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Influence of different types of sterile cytoplasms (A3, A4, 9E) on the combining ability of CMS lines of sorghum

O.P. Kibalnik[1] ✉, L.A. Elkonin[2]

[1] Russian Research and Project-technological Institute of Sorghum and Maize, Saratov, Russia
[2] Agricultural Research Institute of the South-East Region of Russia, Saratov, Russia
✉ e-mail: kibalnik79@yandex.ru

Abstract. Investigation of the effect of the cytoplasm on the combining ability (CA) of lines with cytoplasmic male sterility (CMS) is of considerable interest in terms of understanding the genetic functions of the cytoplasm and for practical purposes to create hybrids with improved economically valuable traits. In order to investigate the effect of different types of sterile cytoplasm (A3, A4, 9E) on CA in sorghum, we studied the manifestation of a number of biological and agronomic traits in 54 F_1 hybrid combinations obtained using iso-nuclear CMS lines with the nuclear genome of the line Zheltozernoye 10, differing only in the types of sterile cytoplasm (A3, A4 and 9E). Eighteen varieties and lines of grain sorghum developed at the Russian Research and Project-technological Institute of Sorghum and Maize were used as paternal parents. The CA was determined by the topcross method. F_1 hybrids and their parents were grown in 2015–2017 in conditions of insufficient (2015–2016: HTC (hydro-thermal coefficient) = 0.32–0.66), or good water availability conditions (2017: HTC = 1.00). On average, for three years of testing, a positive effect of the 9E cytoplasm on the general combining ability (GCA) (0.63) and negative effects of the A3 and A4 cytoplasms (–0.32 and –0.31) for the inflorescence length were noted. In dry seasons, significant positive effects of the 9E cytoplasm on GCA for the length of the largest leaf, and positive effects of the A3 cytoplasm on GCA for the plant height, and negative effects of the A4 cytoplasm on GCA for these traits were observed. No differences were observed during the wet season. The type of CMS did not affect the GCA for the width of the largest leaf and grain yield. The dispersion of specific combining ability (SCA) in the dry seasons was significant for the following traits: leaf length, plant height, panicle length and width, and grain yield, the 9E cytoplasm had the highest SCA dispersion, whereas the A4 cytoplasm had the smallest one. The data obtained indicate that different types of sterile cytoplasm of sorghum make a different contribution to CA under conditions of drought stress.
Key words: *Sorghum bicolor* (L.) Moench; cytoplasmic male sterility; heterosis; combining ability; cytoplasmic effects; drought.

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Влияние разных типов стерильных цитоплазм (A3, A4, 9E) на комбинационную способность ЦМС-линий сорго

О.П. Кибальник[1] ✉, Л.А. Эльконин[2]

[1] Российский научно-исследовательский и проектно-технологический институт сорго и кукурузы, Саратов, Россия
[2] Научно-исследовательский институт сельского хозяйства Юго-Востока, Саратов, Россия
✉ e-mail: kibalnik79@yandex.ru

Аннотация. Изучение влияния цитоплазмы на комбинационную способность (КС) линий с цитоплазматической мужской стерильностью (ЦМС) представляет значительный интерес в плане понимания генетических функций цитоплазмы у растений и в практических целях для создания гибридов с улучшенными хозяйственно ценными признаками. С целью выяснения характера влияния разных типов стерильных цитоплазм (A3, A4, 9E) на КС у сорго исследовали проявление ряда агрономически ценных признаков у 54 гибридных комбинаций F_1, полученных с использованием в качестве материнских родителей изоядерных ЦМС-линий, созданных на основе линии Желтозерное 10 и различающихся только типами стерильных цитоплазм (A3, A4 и 9E). В качестве отцовских родителей были 18 сортов и линий зернового сорго селекции Российского НИИ сорго и кукурузы. Комбинационную способность определяли методом топкросса. Родительские компоненты и гибриды F_1 выращивали в 2015–2017 гг. в условиях недостаточной (2015–2016 гг.: гидротермический коэффициент (ГТК) = 0.32–0.66) либо хорошей (2017 г.: ГТК = 1.00) влагообеспеченности. В среднем за три года испытаний выявлены положительное влияние цитоплазмы 9E на общую комбинационную способность (ОКС) по длине соцветия (0.63) и отрицательные эффекты цитоплазм A3 и A4 (–0.32 и –0.31) на ОКС по этому признаку. В засушливые сезоны отмечены значимые положительные эффекты цитоплазмы 9E на ОКС по длине наибольшего листа, цитоплазмы A3 – на ОКС по высоте растений и отрицательное влияние цитоплазмы

