment of donor DNA has already presented in the region being edited.

The key element leading to successful genome edition via CRISPR/Cas9 has been selecting a gRNA for a target gene. The site of interaction with gRNA does not usually exceed 30 bp. The presence of PAM at the 3′-end of this region is an important condition for selecting a site to be edited. Another important criterion for gRNA selection is the number and localization of the sites for off-target editing, whose search in a genome is performed individually for each particular gRNA using special software solutions like those available on http://crispr.mit.edu/.

Lately, the GE technique using ribonucleoprotein (RNP) complexes has been actively developed. In this case, the transforming agent is not a vector (plasmid RNA) but a ready-to-use complex including Cas9 and a gRNA. This approach has proved its efficacy when editing the genomes of corn, wheat and potato via bombarding the embryonal cells with gold microparticles (Martin-Ortigosa et al., 2014; Woo et al., 2015; Svitashev et al., 2016; Liang et al., 2017; Andersson et al., 2018).

It is noteworthy that this alternative to using an agrobacterium, which by itself can cause an undesirable genetic effect, allows CRISPR/Cas9 to go beyond the GMO approach and overcome the forbiddance against its application in the agricultural industry. Its other advantage is the reduced likelihood of DNA cutting in off-target sites because the lifetime of a delivered RNP complex is much shorter than its DNA expression. At the same time, employing bioballistics for delivering RNP complexes has a number of drawbacks related to the technique's excessive traumaticity for plant tissues, complexity of transformation and regeneration, and low editing frequency. For that reason, vector-based agrobacterial transformation still remains a leading approach to CRISPR/Cas9.

## Using CRISPR/Cas9 for producing new cultivars

Genome editing is a technology that can serve both applied – obtaining plants with new useful properties – and fundamental – studying the functions of genes – purposes. The fundamental tasks are solved using the methods of inverted genetics when scientists manipulate genetic sequencies knocking out this or that gene to see what consequences it will cause in the phenotype.

As for applied problems they are quite diverse and in what follows, the main directions of CRISPR/Cas9 application for breeding will be considered.

### Resistance to pathogens

The Table displays the studies aimed at creating the plants resistant to different pathogens. For instance, in rice (*Oryza sativa* L.) applying CRISPR/Cas9 resulted in its resistance to three pathogens: bacterial blight, tungro spherical virus and blast fungus. In the first case, the resistance was achieved after knocking out one of the *S* genes responsible for sensitivity to bacterial blight (sucrose transportation gene *OsSWEET13* being a target for a bacterial TAL effector (Zhou et al., 2015). In the second case, the host's *eIF4G* gene was knocked out whose product controlled the initiation of viral RNA translation (Macovei et al., 2018). And finally, in case of fungal pathogen, it was the *OsERF922* gene that was knocked out and it led to the reduction in ethylene hormone level in the cells and increased resistance (Wang et al., 2016).

In *T. aestivum*, fungal pathogen *Blumeria graminis* f. sp. *tritici* causes the so-called powdery mildew that significantly reduces the yield of common wheat in many regions. Currently, the *S* genes responsible for the sensitivity to the fungus have been edited. In one of such studies, the MLO genes were knocked out (Wang et al., 2014), in another – the EDR1 (enhanced disease resistance) genes (Zhang et al., 2017). It has been shown that in both cases, a knockout of all three homoelogical copies of the gene is to be achieved since knocking out only one or two copies has only resulted in partial resistance to the disease.

In *Solanum lycopersicum* L., application of CRISPR/Cas9 has made it possible to obtain tomato cultivars resistant to bacterial speck, yellow leaf curl virus and powdery mildew. In the first case, to enhance the barrier preventing bacterial infiltration in the cells, the *SlJAZ2* gene to control stoma closure was mutated to foster the gain of function (Ortigosa et al., 2018). In the case of viral disease, these were the pathogen's genes that were targeted, namely, the viral envelope (*CP*) and replicase (*Rep*) genes. As a part of T-DNA, their short sequencies were built in the plant's nuclear genome to enable their constitutive expression as RNA molecules, which in combination with Cas9 could effectively interfere the viral DNA (Tashkandi et al., 2018).

### Resistance to abiotic stress

A number of studies aimed at developing the cultivars resistant to abiotic stresses are listed in the Table. For instance, applying the protoplast technique in wheat led to mutating two genes related to drought stress (*TaDREB2* and *TaERF3*) (Kim D. et al., 2017). A similar study was performed in soybean (*Glycine max* L.) in which two genes related to the plant's resistance to drought and salinity (Curtin et al., 2018).

In this field, not only applied but also fundamental research has been performed. Hence, it was found out that mitogenic-activated protein kinase (MAPK) reacted to drought by protecting a cell membrane from oxidation and regulating the transcription of other genes. The role of one of *MAPK* genes was determined using CRISPR/Cas9 for creating the knockout mutants of this gene (Wang et al., 2017). In a similar way, the effect of three genes on rice resistance to abiotic factors was determined. It turned out, the genes coded MAPK (*OsMPK2*), phytoene desaturase (*OsPDS*) and betaine aldehyde dehydrogenase (*OsBADH2*) (Shan et al., 2013).

### Yield

The studies applying CRISPR/Cas9 to increase a plant's yield are listed in the Table. The kernel size and thousand-kernel

Summary of CRISPR/Cas9 applications in major crops

| Species | Target gene | Trait study | Editing result | Delivery technique | Reference |
|---|---|---|---|---|---|
| | | Resistance to pathogens | | | |
| *O. sativa* | *OsSWEET13* | Resistance to *X. oryzae* (bacterial blight) | Knock-out | *Agrobacterium*-mediated transformation | Zhou et al., 2015 |
| *O. sativa* | *eIF4G* | Resistance to tungro spherical virus | Knock-out | *Agrobacterium*-mediated transformation | Macovei et al., 2018 |
| *O. sativa* | *OsERF922* | Resistance to *Magnaporthe oryzae* (blast fungus) | Knock-out | *Agrobacterium*-mediated transformation | Wang et al., 2016 |
| *T. aestivum* | *MLO* | Resistance to *Blumeria graminis* (powdery mildew) | Knock-out | *Agrobacterium*-mediated transformation | Wang et al., 2014 |
| *T. aestivum* | *EDR1* | Resistance to *Blumeria graminis* (powdery mildew) | Knock-out | *Agrobacterium*-mediated transformation | Zhang et al., 2017 |
| *S. lycopersicum* | *SlJAZ2* | Resistance to *Pseudomonas syringae* (bacterial speck) | Mutation "gain of function" | *Agrobacterium*-mediated transformation | Ortigosa et al., 2018 |
| *S. lycopersicum* | *CP*- and *Rep*-genes | Resistance to yellow leaf curl virus | Interference with virus DNA | *Agrobacterium*-mediated transformation | Tashkandi et al., 2018 |
| *S. lycopersicum* | *SlMlo1* | Resistance to *Oidium neolycopersici* (powdery mildew) | Knock-out | *Agrobacterium*-mediated transformation | Nekrasov et al., 2017 |
| *Vitis vinifera* | *VvWRKY52* | Resistance to *Botrytis cinerea* | Knock-out | *Agrobacterium*-mediated transformation | Wang X. et al., 2018 |
| *Gossypium hirsutum* | *Gh14-3-3d* | Resistance to *Verticillium dahliae* (verticillium wilt) | Knock-out | *Agrobacterium*-mediated transformation | Zhang Z. et al., 2018 |
| *Citrus sinensis* | *CsLOB1* | Resistance to *Xanthomonas citri* (citrus canker) | Knock-out | *Agrobacterium*-mediated transformation | Jia et al., 2017 |
| *Cucumis sativus* | *eIF4E* | Broad resistance to viruses | Knock-out | *Agrobacterium*-mediated transformation | Chandrasekaran et al., 2016 |
| | | Resistance to abiotic stress | | | |
| *T. aestivum* | *TaDREB2*, *TaDREB3* | Drought tolerance | Knock-out | PEG-mediated transformation | Kim D. et al., 2017 |
| *Glycine max* | *Drb2a*, *Drb2b* | Drought tolerance | Knock-out | *Agrobacterium rhizogenes*-mediated transformation | Curtin et al., 2018 |
| *S. lycopersicum* | *SlMAPK3* | Drought tolerance | Knock-out | *Agrobacterium*-mediated transformation | Wang et al., 2017 |
| *O. sativa* | *OsMPK2*, *OsPDS*, *OsBADH2* | Multiple stress tolerance | Knock-out | Particle bombardment | Shan et al., 2013 |
| *O. sativa* | *SAPK2* | Drought and salinity tolerance | Knock-out | *Agrobacterium*-mediated transformation | Lou et al., 2017 |
| *O. sativa* | *SAPK1*, *SAPK2* | Salinity tolerance | Knock-out | *Agrobacterium*-mediated transformation | Lou et al., 2018 |
| *O. sativa* | *OsNAC14* | Drought tolerance | Overexpression | *Agrobacterium*-mediated transformation | Shim et al., 2018 |
| *O. sativa* | *OsRR22* | Salinity tolerance | Knock-out | *Agrobacterium*-mediated transformation | Zhang A. et al., 2019 |