А4 на эти признаки. Во влажный сезон различия отсутствовали. Тип ЦМС не оказывал влияния на ОКС по ширине наибольшего листа и урожайности зерна. Дисперсия специфической комбинационной способности (СКС) в засушливые сезоны оказалась значимой для следующих признаков: длина листа, высота растений, длина и ширина метелки, урожайность. При этом линия с цитоплазмой 9E отличалась наиболее высокими показателями дисперсии СКС, тогда как линия с цитоплазмой А4 – наименьшими. Полученные данные свидетельствуют, что разные типы стерильных цитоплазм сорго вносят различный вклад в КС в условиях засухи.
Ключевые слова: *Sorghum bicolor* (L.) Moench; цитоплазматическая мужская стерильность; гетерозис; комбинационная способность; цитоплазматические эффекты; засуха.

Introduction

The cytoplasm as the environment for the functioning of the nuclear genes plays an important role in the genetic control of many plant traits. Along with the well-known, and in some cases well-studied mutations of variegation and cytoplasmic male sterility (CMS) that arise as a result of rearrangements in the chloroplast and mitochondrial genomes, there are many examples of the influence of the cytoplasmic environment on the manifestation of many plant traits, including those with important biological and economic value. This effect of the cytoplasm may be caused by retrograde regulation of nuclear gene expression by signals produced by cytoplasmic organelles under the influence of environmental factors (Fujii, Toriyama, 2008). The genetically different plastomes and mitochondrioms can respond differently to environmental signals and affect the expression of nuclear genes. In addition, the cytoplasm is capable of causing inherited changes in the nuclear genome by paramutations (Zavalishina, Tyrnov, 2003, 2010), and changing the methylation of nuclear gene sequences (Xu et al., 2013; Ba et al., 2014) including nucleotide sequences of mobile genetic elements (Elkonin et al., 2018), that can alter the expression level of nuclear genes and have significant genetic effects, since alteration of transposon methylation is one of the key factors of their mobility and, as a consequence, the occurrence of mutations (Yaakov, Kashkush, 2011).

Majority of agronomically valuable plant traits are polygenic and are formed as a result of the interaction of many nuclear genes among themselves and with environmental factors. In this regard, the cytoplasm can have a significant impact on the manifestation of these traits. There is a lot of data in the literature confirming the effect of the cytoplasm on agronomically valuable traits in wheat (Atienza et al., 2007), rice (Tao et al., 2011), cotton (Tuteja, Banga, 2011), pearl millet (Amiribehzadi et al., 2012), winter rye (Urban, Gordey, 2013), sorghum (Aruna et al., 2013), sunflower (Jan et al., 2014), maize (Kabanova et al., 2015), and mustard (Chakrabarty et al., 2015). Assuming that the manifestation of heterosis in F_1 hybrids is determined, in considerable extent, by the combining ability (CA) of maternal lines, investigation of the effect of cytoplasm on CA is of significant interest. However, there are few studies on the effect of cytoplasm on CA. In pearl millet, the A4 and A5 cytoplasms caused positive effect on grain yield in comparison with A1 cytoplasm (Chandra-Shekara et al., 2007; Pujiar et al., 2019). Tests of new CMS sources of sunflower (XA, E002-91A, PKU-2A, ARG-2A, ARG-3A, ARG-6A, DV-10A, PHIR-27A, PRUN-29A) showed a positive effect of sterile cytoplasms E002-91A (*Helianthus annuus*), ARG-3A (*H. argophyllus*) and ARG-6A (*H. argophyllus*) on the combining ability of maternal lines in seed productivity compared to normal cytoplasm NC-41B (Tyagi, Dhillon, 2016). A similar effect of A4 and A8 cytoplasms on the overall combining ability of lines has been described in rice (Young, Virmani, 1990).

In sorghum, there are contradictory data in the literature. The positive effect of A2 cytoplasm on the general combining ability (GCA) of CMS lines for the duration of the seedling-flowering interphase period, grain yield, grain weight per panicle and 100 grains, in comparison with A1 cytoplasm, has been described (Kishan, Borikar, 1989; Ramesh et al., 2006; Reddy et al., 2007, 2009). On the contrary, the lack of effects of A1 and A2 cytoplasms on heterosis was reported (Williams-Alanís, Rodríguez-Herrera, 1994).

The aim of this work was to study the effect of different sterile cytoplasms (A3, A4, 9E) on CA in sorghum using isonuclear CMS lines that differ only in types of sterile cytoplasm.

Materials and methods

To identify cytoplasmic effects on combining ability, we used the early maturing alloplasmic iso-nuclear CMS lines of grain sorghum (*Sorghum bicolor* (L.) Moench) (Elkonin et al., 1997). These lines were obtained by consecutive backcrosses of fertile line Zheltozernoye 10 (Z10) to CMS lines A3 Tx398, A4 Tx398, 9E Tx398 (provided by Dr. K.F. Schertz, Texas Agricultural Experimental Station, USA), carrying cytoplasms of the following accessions: IS1112C (A3), IS7920C (A4), and IS17218 (9E). In this study, maternal plants from the BC_{18} were used. As a pollen parents, early maturing varieties – Perspectivnoye 1, Mercury, Ogonek, Avans, Fakel, Azart, Garant, Topaz, Volzhskoye 615, and mid-early maturing varieties and lines – Start, L-KSI 28/13, Kamelik, Geleofor, Kremovoye, Pishchevoye 614, Sarmat, Vostorg, Pishchevoye 35 were used (18 in total). These pollen parents differed in manifestation of agronomically valuable traits and characterized by high adaptive ability to agro-climatic conditions of the region (Kibalnik et al., 2010, 2017). F_1 hybrids obtained using these pollinators were characterized by mid-early maturity (110–117 days to full maturity).

Pollen parents were grown under strict isolation (the panicles were isolated with parchment bags before flowering) for 8–25 generations. All pollinators were sterility maintainers for the studied types of CMS, with the exception of Perspectivnoye 1 and L-KSI 28/13, which are the restorers of fertility for A4 and 9E CMS and provided 80–100 % seed set in conditions of strict isolation with parchment bags (Kibalnik, Semin, 2018).

The following traits were analyzed: plant height; the length and width of the largest leaf, the length and width of the inflorescence, mass and number of grains per panicle, and grain yield. Since paternal parents were not universal fertility

restorers, and the majority of the studied hybrids were male sterile, in order to register the traits associated with grain productivity, the open-pollinated panicles were used. As far as F_1 hybrids were grown in experimental field among hundreds of thousands of fertile plants, free pollination ensured 100 % seed setting all panicles of the studied hybrids. This approach has already been used to study the grain yield of hybrids in A3 cytoplasm (Moran, Rooney, 2003).

F_1 hybrids (54 in total) were sown in the experimental field of the Russian Research and Project-technological Institute of Sorghum and Maize; in 2015–2017 in the third decade of May. The soil of the experimental plot was represented by medium loamy southern chernozem. The humus content in the arable layer was 3.5 %, nitrification ability – 7.7 mg/kg; phosphorus – 34.2–35.7 mg/kg, potassium (in a carbon ammonium extract) – 349–378 mg/kg. In each season, zonal sorghum cultivation technology was used that did not include artificial irrigation (Gorbunov et al., 2012). The predecessor is steam field. The plots (7.7 m^2) were allocated randomly in three replications. The plant standing density was set manually (100 thousand plants per ha). Evaluation of traits and yield was carried out according to methodology of state testing of crops (Metods of State Variety…, 1989). The combining ability of lines was determined by the topcross method (Savchenko, 1973). For statistical analysis of the experimental data Agros 2.09 software was used (Martynov, 1999).

Weather conditions varied over the seasons of the study. The 2017 season was characterized by high moisture supply: the hydrothermal coefficient (HTC) was 1.00 (the sum of active temperatures was 1072.3 °C and the amount of precipitation was 107.1 mm). In 2015 and 2016, during the "sprouting–flowering" period, arid conditions were observed (HTC was 0.66 and 0.32, respectively). The sum of active temperatures was 1144.9–1167.9 °C, the amount of precipitation was 75.2 and 37.3 mm, respectively.

Results

Analysis of variation of agronomically-important traits in F_1 hybrids. To study the effect of cytoplasm on the combining ability of iso-nuclear CMS lines, a preliminary assessment of variation of the studied traits in 54 F_1 hybrids was made (Table 1).