**End of table**

| Species | Target gene | Trait study | Editing result | Delivery technique | Reference |
|---|---|---|---|---|---|
| | | | Yield | | |
| *T. aestivum* | *TaGW2* | Grain weight | Knock-out | Particle bombardment | Wang W. et al., 2018 |
| *T. aestivum* | *TaGW7* | Grain weight | Knock-out | *Agrobacterium*-mediated transformation | Wang et al., 2019 |
| *T. aestivum* | *CKX2-1*, *GLW7*, *GW2*, *GW8* | Grain yield | Knock-out | *Agrobacterium*-mediated transformation | Zhang Z. et al., 2019 |
| *O. sativa* | *OsAAP3* | Number of shoots | Knock-out | *Agrobacterium*-mediated transformation | Lu et al., 2018 |
| *O. sativa* | *OsDEP1*, *OsGS3*, *OsGn1a* | Panicle size, grain size, grain yield | Knock-out | *Agrobacterium*-mediated transformation | Li S. et al., 2016 |
| *O. sativa* | *GW5* | Grain weight | Knock-out | *Agrobacterium*-mediated transformation | Liu et al., 2017 |
| *O. sativa* | *OsGRF4* | Grain size | Overexpression | *Agrobacterium*-mediated transformation | Li M. et al., 2016 |
| *Zea mays* | *ARGOS8* | High yield under drought | Overexpression | Particle bombardment | Shi et al., 2017 |
| | | | Nutritional value | | |
| *Zea mays* | *ZmIPK* | Decreased phytic acid | Knock-out | *Agrobacterium*-mediated transformation | Liang et al., 2014 |
| *Zea mays* | *PPR*, *RPL* | Increased lysine and tryptophan | Knock-out | *Agrobacterium*-mediated transformation | Qi et al., 2016 |
| *T. aestivum* | α-*gliadin* | Low gluten | Knock-out | Particle bombardment | Sánchez-León et al., 2018 |
| *T. aestivum* | α-*gliadin*, γ-*gliadin* | Low gluten | Knock-out | *Agrobacterium*-mediated transformation | Jouanin et al., 2019 |
| *O. sativa* | *Waxy* | Decreased amylose | Knock-out | *Agrobacterium*-mediated transformation | Zhang J. et al., 2018 |
| *O. sativa* | *SBEIIb* | Increased amylose | Knock-out | *Agrobacterium*-mediated transformation | Sun et al., 2017 |
| *S. tuberosum* | *GBSS* | Decreased amylose | Knock-out | PEG-mediated transformation | Andersson et al., 2017 |
| *Glycine max* | *FAD2-1A*, *FAD2-1B* | Increased oleic acid | Knock-out | PEG-mediated transformation | Kim H. et al., 2017 |
| *Sorghum bicolor* | *k1C* genes | High lysine content and protein digestibility | Knock-out | *Agrobacterium*-mediated transformation | Li A. et al., 2018 |
| *Brassica napus* | *FAD2* | Increased oleic acid | Knock-out | *Agrobacterium*-mediated transformation | Okuzaki et al., 2018 |
| *S. lycopersicum* | *ncRNA1459* | Long shelf life | Knock-out | *Agrobacterium*-mediated transformation | Li R. et al., 2018 |
| *S. lycopersicum* | *SGR1*, *LCY-E*, *Blc*, *LCY-B1* | Increased lycopene | Knock-out | *Agrobacterium*-mediated transformation | Li X. et al., 2018 |
| *S. lycopersicum* | *SlGAD2*, *SlGAD3* | Enhance γ-aminobutyric acid | Knock-out | *Agrobacterium*-mediated transformation | Nonaka et al., 2017 |
| *Glycine max* | *GmGOLS1A*, *GmGOLS1B* | Decreased raffinose in beans | Knock-out | *Agrobacterium*-mediated transformation | Le et al., 2020 |
| *Glycine max* | *F3H1*, *F3H2*, *FNSII-1* | Increased isoflavonoid compounds | Knock-out | *Agrobacterium*-mediated transformation | Zhang P. et al., 2019 |

weight in common wheat were increased by provoking nonsense mutations in the homeological copy of the *GW2* gene being a negative regulator of these traits. The degree of the increase was determined by a portion of mutated homoeological genes (Wang W. et al., 2018). Later, the same authors could change the size and weight of a wheat kernel by mutating the sequence of another gene to belong to the same group: *GW7* in subgenomes B and D (Wang et al., 2019).

The number of kernels in an ear was increased by editing four target genes: *CKX2-1*, *GLW7*, *GW2* and *GW8* (Zhang A. et al., 2019). In this case, the line homozygotic to the large deletion in the *CKX2-1* gene demonstrated the maximum increase of the ear kernel number as well as maximum ear density, which has confirmed the gene is a negative regulator affecting the number of kernels in an ear.

A whole set of genes was knocked out in rice. These were negative regulators of controlling such traits as tiller number (*OsAAP3*), ear size (*OsDEP1*), kernel weight (*OsGW5*) and size (*OsGS3*, *OsGRF4*) and the number of kernels in an ear (*OsGn1a*) (Li M. et al., 2016; Li S. et al., 2016; Liu et al., 2017; Lu et al., 2018). Additionally, the rice model has been applied to integrate whole-genome sequencing, genealogy analysis and CRISPR/Cas9 for full-scale identification of the target genes that affect quantitative traits including yield (Huang et al., 2018).

At the first stage, the genealogy analysis detected multiple quantitative trait loci (QTL) associated with yield to carry out their association mapping. Comparison of the obtained map against the rice's whole-genome sequence enabled for selecting candidate genes to be knocked out using CRISPR/Cas9 for estimating their phenotypical effect. As a result, a whole set of the genes crucial for yield was found.

A study to preserve the yield in presence of stress factors by mutating the *ARGOS8* gene was carried out in corn (*Zea mays* L.) by J. Shi et al. (2017). The authors applied CRISPR/Cas9 to replace this negative regulator of ethylene response by a promotor of another gene to increase *ARGOS8* expression. Field studied have demonstrated that the CRISPR-edited plants had higher yield in drought condition than their parents.

**Nutritional value**

High amounts of phytic acid present in the grains of cereal, legume and oil crops. This acid is antinutrient and cannot be digested by animals with single-chamber stomach and can cause environmental pollution. To reduce the acid's content in corn, CRISPR/Cas9 was applied to knock out the gene of the enzyme catalyzing the stages of phytic-acid biosynthesis, so its production was blocked in the mutant line (Liang et al., 2014). The same corn was used to obtain cultivars with higher level of essential amino acids – lysin and tryptophane – by knocking out the genes having a negative effect on their biosynthesis (Qi et al., 2016).

Changing gluten content and composition in wheat has been another topical issue due to the high spread of gluten intolerance in people. The results of two studies using CRISPR/Cas9 and aimed at reducing in wheat the content of α- and γ-gliadins causing pathological reactions have recently been published. One group obtained the mutant lines with significantly reduced α-gliadin content (Sánchez-León et al., 2018). The other group created lines with low α- and γ-gliadins (Jouanin et al., 2019). The obtained wheat lines may become a start for new elite wheat cultivars to produce low-gluten products.

In rice, application of CRISPR/Cas9 has led to the plant's improved nutritive and culinary qualities. It was achieved by mutating the *Waxy* gene to change the amylose/amylopectin ratio in starch in the favor of amylopectin (Zhang J. et al., 2018). This component determines the waxlike (sticky) qualities of starch in rice grains, which is very important for making sushi. In another study, the opposite result was obtained, so the gene responsible for suppressing amylose synthesis was knocked out (Sun et al., 2017).

In potato (*Solanum tuberosum* L.), the gene encoding granule-bound starch synthase (GBSS) was knocked out, so the obtained lines demonstrated a reduced level of amylose (Andersson et al., 2017).

To improve the quality of soybean oil, CRISPR/Cpf1 was used to knock out genes *FAD2-1B* and *FAD2-1A* and produce high-yield soy plants with high content of oleic acid (Kim H. et al., 2017).

In sorgo (*Sorghum bicolor* L.), GE techniques were applied to knock out the genes responsible for improper digestibility and essential amino acids suppression (Li A. et al., 2018).

Using CRISPR/Cas9 the cultivars of rape (*Brassica napus* L.) were obtained with high content of oleic acid (Okuzaki et al., 2018) as well tomato cultivars with increased storability (Li R. et al., 2018) and increased content of lycopene, a vitamin A precursor of powerful antioxidation effect (Li X. et al., 2018). These and many other studies are listed in the Table.

***De novo* domestication**

The essence of the *de novo* domestication approach is speeding up a domestication process for a wild relative of an agricultural plant. The wild relatives are widely used in selection as donors of the genes responsible for a plant's resistance to biotic and abiotic stresses. However, a simple crossing with a wild species only produces 'half-cultivars' that often lose the features of a cultural plant as well as the many qualities useful for humans.

Studies into the genes of wild and domestic plants have found the so-called 'domestication genes', in other words, mutations that transform a wild plant into one applicable for farming.

The idea behind *de novo* domestication is dedicated introduction of necessary genes into the domestication genes of a cultural plant's wild relative. Such boosted domestication made the headlines in 2018, when CRISPR/Cas9 was applied to convert a wild tomato into an almost cultural plant in a singe generation. To do so, a list of genes to be modified to obtain the plant's *de novo* version had been composed (Zsögön et al., 2017).

Comparing the genetic sequences in both wild and cultural tomato enabled one to determine the structural modifications to be implemented in the wild plant. At the final stage of the experiment, multiplex editing of four genes (*SP*, *SP5G*, *SlCLV3* and *SlWUS* was performed. These genes controlled

the plant's architecture (transition to the determinate type), heading time and fruit size (Li T. et al., 2018).

Another example of such research is changing the morphology of a barley ear. The naked kernel, unlike the rough one, has always been a sign of the crop's domestication. Naked barley is a traditional food and currently considered as a dietary component of functional nutrition. In nature, this transition from chuffy to naked kernel was determined by the *NUD* gene losing its function due to deletion of 17 kb in a corresponding locus. Using CRISPR/Cas9, a naked-barley cultivar has been produced experimentally by knocking out *NUD* in a wild rough variety (Gerasimova et al., 2020).

Thus, *de novo* domestication opens huge perspectives for selective breeding, enabling one to obtain the results of hundreds and thousands of years of evolution in one generation.

## Conclusion

Intensely developing GE technologies will soon see lifting many of the limitations for their wide practical application. The development goes in the direction of higher modification specificity and off-target effects elimination by using new-type nucleases such as the Cas9 orthologs interacting with different PAMs (Fonfara et al., 2014) or completely new nucleases such as Cas12a (Zetsche et al., 2015).

Moreover, there are approaches that go beyond gene knockouts and include other modifications as changing a nucleotide or a whole sequence. This method has proved effective when editing a single DNA base to perform cytosine/thymine or adenine/guanine replacement. Such changes have become possible thanks to using specific enzymes being a combination of cytosine deaminase, adenosine desaminase and nickase (Zong et al., 2017; Li C. et al., 2018).

Another technique that is developing fast is homological recombination when an expressing vector is delivered in a cell together with a donor DNA flankered by the sequencies homologous to the site where endogenous DNA is replaced by a donor's one (Jasin, Haber, 2016).

In addition, transformation techniques are developing since the classical methods such as agrobacterial transformation and particle bombardment in many ways produce low output of transformants. Hence, a possibility to use modified viral genomes has been demonstrated for transition of expression cassettes, geminiviruses in particular, and proved effective for a number of cultures (Baltes et al., 2014; Čermák et al., 2015; Butler et al., 2016).

Along with technological advancements, the development of bioinformatic approaches, in particular, enlargement of genetic databases and enhancing of genetic network analysis will become the basis for multiplex GE to modify several traits at once.