Table 1. Variation of agronomically valuable traits in F_1 hybrids obtained with iso-nucelar CMS lines with genetically different types of sterile cytoplasms (A3, A4, 9E) and nuclear genome of Zheltozernoye 10

Trait, statistical indicator	Trait value (min…max)[1]			
	2015	2016	2017	Mean[2]
Plant height, cm	148.8–258.9	139.5–243.4	159.3–215.3	154.8–219.2
Coefficient of variation, %	11.7	9.1	7.4	7.4
F	8.93*	5.80*	3.54*	2.41*
Panicle length, cm	15.6–26.5	13.8–27.2	16.5–32.8	17.6–25.4
Coefficient of variation, %	11.0	8.9	9.8	7.7
F	5.89*	4.92*	2.74*	1.99*
Panicle width, cm	4.6–15.0	3.8–11.0	7.8–17.8	5.9–13.1
Coefficient of variation, %	25.7	19.7	21.1	19.3
F	9.71*	5.35*	2.21*	3.20*
Length of the largest leaf, cm	54.8–86.1	48.2–74.1	55.8–77.6	54.3–77.1
Coefficient of variation, %	8.8	11.2	8.6	7.3
F	5.46*	4.45*	3.39*	2.85*
Width of the largest leaf, cm	4.1–8.2	3.6–7.0	4.7–7.5	4.7–6.8
Coefficient of variation, %	15.1	13.6	10.5	9.4
F	6.25*	2.76*	2.08*	2.06*
Grain yield per panicle, g	5.9–45.5	5.6–27.4	27.7–70.6	17.0–39.5
Coefficient of variation, %	45.4	38.8	22.1	20.1
F	10.16*	10.88*	2.27*	1.31
Number of grains per panicle	174–1308	234–1159	804–2336	503–1430
Coefficient of variation, %	44.4	35.6	19.8	18.9
F	8.45*	6.67*	1.94*	1.65*
Grain yield, t/ha	1.09–7.53	0.93–4.33	3.41–8.49	2.34–5.59
Coefficient of variation, %	48.6	31.4	19.9	22.4
F	12.42*	5.08*	1.48*	2.14*

[1] min and max – minimum and maximum value of the trait; [2] mean for 2015–2017; * $p > 0.95$.

The traits "plant height" (CV = 7.4–11.7 %), "inflorescence length" (CV = 7.7–11.0 %), "length of the largest leaf" (CV = 7.3–11.2 %) were characterized by low variation (see Table 1). The average variation was found for the width of the largest leaf (CV = 10.5–15.1 %), while for other traits high variation was observed. Higher coefficients of variation of the studied traits were noted in 2015, with the exception of the length of the largest leaf.

The analysis of variance confirmed the differences between the tested F_1 hybrids for majority of agronomically valuable traits ($F_{observed} > F_{expected}$). For the grain yield per panicle, on average, over three years of testing, no significant differences between hybrids were revealed at the 5 % level; therefore, the combining ability for this trait was not determined.

Combining ability of iso-nuclear CMS lines

Vegetative traits. Cytoplasms A3 and 9E significantly increased GCA effects of the CMS lines for plant height in 2015 (2.08–2.71), and SCA dispersions in 2015 (253.47–305.75), and in 2016 (75.16–109.25), in comparison with A4 cytoplasm (Fig. 1).

Differences in the effects of the GCA of the CMS lines for parameters of the largest leaf were observed only in 2016. The effects of the GCA of the CMS-line with 9E cytoplasm (1.78) were significantly higher than with CMS-line with A4 cytoplasm (–2.22). The cytoplasmic effect on the combining ability of CMS lines for the width of the largest leaf was not detected. At the same time, there is a tendency towards the manifestation of higher GCA effects of the line 9E Zheltozernoye 10 (annually). The analysis of SCA dispersion showed the influence of the CMS type on parameters of the largest leaf in 2015–2016, the A3 cytoplasm caused the most strong effect on the leaf width: SCA dispersions were 0.27–0.36. A4 cytoplasm reduced SCA dispersions according to the parameters of the largest leaf (Table 2).

Generative organ traits. A significant influence of the 9E cytoplasm on the GCA effects for the length of inflorescence was recorded in each year (Fig. 2). Higher GCA effect for the width of inflorescence was also detected in 2015 for the 9E cytoplasm: 0.32 versus –0.29 and –0.03 in the A3 and A4 cytoplasms, respectively. The dispersion of SCA for panicle parameters turned out to be significantly higher for the CMS line 9E Zh10: for the inflorescence length in each growing season, and for the inflorescence width in 2015–2016 seasons (see Fig. 2).

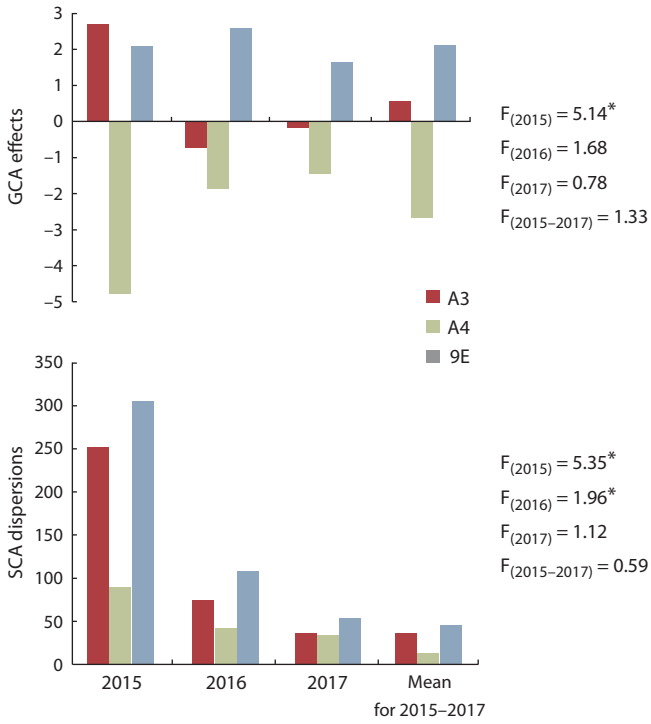

Fig. 1. Influence of the type of sterile cytoplasm on the combining ability of iso-nuclear CMS-lines for the plant height.

* $p > 0.95$.

Table 2. The combining ability of iso-nuclear CMS lines of sorghum Zheltozernoye 10 with genetically different types of sterile cytoplasms (A3, A4, 9E) for the parameters of the largest leaf

CMS type	Lenght				Width			
	2015	2016	2017	Mean	2015	2016	2017	Mean
				GCA effects				
A3	0.96	0.44	–0.90	0.16	–0.07	–0.12	–0.20	–0.12
A4	–1.03	–2.22	–0.11	–1.12	–0.01	0.04	0.07	0.03
9E	0.07	1.78	1.01	0.95	0.07	0.08	0.14	0.09
F	2.38	6.92*	1.62	2.34	0.65	1.11	2.85	1.52
				SCA dispersion				
A3	15.94	23.81	10.02	6.31	0.36	0.27	0.22	0.07
A4	13.51	10.41	5.20	2.48	0.23	0.14	0.13	0.03
9E	20.34	18.22	6.19	6.87	0.22	0.17	0.11	0.04
F	3.31*	2.44*	1.04	0.93	2.99*	1.69*	1.13	0.48

* $p > 0.95$.