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# Agrobacterium-mediated transformation of *Nicotiana glauca* and *Nicotiana sylvestris*

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**Abstract.** Agrobacterium-mediated transformation is the most popular approach for obtaining transgenic plants nowadays. There are plenty of protocols developed for different plant species. These protocols usually include the medium composition, the technology for preparing plant explants and cultivation conditions, as well as the choice of agrobacteria strains. *Nicotiana tabacum*, or cultivated tobacco, was one of the first successfully transformed plant species. *Nicotiana tabacum* is a model object in plant genetics, particularly due to its ability for transformation and regeneration. *N. tabacum* is a naturally transgenic plant since its genome contains a cellular T-DNA acquired from *Agrobacteria*. The significance of cT-DNA for plants has not yet been established. Some assume that cT-DNA can increase the ability of plants to regenerate due to some of the genes they contain. For example, *rolC* has been shown to affect the hormonal balance of plants, but the molecular mechanisms underlying this have yet to be found. *RolC* is also somehow involved in the secondary metabolism of plants. Like *N. tabacum*, *Nicotiana glauca* produces a wide range of secondary metabolites and contains an intact *rolC* gene in its genome. At the same time, unlike *N. tabacum*, *N. glauca* is a diploid species, which makes it more suitable for genetic engineering approaches. *Nicotiana sylvestris* is one of the ancestral species of *N. tabacum* and does not contain cT-DNA. The aim of this work was to develop a protocol for transformation and regeneration of *N. glauca* and *N. sylvestris*. We managed to find an optimum ratio of auxins and cytokinins that promotes both active callus formation and organogenesis in *N. glauca* and *N. sylvestris* leaf explants. The developed technique will be useful both for fundamental research that includes the *N. glauca* and *N. sylvestris* species, and for practical application in the pharmaceutical industry and biosynthesis.
Key words: agrobacterium-mediated transformation; regeneration; *Nicotiana*.

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# Агротрансформация видов *Nicotiana glauca* и *Nicotiana sylvestris*

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**Аннотация.** Агробактериальная трансформация – наиболее популярный метод получения трансгенных растений. Для многих видов растений разработаны протоколы, включающие описание условий трансформации, состав питательных сред, методику подготовки растительных эксплантов и выбор штаммов агробактерий, а также соотношение растительных гормонов, необходимых для последующей регенерации эксплантов. Одним из первых успешно трансформированных видов стал культурный табак, *Nicotiana tabacum*, который сегодня служит модельным объектом генетики растений. *Nicotiana tabacum* эффективно трансформируется и легко регенерирует, что делает его удобным для генно-инженерных манипуляций. При этом *N. tabacum* относится к природно-трансгенным видам, поскольку содержит в своем геноме последовательности агробактериального происхождения, клеточную Т-ДНК, значение которой для растений пока не установлено. Одним из предковых видов для *N. tabacum* является *N. sylvestris*, геном которого не содержит клТ-ДНК. Предполагают, что клТ-ДНК может повышать регенерационные способности растения за счет генов, входящих в ее состав, таких как, например, *rolC*. Для *rolC* действительно показано влияние на баланс растительных гормонов, однако стоящие за этим молекулярные механизмы остаются неизвестными. Помимо участия в морфогенезе, *rolC* влияет на биосинтез вторичных метаболитов в растении. Вид *N. glauca*, как и *N. tabacum*, считается природно-трансгенным, несет в клТ-ДНК интактный *rolC* и содержит широкий спектр вторичных метаболитов. При этом, в отличие от *N. tabacum*, *N. glauca* –диплоидный вид, что делает его гораздо более удобным объектом для проведения генно-

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инженерных работ. Целью данной работы была разработка протокола трансформации и регенерации для видов *N. glauca* и *N. sylvestris*. На основании уже известных протоколов для других представителей рода *Nicotiana* нами было подобрано такое соотношение ауксинов и цитокининов, при котором листовые экспланты *N. glauca* и *N. sylvestris* переходят к активному каллусообразованию, а затем к органогенезу. С использованием разработанной методики получены трансгенные растения этих видов. Разработанная методика трансформации и регенерации полезна как для фундаментальных исследований, затрагивающих виды *N. glauca* и *N. sylvestris*, так и для практического применения в области фарминдустрии и биосинтеза.
Ключевые слова: агробактериальная трансформация; регенерация; *Nicotiana*.

## Introduction

Agrobacterium-mediated transformation has been the main approach for obtaining transgenic plants in laboratories for over 30 years (Sawahel, Cove, 1992). To date, transformation protocols have been developed for many plant species represented by various life forms, such as herbs, shrubs, and trees (Wang, 2015). The main differences between these protocols are determined by the choice of the agrobacterium strain, the vector, the type of plant explant and the way to prepare it for transformation. It is also necessary to select certain conditions of inoculation and co-cultivation processes, such as their duration, as well as lighting and temperature values. Due to various modifications of the protocols, it was possible to significantly increase the efficiency of agrobacterium-mediated transformation of various plant species, including economically important crops (Cheng M. et al., 2004). The process of plant regeneration that usually follows transformation also requires specific conditions for each particular species.

*Nicotiana tabacum* is one of the first transformed species. Development of the transformation and regeneration protocols for *N. tabacum* led to the first transgenic tobacco plants resistant to antibiotics (Herrera-Estralla et al., 1983). To date, *N. tabacum* is a classic model object of plant genetics and is widely used in genetic engineering. Since *N. tabacum* is a valuable agricultural crop, it is well studied, and a high-quality reference genome is available in the open database (Edwards et al., 2017). The *N. tabacum* species includes many cultivars that differ in a number of ways, including the efficiency of the regeneration process (Ali et al., 2007), which allows researchers to choose the most suitable ones for the transformation process.

The genome of *N. tabacum* contains DNA sequences acquired from *Agrobacteria*. These sequences, homologous to the agrobacterial T-DNA, are called cellular T-DNA (cT-DNA) (White et al., 1983). Plants that carry cT-DNA are considered natural transgenic species or natural genetically modified organisms (nGMOs) (Matveeva, 2018). To date, the list of nGMOs includes more than 40 genera of angiosperms (Matveeva, 2021). The function of cT-DNA for plants has yet to be established. Several hypotheses are discussed in the literature, such as increasing the adaptive capacity to arid conditions, impact on the microbial communities of the rhizosphere, enhancing regenerative abilities and resistance to subsequent agrotransformation (Chen, Otten, 2017; Matveeva, Sokornova, 2017). In addition, an increased sensitivity of natural transgenic plants to agrobacterium-mediated transformation is assumed. The experimental data obtained for different nGMO species do not add up to a unified picture. However, to date, only five natural transgenic species belonging to the genus *Nicotiana* have been studied for the transformation efficiency (Matveeva, Sokornova, 2017). Expanding the list of studied species may clarify this issue.

Some of cT-DNA genes have retained their activity in natural transgenic species for generations, suggesting the importance of these genes for the plant. One of these genes is *rolC* in the cT-DNA of *N. tabacum* (Chen et al., 2014) and *N. glauca* (Intrieri, Buiatti, 2001). The *rolC* gene activity is known to affect morphogenetic processes, as well as the secondary metabolism of plants, although the molecular mechanisms underlying its effects have not yet been elucidated (Khafizova, Matveeva, 2021). For studying gene activity, various approaches can be used. Silencing and controlled gene activation are the most popular among them. However, they require developed methods of transformation and regeneration under *in vitro* conditions for specific plant species.

This work is devoted to the development of transformation and regeneration methods for the *N. glauca* and *N. sylvestris* species. *N. glauca*, like *N. tabacum*, is a natural transgenic plant carrying an intact *rolC* in its cT-DNA. And, like *N. tabacum*, it contains a wide range of secondary metabolites (Long et al., 2016). At the same time, *N. glauca* is a diploid, which makes it a much more convenient object for genetic engineering manipulations comparing to allotetraploid *N. tabacum*. *N. sylvestris* is one of the ancestral species of *N. tabacum* and its genome does not contain cT-DNA (Yukawa et al., 2006). Like *N. glauca*, *N. sylvestris* is a diploid species.

Two plasmids were constructed in this work: the first contains *rolC* under an inducible promoter to create *N. sylvestris* plants with controlled expression of *rolC*. The second plasmid contains a CRISPR/Cas9 cassette with 2 guide RNAs targeting *rolC* aiming to "turn off" this gene in *N. glauca*. However, protocols for the transformation and regeneration of *N. sylvestris* and *N. glauca* have not previously been developed. Therefore, it was necessary to design such protocols based on existing ones. The possibility to create transgenic *N. sylvestris* and *N. glauca* plants will expand the range of research involving these species. For example, *N. glauca* mutants for various genes in biosynthesis pathways will contribute to the study of the molecular mechanisms of secondary metabolism. *N. glauca* plants with inactivated *rolC* and *N. sylvestris* plants carrying *rolC*, obtained in this work, will be further used to investigate the functions of the *rolC* gene, contributing to fundamental studies in horizontal gene transfer from agrobacteria to plants.

## Materials and methods

Aseptic plants *Nicotiana glauca* (var. 359 from the Federal state budget scientific institution "All-Russian Scientific Research Institute of Tobacco, Makhorka and Tobacco Products" collection) and *Nicotiana sylvestris* (obtained from the Federal

state budget scientific institution "All-Russian Scientific Research Institute of Tobacco, Makhorka and Tobacco Products" collection) were used in this work. Plants were grown *in vitro* and maintained by cuttings on Murashige–Skoog (MS) medium (Murashige, Skoog, 1962) with 20 g/L sucrose at 23 °C and a photoperiod of 16 hours day/8 hours night.

For the transformation of *N. glauca* plants, a pHSE401_roC vector was prepared. It contained a cassette for the *rolC* gene editing: 2 guide RNAs and *Cas9* under the control of CaMV 35S; as well as kanamycin and hygromycin resistance genes. For the transformation of *N. sylvestris* plants, the pB7WG2D_PdexA4rolC vector was prepared. The pB7WG2D_PdexA4rolC vector contained the *rolC* gene sequence from *A. rhizogenes* under a dexamethasone-inducible promoter along with spectinomycin and glufosinate resistance genes.

**Vector design.** The sequence of the *rolC* gene and the dexamethasone-inducible promoter was obtained from transgenic plants previously created by colleagues (Mohajjel-Shoja et al., 2011). PCR was carried out in a volume of 20 μL using DreamTaq PCR master mix (Thermo Scientific) according to the prescription into a Tertsik amplifier (DNA-technology) by the following program: 95 °C – 5 minutes, 40 cycles (95 °C – 20 sec, 60 °C – 30 sec, 72 °C – 90 sec), 72 °C – 5 minutes. To obtain the sequence "promoter + *rolC*", the following primers were used, DexF: CGCTACTCTCCCAAAACCAA, DexR: GGCCAGTGAATTCTCGACTC. Primers were synthesized by Evrogen. The resulting sequence was placed into the pENTR/D-TOPO cloning vector (https://www.addgene.org/vector-database/2519/), which was used to transform *E. coli* Top10 strain. Bacteria grew on LB medium with kanamycin (100 mg/L) at 37 °C for 14 hours. Isolation of plasmid DNA from the resulting colonies was carried out using a Plasmid Miniprep Kit (Evrogen). PCR with primers DexF and DexR was performed to detect the insertion of *rolC* with an inducible promoter. The insert was then cloned into the destination vector pB7WG2D (https://gatewayvectors.vib.be/collection/pb7wg2d) using the Gateway system (Invitrogen, USA). The resulting plasmids were tested by PCR with primers DexF and DexR. After plasmid verification, the *Agrobacterium* EHA105 strain was transformed with pB7WG2D_PdexA4rolC.

The pHSE401_roC vector was created using the pHSE401 plasmid (https://www.addgene.org/62201/), according to the protocol described by Xing (Xing et al., 2014). The pHSE401 vector was kindly provided by the senior researcher of the Department of Genetics and Biotechnology, St. Petersburg State University, Tvorogova V.E. The *NgrolC* gene (Acs. X03432.1; 145–687) was chosen as a target. The selection of 19-nt target sequences and the final verification of the vector by PCR and restriction methods were carried out according to the protocols described by Xing (Xing et al., 2014). After verification, pHSE401_roC was used to transform the *Agrobacterium* strain AGL1.

**Plants transformation.** For plant transformation, overnight cultures of agrobacteria were prepared. Young leaves (3–4 upper leaves) were selected from aseptic plants in laminar box. Along the perimeter of the leaf blade, incisions 2–3 mm long were made with a sterile scalpel. The cuts crossed the leaf vein. The leaves were then placed in a mixture of liquid Murashige–Skoog medium (MS without agar) and overnight bacteria culture in a ratio of 1:1 for 2 hours. At the end of cultivation, the liquid from the leaves surfaces was removed with sterile filter paper, and the leaves were transferred to a solid MS medium.

**Plants regeneration.** Plates with leaf explants were kept for 2 days at 23 °C in the dark. Then the explants were transferred to MS medium containing 250 mg/L of cefotaxime, 2 mg/L of 6-benzylaminopurine (BAP), and 1 mg/L of naphthylacetic acid (NAA). Every 8–10 days the explants were transplanted onto a fresh medium containing hormones and an antibiotic. After the formation of organogenic calli and the initiation of shoot formation (4–6 weeks from the moment of transformation), the calli were placed on a hormone-free MS medium containing an antibiotic. The grown shoots were separated from the calli and placed on the MS medium with a mixture of antibiotics: 50 mg/L of cefotaxime, 10 mg/L of a selective antibiotic. Cefotaxime was used to kill agrobacteria. A selective antibiotic was used to select transformants.

Hygromycin was used for *N. glauca* because pHSE401_roC contains the *HygR* gene providing hygromycin resistance. For *N. sylvestris*, glufosinate was used as a selective antibiotic, since the pB7WG2D_PdexA4rolC vector contains the *BAR* gene.

**Transformants analysis.** Those shoots that remained green on the selecting medium were checked by PCR for the presence of a transgenic insert. DNA was isolated using the CTAB method (Murray, Thompson, 1980). For *N. glauca*, PCR analysis was carried out with primers pHSE401RolCF (5′TGTCCCAGGATTAGAATGATTAGGC) and pHSE401RolCR (5′AGCCCTCTTCTTTCGATCCATCAAC) to a CRISPR cassette. PCR was performed in a volume of 20 μL using DreamTaq PCR master mix (Thermo Scientific) according to the prescription in the Tertsik amplifier (DNA-technology) by the following program: 95 °C – 5 minutes, 40 cycles (95 °C – 10 sec, 58 °C – 30 sec, 72 °C – 30 sec), 72 °C – 5 minutes. The amplicons were visualized and separated on a 1 % agarose gel in TAE buffer.

Validation for *N. sylvestris* was performed by real-time PCR with primers for the *BAR* gene contained in the vector (BarF: AGCCCGATGACAGCGACCAC; BarR: CGCCGATGACGCGGGACAA). The DNA of the transgenic *N. tabacum* plant containing the *rolC* gene was used as a positive control. A sample without DNA was used as a negative control. PCR was performed in a volume of 20 μL using Fast SYBR Green master mix (Thermo Scientific) according to the prescription in ANK-32-M amplifier (Synthol) by the following program: 95 °C – 5 minutes, 40 cycles (95 °C – 10 sec, 58 °C – 30 sec, 72 °C – 30 sec), 72 °C – 5 minutes. Primers were synthesized by Evrogen.

## Results

Transformation and regeneration protocols for *N. sylvestris* and *N. glauca* were developed by optimization of existing protocols for different *Nicotiana* species. We conducted a preliminary experiment to evaluate the regeneration efficiency of *N. sylvestris* and *N. glauca* explants. The explants were put on media with different hormone ratios: 2 mg/L BAP and 1 mg/L NAA, 1 mg/L BAP and 1 mg/L NAA, 0.1 mg/L BAP and 1 mg/L NAA. At first, all explants actively formed

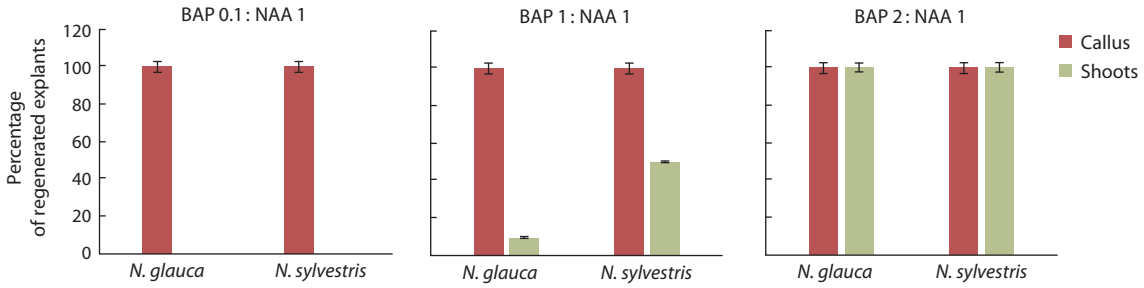


**Fig. 1.** Results of a preliminary experiment on the regeneration of *N. sylvestris* and *N. glauca* leaf explants.

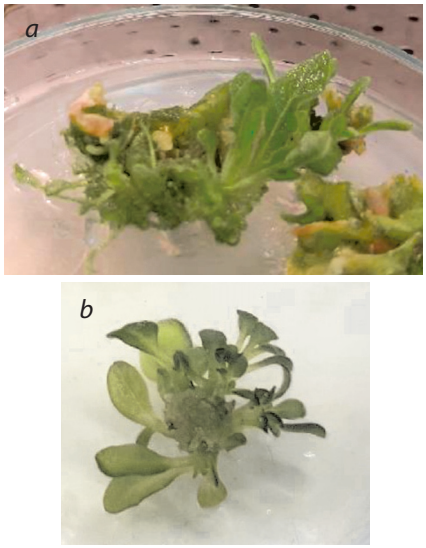


**Fig. 2.** Regeneration of shoots from leaf explants after co-cultivation with agrobacteria, *N. sylvestris* (*a*) and *N. glauca* (*b*).

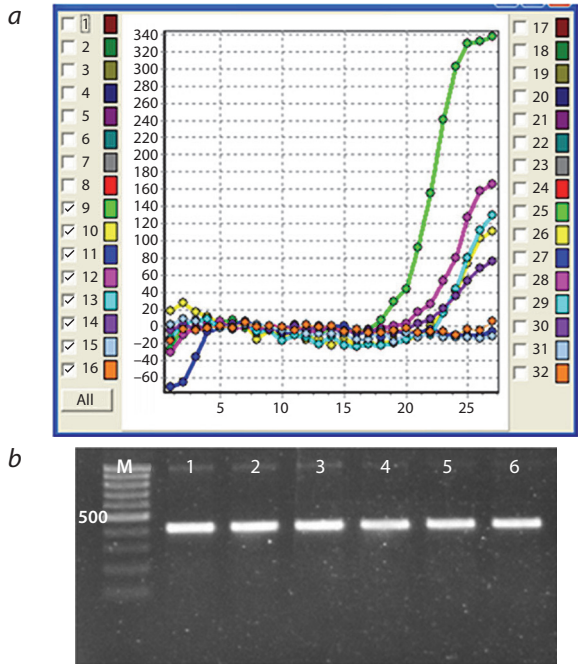


**Fig. 3.** Confirmation of transgenic inserts in regenerated plants:

*a*, *N. sylvestris* (9 – positive control, 10–15 – regenerated plants, 16 – negative control); *b*, *N. glauca* (1–6 – regenerated plants).

callus, no differences were observed on various media at this stage. However, upon transition to organogenesis, the amount of cytokines began to affect the efficiency of regeneration (Fig. 1). While 100 % of explants of both *N. sylvestris* and *N. glauca* formed shoots on a medium with a high content of cytokinins, only 50 % of explants of *N. sylvestris* and 10 % of *N. glauca* switched to organogenesis on 1 mg/L BAP and 1 mg/L NAA medium. Shoots did not develop on the medium 0.1 mg/L BAP and 1 mg/L NAA.

Thus, in a preliminary experiment, we noted active regeneration and shoot formation processes on leaf explants when 2 mg/L BAP and 1 mg/L NAA were added to the medium. For comparison, the traditional medium for the induction of callus formation in *N. tabacum* contains 0.5 mg/L BAP and 2 mg/L NAA (Draper et al., 1991), and the medium for *N. benthamiana* contains 1 mg/L BAP and 0.1 mg/L NAA (Hasan et al., 2014). Analyzing the literature, we also noticed different ways of preparing explants: in the classic version of "leaf disk transformation" cut out fragments of the leaf blade that do not contain veins are used (Wang, 2015). There are also options for cutting the leaf blade into pieces (Draper et al., 1991) and the deep vein incision method. We used the cutting method and the deep vein incision method in the preliminary experiment. The way of the explant preparation did not affect the efficiency of transformation.

Using the developed protocols we performed the transformation of *N. sylvestris* and *N. glauca* leaf explants, 500 for each species. As a result, regenerated plants were obtained from 498 *N. sylvestris* explants and 491 *N. glauca* explants (Fig. 2). Several explants (2 and 9, respectively) were contaminated during transplantation and removed from the experiment. Thus, the results obtained on a large sample are consistent with the results of the preliminary experiment.

The shoots formed on the medium with BAP and NAA were transplanted onto the medium with a selective antibiotic. For *N. sylvestris*, 15 plants were obtained on the medium with glufosinate, and 12 plants for *N. glauca* on the medium with hygromycin, which is 3 and 2.4 % of the regenerated explants. Plants that remained green and rooted on the selective medium were tested for the presence of the transgenic insert. Positive results were obtained for all *N. glauca* regenerants and 12 *N. sylvestris* regenerants (Fig. 3). For *N. sylvestris*, real-time PCR was used to detect a signal in transgenic plants, indicating the presence of the *BAR* gene contained in the vector (see Fig. 3, *a*). In the case of *N. glauca*, 423 bp sequences corresponding to the fragment of the CRISPR cassette were obtained (see Fig. 3, *b*).