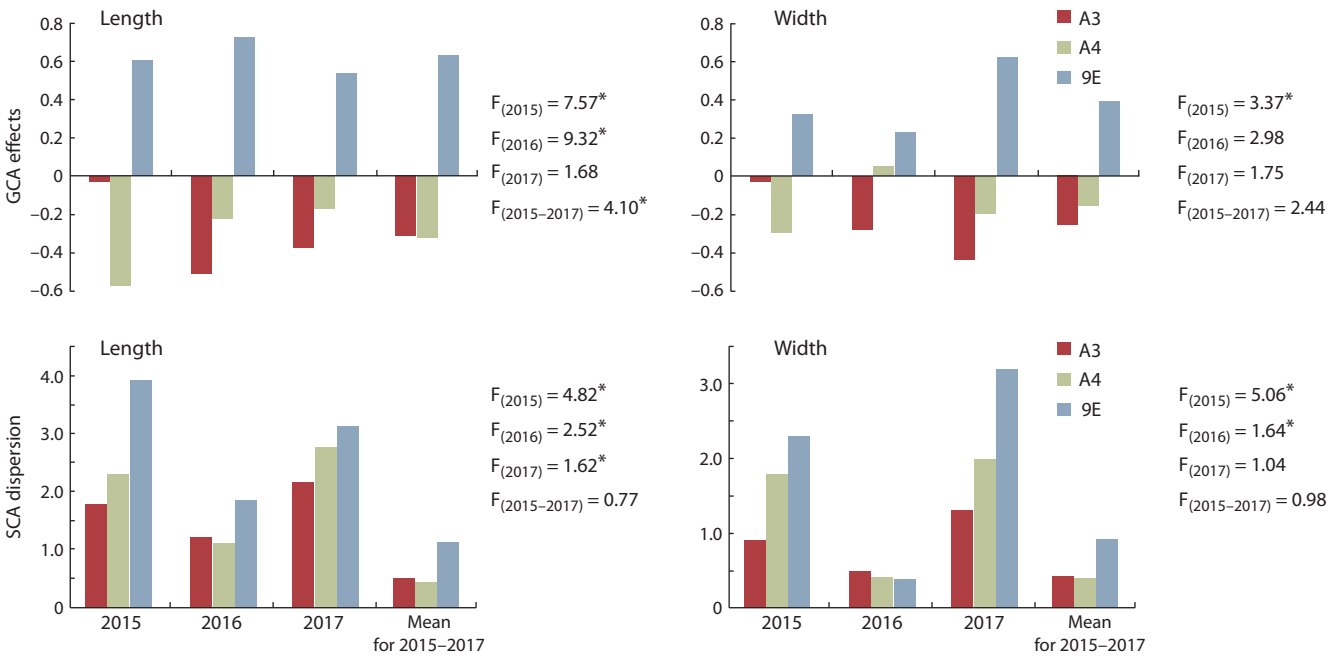

Fig. 2. The influence of the type of cytoplasm (A3, A4, 9E) on the GCA and SCA of the iso-nuclear CMS lines of sorghum for the length and width of inflorescence.

* $p > 0.95$.

Table 3. The combining ability of the iso-nuclear CMS lines of sorghum Zheltozernoye 10 with genetically different types of sterile cytoplasms (A3, A4, 9E) for panicle mass and number of grains per panicle

CMS type	Panicle mass			Number of grains per panicle			
	2015	2016	2017	2015	2016	2017	Mean
				GCA effects			
A3	–0.64	1.24	0.22	–11.96	43.19	10.87	14.63
A4	1.19	–0.02	–2.66	27.04	–6.33	–76.41	–17.95
9E	–0.55	–1.22	2.44	–15.07	–36.85	65.55	3.32
F	2.33	8.17*	2.94	1.33	4.37*	2.10	0.27
				SCA dispersion			
A3	26.06	8.68	17.88	18994.14	13808.67	15072.85	5839.94
A4	18.40	3.59	32.82	12129.40	9154.16	23906.35	6353.38
9E	22.99	8.47	31.82	17221.55	15883.96	23156.41	7521.93
F	4.09*	3.11*	1.03	3.24*	2.89*	0.71	0.53

* $p > 0.95$.

A stimulating cytoplasmic effect on CA of CMS lines for the panicle mass and number of grains per panicle was established in 2015–2016, i. e. under drought conditions of the cultivation of F_1 hybrids. At the same time, the effects of GCA for weight and number of panicle mass were significantly higher in A3 Zh10 (1.24 and 43.19, respectively), and the SCS dispersion was lower in A4 Zh10 (in different seasons: 3.59–18.40 and 9154.16–12129.40, respectively) (Table 3).

The GCA effects of maternal lines for grain yield did not differ significantly (Fig. 3). On average for three-year trails, indicators of the A3 cytoplasm were slightly higher than for A4 and 9E cytoplasms (0.06 vs. –0.10 and 0.03, respectively). Cytoplasmic effects on SCA dispersion for grain yield were noted only in 2015: cytoplasm A3 significantly increased it in comparison with A4 and 9E cytoplasms.

Discussion

The analysis of the combining ability of CMS lines is the most important step in sorghum hybrid breeding. One of the effective methods for analysis of CA is the topcross method.