Therefore, *N. sylvestris* plants with the *rolC* gene under a dexamethasone-inducible promoter were created, as well as *N. glauca* plants carrying a CRISPR cassette for "turning off" the *rolC* gene. Currently, some plants are planted in a greenhouse and some are grown *in vitro*. Next, they will be used to study the function of the *rolC* gene by controlled activation of its expression in *N. sylvestris* and by gene silencing in *N. glauca*.

Agrobacterium-mediated transformation and regeneration protocols for *N. sylvestris* and *N. glauca* were developed in this study. We have shown transformation efficiencies of 3 and 2.4 % for these species, respectively. The efficiency can be increased by adding acetosyringone to the medium. However, the described protocol is sufficient to obtain transgenic *N. sylvestris* and *N. glauca* plants, which was successfully demonstrated in this work.

## Discussion

Agrobacterium-mediated transformation is the most common way to obtain transgenic plants today. For many plant species, protocols for transformation and regeneration have been developed (Wang, 2015). While some species regenerate easily through the stages of organogenic callus and shoot development, other species show low regeneration efficiency. For example, in *Pisum sativum*, less than half of the somatic embryos develop into plants (Loiseau et al., 1995).

Many *Nicotiana* species regenerate easily and can also be grouped according to their tendency to shoot formation or root formation when regenerating (Matveeva, Sokornova, 2017). *N. tabacum* actively forms both roots and shoots. At the same time its ability to form callus on media with different ratios of hormones is of particular interest. There are protocolos describing *N. tabacum* callus formation on both auxin-dominated media (Draper et al., 1991; Ali et al., 2007) and cytokinin-dominated media (Horsch et al., 1985; Otten, Helfer, 2001). Researchers note the active formation of calli on explants, regardless of the protocol chosen. A similar picture is shown for *N. rustica*, which equally forms both roots and shoots during regeneration (Gill et al., 1979; Furze et al., 1987; Tinland et al., 1992). For other *Nicotiana* species, this feature has not been noted in the literature.

Cellular T-DNA was named as a possible explanation of the increased ability to regenerate, since T-DNA contains genes that affect the plant hormonal balance (Ichikawa et al., 1990). At the same time, those genes, which are called plast genes, differ in their effects (Otten, 2018). Therefore, it is important which plast genes carry cT-DNA and whether their reading frames remain intact. The *N. tabacum* genome holds three cT-DNAs of different composition, containing plast genes with intact frames (Chen et al., 2014). However, the *N. rustica* genome lacks cT-DNA (Intrieri, Buiatti, 2001). Our results also refute this hypothesis. Likewise, we did not confirm the assumption about the increased sensitivity of natural transgenic species to agrotransformation. The transformation efficiency turned out to be about the same for *N. glauca* containing cT-DNA and for *N. sylvestris* that does not contain cT-DNA in the genome. A similar trend was noted at the stages of callusogenesis and subsequent organogenesis. While *N. glauca* contains an intact *rolC* gene, which affects the balance of cytokinins in the plant (Schmulling et al., 1988), and *N. sylvestris* does not contain cT-DNA (Intrieri, Buiatti, 2001), regeneration in these species is triggered by the same ratio of hormones.

At the same time, differences in the efficiency of regeneration were noted at the intraspecific level. For example, *N. tabacum* cultivar SPTG-172 regenerates better on a medium containing 0.2 mg/L BAP and 2 mg/L NAA, while for cultivar K-399, the ratio of 0.2 mg/L BAP and 1 mg/L NAA is preferable. But even on a more suitable medium, K-399 forms less callus and shoots than SPTG-172 (Ali et al., 2007). Ali and colleagues explain such differences by the genotype influence. The effect of genotype on callusogenesis and organogenesis has already been shown for peas (Lutova et al., 1994; Saschenko, 2014) and cruciferous plants (Ockendon, Sutherland, 1987; Narasimhulu, Chopra, 1988). Particular qualities of the genotype, which determine the response to the medium and cultivation conditions, are often called the main factor that affect the regeneration efficiency (Pang et al., 2000). Ali and colleagues also noted that auxin-rich media are preferred for some tobacco cultivars, while cytokinin-rich media are preferable for others (Ali et al., 2007). In order to confirm or refute this hypothesis, it is necessary to conduct a study on a larger number of *N. tabacum* cultivars.

Despite a significant number of developed protocols for regeneration and studies on this topic (Wang, 2015), the genetic mechanism responsible for morphogenetic reactions remains unknown. In an attempt to establish it, geneticists and biochemists are actively studying both the biosynthesis pathways of plant hormones and their signaling, as well as the mutual influence of various hormones on regeneration processes in different plant species (Su, Zhang, 2014). More and more specific points of hormones interaction are being identified. For example, it has been shown that a participant in the auxin signaling pathway ARF3 (AUXIN RESPONSE FACTOR3) directly suppresses cytokinin biosynthesis during shoot regeneration by binding the *AtIPT5* gene promoter (Cheng Z.J. et al., 2013). However, the questions of why some plants regenerate more easily than the others, and what factors directly affect these processes, have yet to be answered.

The techniques we have developed for *N. glauca* and *N. sylvestris* expand our knowledge of the regeneration conditions of various *Nicotiana* species. In the case of *N. glauca*, this technique can contribute to the development of the pharmaceutical industry, since it allows to create various mutants for studying the biosynthesis of secondary metabolites. Both the transgenic *N. glauca* and *N. sylvestris* created in this work will advance fundamental studies of horizontal gene transfer. Despite the fact that the species *N. glauca* and *N. sylvestris* are not closely related (Clarkson et al., 2004), the same ratio of hormones triggers the induction of callusogenesis and regeneration. In this regard, the proposed technique can become a starting point for the development of new protocols based on it, as was described in this article.

## Conclusion

A technique for agrobacterium-mediated transformation and effective regeneration of *N. glauca* and *N. sylvestris* species has been developed. On its basis, it is possible to create protocols for other *Nicotiana* species by varying the ratio of the main exogenous plant hormones added to the medium. The

technique includes agrotransformation of plant explants using the leaf disk method, followed by cultivation on MS nutrient medium containing antibiotics as well as plant hormones in the amount of 2 mg/L BAP and 1 mg/L NAA. The choice of antibiotic is determined by the resistance genes in the vector used for the transformation. On this medium, leaf explants of *N. glauca* and *N. sylvestris* actively regenerate with the formation of first callus mass, and then with the development of shoots. The designed protocol will be useful both for fundamental research involving *N. glauca* and *N. sylvestris* species and in practical areas. For example, to study the phenomenon of horizontal gene transfer and related changes in the plant genome, as well as in the researches related to the biosynthesis of various metabolites, a wide range of which is synthesized in *N. glauca*.

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# The use of maize haploidy inducers as a tool in agricultural plant biotechnology

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**Abstract.** The discovery of the ability of some mutations to stimulate haploidy during hybridization made it possible to create one of the most promising and sought-after trends in the field of reproductive biology. Haploid inducers created on their basis are capable of increasing the frequency of haploidy up to 15 %. The improvement of the existing haploid inducer lines and the search for new genes that contribute to a high frequency of haploidy are underway. Along with these studies, the field of application of haploid inducers in genetics and plant breeding is expanding. Haploid inducers carrying *R1-nj* genes for anthocyanin pigmentation of the seed and embryo are able not only to mark the hybrid embryo and identify haploid genotypes, but also to detect genes that suppress the anthocyanin color of the grain, like *C1-I*, *C2-Idf*, and *In1-D*. Depending on their quantity, the phenotypic manifestation of the gene in the seed varies. Haploidy is widely used for accelerating hybrid breeding and obtaining both new maize lines with improved traits and their sterile counterparts. By introducing certain genes into the genome of the improved line, breeders can use the doubled haploid (DH) breeding technology to accelerate the creation of pure lines carrying the desired gene. Haploid inducer maize lines and their tetraploid analogs are used in the selection of rediploid maize lines by their resynthesis from tetraploid genotypes. In 2019, Syngenta Company synthesized a haploid inducer maize line carrying a CRISPR/cas construct capable of simultaneously stimulating haploidy and editing the genome at a specified DNA site. Thanks to this technology, it became possible to improve haploid inducers by introducing various CRISPR/cas constructs into the haploid inducer genome for editing any DNA site. Maize haploid inducers are widely used in doubled haploid wheat breeding. The first experiments showed that the most effective haploid inducer for stimulating haploidy in wheat is maize pollen. Researchers are intensively searching for other ways of using maize haploid inducers in plant breeding.
Key words: maize; haploidy; haploid inducer; haploid; doubled haploid; tetraploid; rediploid.

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# Использование гаплоиндукторов кукурузы как инструмента в биотехнологии сельскохозяйственных растений

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**Аннотация.** Использование гаплоиндукторов в гибридной селекции растений является одним из перспективных и востребованных направлений в области репродуктивной биологии. Продолжается совершенствование уже существующих линий-гаплоиндукторов и поиск новых генов, способствующих повышению частоты гаплоидии. Наравне с этими исследованиями расширяется область применения гаплоиндукторов в генетике и селекции растений. Гаплоиндукторы, несущие гены *R1-nj*, которые маркируют антоциановую окраску верхушки зерновки и зародыш, используются не только для выявления гибридных зародышей (окрашенный зародыш) и гаплоидных генотипов (неокрашенный зародыш), но и для обнаружения генов, репрессирующих антоциановую окраску зерна, таких как как *C1-I*, *C2-Idf*, *In1-D*. В зависимости от количества генов изменяется их фенотипическое проявление в зерновке. Гаплоидия широко применяется для ускорения гибридной селекции и получения новых линий кукурузы с улучшенными признаками и их стерильных аналогов. Вводя те или иные гены в геном улучшаемой линии, селекционеры могут ускорить создание чистых линий, несущих нужный ген, методом дигаплоидной (DH) селекции. Гаплоиндукторные линии кукурузы и их тетраплоидные аналоги используются в селекции редиплоидных линий кукурузы методом ресинтеза из тетраплоидных генотипов. Фирма Syngenta в 2019 г. синтезировала гаплоиндукторную линию кукурузы, несущую в спермиях пыльцевого зерна конструкцию CRISPR/cas, которая

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способна к одновременному стимулированию гаплоидии и редактированию генома на заданном участке ДНК. Благодаря этой технологии стало возможным совершенствование линий гаплоиндукторов кукурузы с помощью введения различных конструкций CRISPR/cas в ее геном для редактирования на любом участке ДНК. Гаплоиндукторы кукурузы широко применяются в селекции дигаплоидной пшеницы. Первые опыты показали, что наиболее эффективным гаплоиндуктором для стимулирования гаплоидии на пшенице является пыльца кукурузы. Исследователи ведут интенсивный поиск других возможностей использования гаплоиндукторов кукурузы в селекции растений. В данном обзоре рассмотрено современное состояние в технологии гаплоиндукции у растений.
Ключевые слова: кукуруза; гаплоидия; гаплоиндуктор; гаплоид; дигаплоид; тетраплоид; редиплоид.