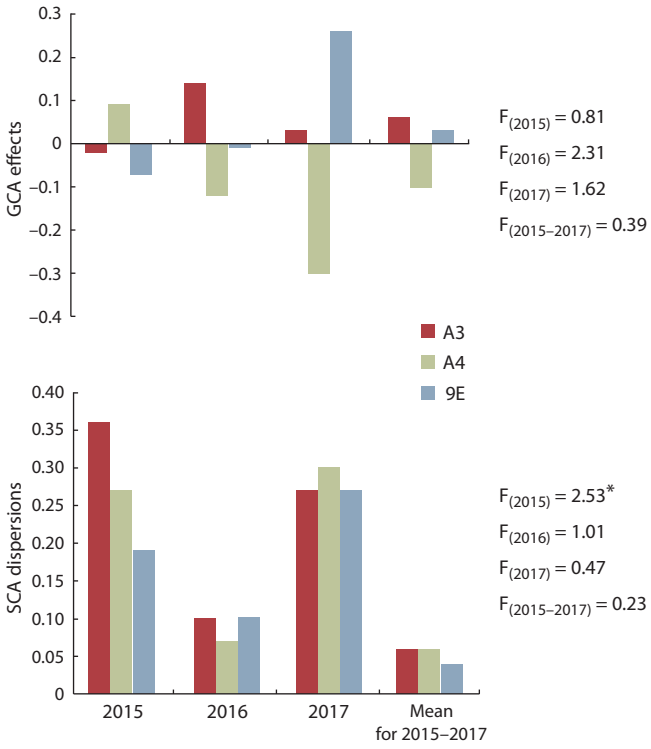

Fig. 3. The influence of the type of cytoplasm (A3, A4, 9E) on the combining ability of iso-nuclear CMS lines of sorghum for grain yield.

* $p > 0.95$.

According to this method, all the studied lines are crossed with several tester lines (Kilchevsky et al., 2008). The GCA of parental line is measured by the average deviation of the trait for all hybrids with the line from the total average for all hybrids (Khotyleva et al., 2016). This method allows comparing different lines with each other, and the more testers involved in hybridization, the more accurate the results of such a comparison. In our study, iso-nuclear CMS lines that differ from each other only in the type of cytoplasm were involved in crosses. F_1 hybrids were obtained with each of these lines, and the same lines were used as paternal parents. Therefore, a comparison the sets of F_1 hybrids allows us to identify the presence or absence of the influence of the cytoplasm on the combining ability of the studied CMS lines.

The experimental data presented above demonstrate the effect of the cytoplasm on the CA of iso-nuclear sorghum lines. Over three years of testing, on average, a positive effect of the 9E cytoplasm on GCA for the inflorescence length (0.63) and negative effects of A3 and A4 cytoplasms (–0.32 and –0.31, respectively) on GCA for this trait were found. It should be noted that to study cytoplasmic effect on GCA for the traits determining the grain productivity of hybrids, we used panicles that set seed after free pollination. We used such approach because among the pollen parents used in our experiment, there were no CMS A3 restorers; fertility restorers of this type of CMS are extremely rare (Worstell et al., 1984; Torres-Cardona et al., 1990; Dahlberg, Madera-Torres, 1997). CMS A4 and 9E restorers were few and not capable of restoring CMS A3 fertility. Nevertheless, male-sterile hybrids grown with the free pollination regime among hundreds of thousands of fertile plants in experimental field, had 100 % seed set on all panicles of the studied hybrids. This approach has already been used previously in the study of hybrids with A3 CMS (Moran, Rooney, 2003).

It is noteworthy that the manifestation of cytoplasmic effects depends on the hydrothermal regime of plant growth. For example, significant positive effects of cytoplasms on GCA were found in dry seasons: for 9E (for the length of the largest leaf), and for A3 (for plant height), while there were no differences between them in the wet season. Remarkably, in conditions of drought, the A4 cytoplasm had a negative effect on CA for many traits (leaf length and width, number of grains per panicle, and yield). Apparently, A4 cytoplasm is less resistant to extreme drought conditions (lack of the necessary amount of precipitation, accompanied by high average daily air temperatures). As a result, the combining ability of the CMS line A4 Zheltozernoye 10 for the complex of studied traits turned out to be lower. Perhaps it is for this reason, the significance of the influence of the cytoplasm on GCA and SCA were observed only in a particular season. In addition, the manifestation of the effects of GCA is less dependent on environmental conditions than SCA. For example, CMS lines differ in the SCA for the length of the largest leaf (2015), width of the largest leaf (2015–2016), plant height (2016), panicle mass and number of grains per panicle (2015), grain yield (2015), while the effects of GCA for these traits in these seasons were not significant. A similar dependence of the manifestation of cytoplasmic effects on environmental conditions was found in pearl millet, with cytoplasms A4 and A5 showing greater environmental sustainability compared to cytoplasms A1, A2 and A3 (Chandra-Shekara et al., 2007).