## Introduction

The creation of inbred lines is the main goal of hybrid maize breeding. As a result of long-term self-pollination, breeders obtain homozygous lines for using them as parents in hybrid combinations. This part of the breeding work is the longest and most labor-consuming. Doubled haploid (DH) maize lines are usually created *in vivo* by crossing with haploid inducers that are able to stimulate the formation of haploid embryos in a fraction of the seeds of the mother plant, which serves as a donor of genetic material for the future doubled haploid line. The obtained haploid plants are subjected to the chromosome number doubling to diploid, as a result of which homozygous doubled haploid seeds are formed in the cob. This method made it possible to quickly obtain completely homozygous lines, which, in turn, significantly reduced the time and means spent by breeders on creating a hybrid combination.

The existing haploid inducer lines are characterized by a low frequency of haploid induction and require additional research to improve this trait. The research on improving the frequency of haploid induction in haploid inducer lines has caused interest in the mechanism of this phenomenon and the study of its genetics. The area of haploid inducers and haploidy application has stepped far beyond the limits of only obtaining doubled haploid lines; new technologies in genetics and plant breeding are being elaborated on this basis.

The present paper discusses various applications of maize haploid inducers and haploid induction, which are important for research in the field of basic and applied genetics of maize and other crops. Also, the paper explains their genetic basis, lists known genes and quantitative traits loci, and discusses different approaches to breeding for developing haploid inducer application technologies.

## Progress in the maize haploid inducer breeding

The rapid development of hybrid maize seed production since the middle of the 20th century has contributed to the development of breeding inbred lines with the maximum genome homozygosity. An effective method for improving the quality of seed production and for the accelerated production of inbred lines, as well as for breeding for economically valuable traits, to this day is, and remains, the use of haploid induction for the creation of doubled haploid lines. For a long time, inbred breeding has been the most common method of obtaining inbred lines. This method had many disadvantages, one of which is the impossibility of achieving complete homozygosity of all allele loci in an inbred line even after 10 inbreeding rounds. A high degree of homozygosity is necessary to achieve maximum uniformity of all traits of importance for breeding (uniformity of emergence, dates of flowering and ripening, of grain quality, etc.) and improvement to perfection of the seed production quality, the manufacturability of hybrid combinations obtained on its basis and, accordingly, improvement of the commercial products quality. A breeder spends 10 years to create lines with a fraction of homozygotes equal to 99.90 %, and up to 14 years of breeding work (inbreeding) to achieve the fraction of 99.99 %. For the resulting heterotic hybrids, it is very important to achieve a high degree of homozygosity, since it determines the uniformity of phenotypic traits, the synchronism in the onset of phenophases in plant development and maturation, which, in turn, affects their manufacturability in seed production and industrial production of marketable products.

The introduction of the first haploid inducers into breeding practice made it possible to obtain inbred maize lines with complete homozygosity for all gene loci in a short time. Thanks to making hybrid breeding a common breeding practice, the United States quickly occupied a leading position in the world in the production of hybrid maize in the middle of the 20th century. In addition to improving seed quality in seed production, haploid inducer breeding is widely used for creating inbred maize lines. The rapid production of pure doubled haploid maize lines within 2–3 years allows breeders to qualitatively evaluate more lines and genotypes in a shorter period of time than the previously used inbreeding method (Seitz, 2005; Ravi et al., 2011). Along with that, the possibilities of selecting valuable gene alleles and their rapid introduction into the genome of inbred genotypes have been broadened. The use of doubled haploid breeding technology allows breeders to use haploidy inducer (HI) lines each year to produce millions of recombinant plants based on DH lines derived from $F_1$ or $F_2$ generations. This makes it possible to avoid six or more generations of self-pollination, which are usually required to obtain inbred lines used not only in hybrid breeding, but also for genetic mapping, identification of trait introgression, and genetic studies of quantitative traits (Chang, Coe, 2009). In addition, the technology for obtaining DH lines broadens the possibilities for both improving the quality of selection during breeding and for more accurate phenotyping at different stages of inbreeding (Yan et al., 2017).

Haploid induction methods are widely used in many areas of breeding not only maize, but also other crops. The phenomenon of haploidy has been studied insufficiently

and raises many questions, which researchers have to answer – from understanding the mechanism of haploidy to exploring all the possibilities of its use in genetic and breeding research.

The first description of haploidy was made by Dorothy Bergner in 1922 on Jimson weed (*Datura stramonium*) (Blakeslee et al., 1922). Some time later, haploid genotypes were found in wheat (Gains, Aase, 1926) and tobacco (Clausen, Mann, 1924). Still later, haploidy was described in all major cultivated plants, i. e. wheat, rye, maize, rice, barley, sorghum, potato, tobacco, cotton, flax, beet, cabbage, cucurbits, cucumber, tomato, as well as in such forage grasses as bluegrass, brome grass, timothy, alfalfa, vetch, etc. (Kirillova, 1966). Haploids were also found in many other plants, but the frequency of haploidy remained insignificant (0.001–0.01 %) for its use in breeding.

Haploids can be produced experimentally by disrupting androgenesis or gynogenesis, the fertilization mechanisms of flowering plants (Asadova et al., 2020). The progeny obtained via androgenesis is characterized by haploid or doubled haploid germplasm and inherits only paternal traits (Astaurov, 1977; Darevsky et al., 1985; Golubovsky, 2000). An organism obtained by means of gynogenesis carries the haploid or doubled haploid material of the mother plant and inherits only the characteristics of the mother form (Astaurov, 1966; Strunnikov, 1998). An organism formed by apomixis carries the haploid or doubled haploid material of the mother plant and inherits only characteristics of the mother form (Takhtadzhyan, 1980; Batygina, 2000).

Serious progress in haploid induction technology was made only 40 years after the discovery of haploidy in plants by Guha and Maheshwari, who obtained haploids of Jimson weed (*Datura stramonium*) *in vitro* through anther culture (Guha, Maheshwari, 1964). A real breakthrough in the breeding of hybrids and their parental lines was made thanks to the creation of the first haploid inducers and doubled haploid maize lines, which were first obtained by S. Chase (Chase, 1949). In his studies, he established that the occurrence of haploid plants in crops of commercial inbred maize lines has a frequency of 0.1 % (Chase, 1947). He supposed that diploid pure lines can be obtained with the help of haploids; this suggestion could not but attract interest of breeders and geneticists.

When Chase published the results of his research, work began around the world on the search for maize lines capable of stimulating haploidy with a certain frequency and the development of haploid inducers with anthocyanin marker genes on their basis. The first haploid inducer of this kind, Stock 6, was obtained in 1959 by Ed Coe, and it had a haploid induction frequency of 2–3 %, which at that time was a sufficiently high value to start its introduction into breeding practice and for its commercial use in hybrid maize breeding (Coe, 1959). Subsequently, breeders managed to increase the haploidy frequency up to 7–15 % in newly created haploid inducers derived from Stock 6 by various breeding methods (Eder, Chalyk, 2002; Xu et al., 2013; Asadova et al., 2020). The first studies on maize haploid induction were started in the USSR by V.S. Tyrnov and A.N. Zavalishina at the Saratov University under the guidance of Prof. S.S. Khokhlov (Khokhlov et al., 1976; Tyrnov, Zavalishina, 1984). They created the first domestic haploid inducer maize lines based on the AT-1 line, which served as a source for the creation of most domestic haploid inducers. The number of effective haploid inducers used in the world for producing maize haploids varies within 50–60 lines, most of which were created on the basis of Stock 6 (Hu et al., 2016).

## Physiological mechanisms of haploid induction in maize

Much research has been devoted to exploring the mechanism of haploidy manifestation in plants, which showed that it is based on the phenomenon of parthenogenesis (Shishkinskaya, Yudakova, 2009; Yudakova, 2017), or gynogenesis, when seeds form an embryo that develops from an unfertilized egg, with a triploid endosperm, resulting from the fusion of the sperm with the central cell of the embryo sac (Takhtadzhyan, 1980; Batygina, 2000). The method of obtaining haploids based on the *in vitro* gynogenesis is used in the breeding of onion (*Allium cepa* L.) (Campion et al., 1992), sugar beet (*Beta vulgaris* L.) (Doctrinal et al., 1989), and some trees *in vivo* (Borodina, 1982). In onions (*A. cepa*), gynogenesis occurs in the culture of flower buds or ovaries (Keller, 1990). It should be noted that the temperature regime in which the donor plant grows before flowering, plays a decisive role for the induction of gynogenesis (Michalik et al., 2000).

Haploidy in maize under natural conditions can result from the disruption of one of the two sperms fusion with the egg, or their elimination after penetration into the embryo sac during fertilization (Tyrnov, Khokhlov, 1974, 1976). The mutations accumulated in the genome, which disrupt the fertilization process in maize, contribute to an increase in the frequency of haploidy both in own and in hybrid cobs during cross-pollination. A search for such mutations and identification of their sources are essential for creating haploid inducer maize lines (Khokhlov et al., 1976). It has been established that haploid embryos can form during distant hybridization (Kasha, Kao, 1970; Laurie, 1990).

The mechanisms of haploids appearance during fertilization of the embryo sac in flowering plants are still being elucidated, nevertheless in most cases, normal double fertilization occurs with the formation of a zygote and endosperm, but several subsequent mitotic divisions lead to the elimination of paternal chromosomes, both in the zygote and endosperm cells (Chaikam et al., 2019). To explain this process in the haploid inducer lines obtained from Stock 6, two hypotheses have been proposed, namely the single fertilization hypothesis and the chromosome exclusion hypothesis. The former was put forward first (Sprague, 1929, 1932). According to this hypothesis, one of the sperms cannot fuse with the egg, which triggers the development of the haploid embryo. Bylich and Chalyk found that about 6 % of the pollen in the haploid inducer ZMS (Zarodyshevy [Embryo] Marker Saratovskiy) line had sperms of different

size. The scientists supposed that one of the sperms successfully fertilized the endosperm or egg, while the other was incapable of fertilization due to its abnormality (Bylich, Chalyk, 1996). B. Chen et al. found that poorly developed eggs of immature embryo sacs do not develop a well-defined embryo despite normal endosperm formation, which suggests a single fertilization by only one of the two sperms (Chen B. et al., 2016; Tian et al., 2018).

According to the second hypothesis, which adheres to the theory of chromosome elimination, double fertilization occurs normally, but the haploid inducer chromosomes are eliminated during several zygotic divisions. This hypothesis is corroborated by several facts, one of which is the appearance of several micronuclei in some ovules that were fertilized with haploid inducer pollen followed by elimination of haploid inducer chromosomes (Wedzony et al., 2002; Fischer, 2004; Li L. et al., 2009). Later, X. Li et al. showed that chromosome elimination is directly related to the induction of the haploid genotype in the embryo, and the elimination process occurs gradually, so they proposed two models for the mechanism of haploid induction in maize according to the importance of chromosome elimination in both sperms (Li X. et al., 2017). Model I suggests the fusion of normal or slightly defective sperms with normal eggs to form diploid hybrid kernels in the cob. According to Model II, haploids can be formed only when the sperm fertilizes the egg and undergoes further elimination of chromosomes, while fertilization of the central cell by the same sperm leads to formation of abortive seeds.

## Genetic mechanisms of haploid induction in maize

An important step in the study of haploid induction was the determination of the localization of a group of genes promoting haploidy, which was carried out on the Stock 6 haploid inducer line. It was found that the most frequently used genes in modern maize haploid inducers are the ones located in the *qhir1*, *qhir11*, and *qhir12* regions of chromosome 1 (Hu et al., 2016). However, an assessment of the haploid induction frequency in genotypes containing the *qhir11* and *qhir12* subregions showed that only *qhir11* has a significant effect on haploid induction (Nair et al., 2017). In the *qhir11* region, the gene encoding phospholipase A has been identified as responsible for haploid induction by three independent research groups and named *MATRILINEAL* (*MTL*) (Kelliher et al., 2017), *NOT LIKE DAD* (*NLD*) (Gilles et al., 2017) and *ZmPLA1* (Liu C. et al., 2017). It was shown that the mutant gene *MTL*/*NLD*/*ZmPLA1* has a 4 bp insert in exon 4 (CGAG), which, in turn, has a haploid induction effect. It turned out that this insert shifts the reading frame, thereby changing the sequence of 20 nucleotides and forming a premature stop codon, which shortens the protein by 29 amino acids in comparison with the protein derived from the wild-type gene. This insert was present in all maize haploid inducer lines that originated from Stock 6 (Liu C. et al., 2017). It should be noted that this mutation was not found in teosinte, the ancestral form of maize, which suggests that this mutation appeared after maize domestication (Liu C. et al., 2017).

Another equally interesting discovery is that some gene editing and knockdown events increase the frequency of haploidy, thus indicating the possibility of creating an inducer with a higher haploid induction frequency by modifying the *MTL* gene. This gene encoding patatin-like phospholipase is expressed in maize pollen (Gilles et al., 2017; Kelliher et al., 2017; Liu C. et al., 2017). In turn, the altered phospholipase affects both the rate of pollen germination on maize stigmas and the growth of pollen tubes to the embryo sacs, significantly slowing down their germination (Kim et al., 2011), which explains the haploid inductive ability of maize (Xu et al., 2013; Kelliher et al., 2017). However, the mechanisms of haploidy based on single fertilization (Sarkar, Coe, 1966) or postzygotic genome elimination (Qiu et al., 2014; Li X. et al., 2017) still leave many questions unanswered and remain unclear.

Recent studies by Chinese scientists of the factors affecting the frequency of haploidy have characterized the membrane protein DUF679 (*ZmDMP*) in a line that is not related to the Stock 6 haploid inducer (Zhong et al., 2019). The researchers established that this gene is localized on chromosome 9 in the *qhir8* region. It was proven that a mutation in this gene in combination with a mutant haploid induction *MTL*/*ZmPLA1*/*NLD* gene (containing a 4 bp insert) significantly enhances haploid induction. At the same time, the authors noted that the *ZmDMP* gene mutation itself without interaction with *mlt*/*pla1*/*nld* does not cause an increase in the frequency of haploid induction. A wild-type *ZmDMP* knockout resulted in a haploid induction frequency of 0.1–0.3 % in the absence of a mutation in the *MTL* gene, while in combination with such a mutation it led to a 5–6-fold increase in the frequency of haploid induction. The results show that a mutation in the *MTL* gene is critical for the high frequency of haploids induction due to the effect of *ZmDMP*. The mechanism by which the *MTL* gene determines haploid induction and how the interaction of this gene with *ZmDMP* increases the haploid induction frequency remains to be elucidated.

## The use of maize haploid inducers for marking haploid seeds in genotypes with colored and non-colored seeds

In 1949, S. Chase (Chase, 1949) proposed to introduce genetic markers for haploid inducer maize lines, the meaning of which was in the possibility to observe the phenotypic manifestation of dominant traits in hybrid offspring from such crosses, and of recessive ones in matroclinic haploids (Gutorova et al., 2016). The use of dominant markers in haploid inducer maize lines greatly simplifies the work of culling hybrid diploid seeds and improves the quality of selection of haploid ones, which lack these markers. Thanks to the haploid inducers labeling method proposed by Chase, a variety of created haploid inducers has significantly increased the effectiveness of this technology (Nanda, Chase, 1966; Greenblatt, Bock, 1967; Chase, 1969).

Most of the phenotypic markers used to distinguish haploid seeds from diploid ones are represented by dominant genes with the effects of anthocyanin (purple) markers on various parts of the seed and plant. In the most popular haploid inducer maize lines, the visual differentiation of haploid and diploid seeds is based on the marker of purple color of the embryo, which is encoded by the *R1 – navajo* gene (*R1-nj*), and the absence of a dominant allele blocks the synthesis of anthocyanins in the aleurone layer of the seed (Ford, 2000). It should be noted that the expression of the *R1-nj* anthocyanin marker in hybrid maize seeds can vary significantly depending on the genotype of the maternal line used in the hybrid combination, the genotype of the haploid inducer itself, and environmental factors (Chase, 1952; Röber et al., 2005; Kebede et al., 2011; Prigge et al., 2011).

For a more reliable selection of haploid genotypes, other genes controlling anthocyanin biosynthesis (purple color) were used in addition to the *R1-nj* gene; these are *A1*, *A2*, *C2*, *Bz1*, *Bz2*, and another regulatory gene *C1*, the expression of which was based on the inhibition of anthocyanin synthesis in homozygotes of recessive alleles of any of these genes, i. e. of *A1*, *A2*, *C2*, *Bz1*, or *Bz2* (Chase, Nanda, 1965; Geiger, Gordillo, 2009). In addition, the dominant anthocyanin synthesis inhibiting genes *C1-I*, *C2-Idf*, and *In1-ID* can influence the purple seed color phenotype (Coe, 1994; Eder, Chalyk, 2002). The studies conducted at CIMMYT suggested that the expression of the purple seed pigmentation by the *R1-nj* gene is inhibited in 8 % of crosses of haploid inducers with various maize genotypes (Röber et al., 2005; Prasanna et al., 2012). The *Pl1* (*Purple1*) gene causes the light-dependent production of anthocyanins in roots. This effect provides an additional way to distinguishing between haploid and diploid genotypes. If the anthocyanin synthesis inhibitor genes (*C1-I*, *C2-Idf*, and *In1-ID*) caused the incorrect classification of seeds in the *R1-nj* marker, the effect of the *Pl1* gene makes it possible to distinguish hybrid seeds by the presence of purple pigmentation in the primary (embryo) roots of the putative haploid maize seeds. However, the roots of seedlings of some genotypes can turn purple when exposed to light, which in such a case makes the classification based on *Pl1* erroneous. A combination of *Pl1* with the *B1* (*Booster1*) genes leads to the production of anthocyanins in the coleoptile of the seedling, leaf tip, and primary root. Plants homozygous for alleles of the *B1* and *Pl1* genes develop a dark purple color on the cob and stem envelope (Coe, 1994), but these markers are effective for genotypes that do not contain pigments in the aleurone layer or various parts of the recipient maize plant.

With the advent of the possibility of analyzing the chemical composition of grain by IR spectrometry, the possibilities of labeling haploids have expanded. In 2007, V. Rotarenco suggested using the oil content in the seed embryo as a marker of haploid seeds (Rotarenco et al., 2007, 2010). The analysis of the chemical composition of the embryo by NMR showed significant differences in the oil content in diploid and haploid seeds, which amounted to 5.26 and 3.42 %, respectively (Chen S., Song, 2003). Based on these results, L. Li et al. crossed a high-oil line with the Stock 6 line and used the resulting hybrid for creating the haploid inducer CAUHOI with a haploid induction frequency of up to 2 %, and an oil content in the seed of up to 7.8 % (Li L. et al., 2009). This haploid inducer makes it possible to significantly broaden the use of haploid inducer lines for obtaining haploids of various maize genotypes, characterized by the presence of purple color in various parts of the plant and seed, or inhibitors of the phenotypic manifestation of the *R1-nj* genes. Studies by Z. Liu et al. (2016) proved that the oil content in the seed embryo makes it possible to identify haploid genotypes with an accuracy of at least 90 % (Liu Z. et al., 2016).

## The use of maize haploid inducers for the resynthesis of maize polyploid genotypes

The creation of effective haploid inducers made it possible to shape a new direction in breeding, i. e. the creation of rediploid maize using the resynthesis of polyploid genotypes. This method was first proposed for maize by Shatskaya and Khatefov in 2009. The authors of this method proposed two ways for breeding rediploid maize lines (Khatefov, Shatskaya, 2009). The first option suggested resynthesis from hybrid triploid seeds without the use of a haploid inducer. It was a very cumbersome and laborious technique, which did not find wide application in breeding. The second method proposed the use of diploid and tetraploid analogs of haploid inducer lines in a scheme of crossing with tetraploid sources. This method was much simpler and more convenient to use, provided that a breeder had a tetraploid analog of the haploid inducer line. The use of the method of rediploidization of tetraploid genotypes made it possible to create a series of rediploid maize lines from the tetraploid synthetic population MRPP20.

The further testing of the breeding value of the collection of rediploid lines obtained by the resynthesis from tetraploid populations showed their high effectiveness in hybrid maize breeding (Khatefov, Matveeva, 2018; Khatefov et al., 2019, 2021). The use of this method for the resynthesis of natural tetraploid genotypes and populations of cultivated and wild maize relatives opens up great prospects for broadening the genetic polymorphism of the initial breeding material and maize breeding improvement.

## The use of maize haploid inducers for creating sterile analogs of CMS maintainer lines

Along with the spread of the method for creating doubled haploid lines for hybrid maize breeding, the problem of creating their sterile analogs has appeared over time. This is a complex problem to solve, because a sterile analogue is created from the sterile (S) cytoplasm, which is the maternal form in the hybrid maize breeding. It is obtained through a series of backcrosses of the nuclear material source of normal (N) cytoplasm from a maintainer line serving as the paternal form. In this breeding scheme, it becomes impossible to use a haploid inducer line to obtain a sterile analog by the traditional way, when the haploid inducer serves as

the paternal form, since the doubled haploid analog obtained from sterile cytoplasm will be sterile and impossible to propagate after doubled haploidization.

A method for creating sterile analogs of maize lines through androgenesis using CMS-*ig* haploid inducers carrying the *ig1* mutation was found at the P.P. Lukyanenko National Grain Center (Kermicle, 1969, 1971, 1994). The method is based on the principle according to which the sperm nucleus replaces the egg nucleus. The proposed method stipulates the fusion of one of the sperms from the source of the *rfrf* genes with the egg of the haploid inducer line, which serves as the maternal form created on the basis of M- and S-type CMS. Similar to the haploid inducer lines used as a source of pollen, the haploid inducer maternal lines carry alleles of the *ig1* genes in combination with the *R1-nj*, *Pl1*, and *B1* genes. Maternal forms of haploid inducers, homozygous for the *ig1* mutation, exhibit a long phase of nuclear-free divisions, which ends with the formation of nuclear-free eggs (Evans, 2007). When hybridizing the paternal form, which carries homozygotes of alleles of the *rfrf* nuclear genes (maintainer) and a source of sterile cytoplasm (maternal form), a sterile analog of the paternal line with complete sterility is formed in seeds which set in the cobs of the maternal haploid inducer line (Chumak, 1977; Shatskaya, Shcherbak, 1999). To multiply the sterile analogue in future, only the paternal line, which served as a source of pollen for haploid induction, is used as a pollinator. Thanks to this method, the Maize Department of the P.P. Lukyanenko National Grain Center has been creating sterile analogs of maize lines for two years, it being much more efficient than the traditional method of saturating crosses, which takes 8 or more years of breeding work.

### *CENH3*-mediated haploid induction

In 2010, Ravi and Chan reported that a *CENH3* mutant gene can induce haploidy in crosses with wild-type *Arabidopsis thaliana* (Ravi, Chan, 2010). This *CENH3* mutant was created by replacing the endogenous N-terminal (N-ter) tail of *CENH3* with a fluorescent GFP protein fused with the N-ter tail of a normal histone 3.3, which partially complements the lethal phenotype of the *cenh3* null mutant. Pollination of this GFP-tailswap transgenic line with the wild-type pollen resulted in the induction of ~30 % haploid embryos with only male (wild-type) genome. GFP-tailswap-tagged female chromosomes are lost during early zygote divisions, creating a high frequency of paternal haploid embryos along with aneuploid (~30 %) and normal diploid embryos. In the future, this scheme of haploid induction can be applied to other important agricultural crops (Tek et al., 2015).

Attempts to edit the N-terminal (N-ter) tail of *CENH3* have been successful with maize (Tek et al., 2015), tomato, and rice (Kalinowska et al., 2019). Although the rate of natural haploid induction was relatively low (0.065–0.86 % in maize, 0.2–2.3 % in tomato, and 0.3–1.0 % in rice), these experiments demonstrated the feasibility of haploid induction by changing the N-terminal tail of *CENH3* in cultivated monocots. Sanei et al. (2011) found that *CENH3* of *H. bulbosum* is inactive and/or *CENH3* of *H. vulgare* could not be introduced into the paternal form in a classical barley cross (*H. vulgare* × *H. bulbosum*) because it leads to the formation of haploids with the maternal genome only. Studies by Zhao et al. (Zhao et al., 2013) reported that *CENH3* does not directly contribute to the ability to induce haploids. This finding is consistent with the QTL mapping analysis (Prigge, Melchinger, 2012). However, the authors suggest that *CENH3* may influence the elimination of chromosomes of the haploid inducer line during the formation of haploids in maize. The authors did not find any differences in the coding sequence and the level of mRNA expression between the inducer and non-inducer lines, while the regulatory levels of *CENH3* (splice variants, translation, modification, etc.) may differ. The results of the study showed that *CENH3* acts epigenetically in plants (Han et al., 2009; Ravi, Chan, 2010; Sanei et al., 2011). This new knowledge opens up broad prospects for researchers involved in the development of haploid inducer lines for agricultural crops for which it is difficult to create haploid inducers using classical breeding methods.

### The use of the CRISPR/cas system for generating edited dihaploid lines

Over the past decade, the use of the CRISPR/cas method in agriculture has expanded significantly. To obtain pure lines with an edited genome, various haploid inducers, including those of maize, are widely used, which allows scientists to accelerate the breeding process and create plants with the desired traits in a short time. In 2019, two groups of scientists from America and China led by Kelliher and Wang, respectively, independently of each other decided to combine the CRISPR/cas editing method and *in planta* haploid induction. They used two HI-Edit haploid inducers (Kelliher et al., 2019) and IMGE haploid inducer (Wang et al., 2019). They conducted studies on their transformation with *Agrobacter* carrying a vector that contained the CRISPR/cas sequence. The transformed haploid inducer lines were crossed with elite maize lines. When the hybrid seeds matured, the isolated haploid seeds were tested for the presence of mutations (transformation event markers) in the aimed (target) region of the gene. The studies intended to demonstrate that the CRISPR/cas tool can be expressed or present in the zygote before the elimination of the haploid inducer genome, and carry the transgene necessary for editing the non-transgenic haploid genome of the elite line. Further studies of the mechanisms of the CRISPR/cas system transfer through haploid inducer lines will serve for improving them and expanding the possibility of their use as an effective tool for editing the genome of maize and other agricultural crops. The fact that some maternal haploid plants were modified with the help of a haploid inducer suggests that the sperm fuses with the egg and delivers the CRISPR/cas system there, but the subsequent elimination of male chromosomes leads to the formation of a haploid plant with the edited genome (Kelliher et al., 2019) using the IMGE approach (Wang et al., 2019). The method developed

by the authors makes it possible to obtain doubled haploid lines with the genome edited regarding the target genes during one reproduction cycle.

It is also possible to obtain haploids through interspecific crossing with subsequent elimination of haploid inducer chromosomes. In barley breeding, the method of chromosomes selective elimination is widely used to obtain haploids when crossing *H. vulgare* L. × *H. bulbosum* L. (the 'bulbosum' method). For the first time this interspecific hybrid was obtained back in 1934. It was later established that the appearance of maternal-type haploid plants resulted not from parthenogenesis, but from the selective elimination of *H. bulbosum* chromosomes ($2n = 2x = 14$) in the alien cytoplasm of *H. vulgare* ($2n = 2x = 14$) within 9 days from the date of crossing (Gernand et al., 2006). As a result of this process, the wild-type chromosomes are eliminated and a haploid embryo is formed, while the endosperm remains underdeveloped. Therefore, 12–14 days after pollination the embryo should be transferred to the *in vitro* culture, and the resulting haploid regenerated plants should be colchicine-treated for double haploidization. The studies by A.P. Ermishin (Ermishin, Voronkova, 2015) described a method for obtaining dihaploids of cultivated potato *Solanum tuberosum* L. ($2n = 2x = 24$) by using a primitive cultivated potato species *S. phureja* ($2n = 2x = 24$) as a haploproducer. The method is based on the phenomenon of pseudogamy, in which both sperms of a pollen grain of a diploid species ($n = x = 12$) fuse with the central nucleus of the *S. tuberosum* embryo sac ($2n = 4x = 48$), which leads to the formation of a hexaploid endosperm. In this case, the unfertilized egg ($n = 2x = 24$) begins to differentiate; the forming viable seed combines a hexaploid endosperm with a diploid embryo (Ermishin, Voronkova, 2015).

A rather original use of the haploid induction method in breeding was found in the propagation of the dioecious asparagus (*Asparagus officinalis*) to increase the proportion of genotypes with low fiber content in the stem in the offspring. To this end, female (XX) and male (XY) genotypes were crossed, and the progeny showed a segregation of female fibrous and male low-fiber genotypes in the 1:1 ratio. Haploid induction in male genotypes followed by doubled haploidization of haploid genotypes results in doubled haploid super-male (YY) and female (XX) plants. The use of super-male genotypes as pollinators of female genotype makes it possible to obtain all-male (XY) plants in the $F_1$ progeny (Bhojwani, Razdan, 1996). Haploidy is also used in a similar way to obtain same-sex genotypes for papaya (*Carica papaya*), but with a difference that these are female plants that are of commercial value. In this case, the anther culture method is used to obtain doubled haploid lines, which greatly facilitates the production of female pure lines (Rimberia et al., 2006).

In the doubled haploid wheat (*Triticum aestivum* L.) breeding, pollen from other cereal crops, such as maize, sorghum, teosinte, ornamental millet, and wild barley *H. bulbosum*, is used as a pollen source, however the most effective haploid inducer for wheat and oats (*Avena sativa* L.) remains maize pollen (Laurie, Bennett, 1988; Dziurka et al., 2022). Cytological studies have shown that maize pollen successfully germinates on the stigma of wheat, reaches the embryo sac, in which the wheat egg is fertilized by sperm from the maize pollen grain, and a zygote containing 21 wheat chromosomes and 10 maize chromosomes is formed (Laurie, 1989). The resulting hybrid zygote is karyotypically unstable, since maize chromosomes do not get attached to the division spindle and are eliminated. This process provided a basis for the original method developed by Syngenta, which used it to edit the wheat genome. To this end, a maize line NP2222 was transformed with a vector expressing Cas9 and gRNA targeting putative wheat orthologs *GRASSY TILLER1 TaGT1-4A*, *TaGT1-4B*, and *TaGT1-4D*. As a result, two of the 292 CMS haploids were edited, and the sequencing of the target genes showed that the *JSWER30A22* gene had a 97 bp deletion in *TaGT1-4B* (Kelliher et al., 2019). This success evidences that the Hi-edit editing method can be applied in the future to wheat for simultaneously obtaining haploid embryos with an already edited genome, which, in turn, will significantly accelerate breeding for wheat improvement (Kelliher et al., 2019).

## Conclusion

The development of applied genetics and breeding technologies opens new areas of haploid induction technique application using maize haploid inducer lines. The range of objects for the study and practical application of haploid inducers is expanding, involving not only representatives of the genus *Zea*, which includes its own six species, but also interspecific crosses with representatives of the genera *Triticum* and *Avena*. The first haploid inducer lines served as sources for creating many more effective haploid inducer lines due to the inclusion of various mutations that enhance the haploid induction frequency and marker properties due to the introduction of genes for anthocyanin pigmentation of grain and plants, as well as genes that control the high content of some biochemical components in the embryo. The use of maize haploid inducers for expanding the genetic polymorphism of the initial breeding material has allowed researchers and breeders to develop new technologies and methods for obtaining doubled haploid lines not only from diploid varieties, populations and synthetics, but also from polyploid maize genotypes and its wild tetraploid relatives. This method significantly accelerated hybrid maize breeding by creating new haploid inducer lines from sterile cytoplasm of S- and M-type CMS to create sterile doubled haploid maize lines. The improvement of the CRISPR/cas method using haploid inducer lines opens up great prospects in editing the maize genome, when it becomes possible to combine two elements in one breeding operation, namely genome editing by CRISPR/cas and obtaining a completely homozygous doubled haploid line carrying these genetic changes. The research in the field of maize haploid inducer breeding has long overgrown the boundaries of application in hybrid breeding for obtaining doubled haploid lines, and every year witnesses an improvement and expansion of the area of application in applied genetics and breeding.

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