According to published data, the effect of CMS type on panicle length was observed in maize hybrids (Kabanova et al., 2015); cytoplasmic effects on leaf parameters were revealed in maize hybrids with C- and S-types of CMS: hybrids with C-type CMS had higher leaf length, while S-type hybrids had higher leaf width (Frankovskaya et al., 1995).

In sorghum, the influence of the cytoplasm type on GCA for grain yield and mass of 100 grains was previously noted in the study of Indian researchers, while cytoplasm A2 had an advantage over A1 and A4 cytoplasms (Kishan, Borikar, 1989; Ramesh et al., 2006; Reddy et al., 2007, 2009). In our studies, it was found that 9E cytoplasm increased leaf width in sorghum-sudanense hybrids (Kibalnik, Elkonin, 2012). In grain sorghum hybrids this cytoplasm increased photosynthetic potential during the "heading–full maturity" period (Bychkova, Elkonin, 2016), in comparison with A3 cytoplasm. The effect of a sterile cytoplasm on the CA of sorghum CMS lines for the intensity of the initial plant growth was also found, the 9E cytoplasm contributing to an increase, and A4 cytoplasm contributing to a decrease of GCA effects (Elkonin et al., 2018). The positive effect of the 9E cytoplasm on CA for biomass productivity in dry seasons was also established (Elkonin et al., 2018), while A3 cytoplasm had a stimulating effect on grain yield in the dry and hot season (Bychkova, Elkonin, 2017). The totality of these data indicates that the cytoplasm plays a significant role in the manifestation of many agronomically valuable traits in sorghum, reducing or increasing the resistance of plants to drought stress.

Conclusion

The effect of the cytoplasm on the combining ability of sorghum lines for a number of agronomically valuable traits (plant height, length and width of the largest leaf and of the inflorescence, panicle mass and number of grains per panicle, grain yield) was found. The manifestation of cytoplasmic effects in sorghum hybrids depends on the specific interaction of the genotypes of the parental lines and hydrothermal factors of the growing season. Significant differences in the combining ability of the iso-nuclear lines of Zh10 with the cytoplasms A3, A4 and 9E were observed during the dry seasons of vegetation (2015–2016). A3 Zh10 was distinguished by the highest GCA for the plant height, while 9E Zh10 – by the high SCA dispersion for this trait. For the length and width of the largest leaf, the highest SCA dispersion indicators are characteristic for the A3 Zh10 line. For the length and width of the inflorescence, the highest GCA effect and SCA dispersion were noted in the 9E Zh10 line. For panicle mass and the number of grains per panicle, the highest GCA effects were found in the A3 Zh10 line. The 9E Zh10 line had the highest SCA dispersion for the grain yield. A4 cytoplasm reduced combining ability for majority of the studied traits.

These experimental data can be used in grain sorghum breeding programs aimed at creating drought tolerant F_1 hybrids with improved agronomically valuable traits.

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ORCID ID
O.P. Kibalnik orcid.org/0000-0002-1808-8974
L.A. Elkonin orcid.org/0000-0003-3806-5697

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Молекулярные маркеры в генетическом анализе скрещиваемости мягкой пшеницы с рожью

И.В. Поротников ✉, О.Ю. Антонова, О.П. Митрофанова

Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
✉ e-mail: i.v.porotnikov@gmail.com

Аннотация. Мягкая пшеница (*Triticum aestivum* L.), сорта которой широко используются в мировом производстве зерна, плохо скрещивается с видами других родов Triticeae Dum., что ограничивает возможности введения чужеродного генетического материала в ее генофонд и создания новых сортов, хорошо адаптированных к различным неблагоприятным абиотическим и биотическим факторам внешней среды. Известно, что скрещиваемость мягкой пшеницы с представителями других родов контролируется генами *Kr1–Kr4* (Crossability with Rye, *Hordeum* and *Aegilops* spp.) и геном *SKr* (Suppressor of crossability). Из названных генов наиболее сильное влияние на признак оказывают *SKr* и *Kr1*. В рецессивном состоянии, когда гены не функционируют, может завязываться более 50 % зерновок от числа цветков в колосе при опылении пыльцой чужеродного вида. Оба гена локализованы в хромосоме 5B. Расположение гена *SKr* в коротком плече хромосомы 5B ограничено маркерами GBR0233 и *Xgwm234* в тесном сцеплении с маркерами *Xcfb341*, TGlc2 и gene12. Ген *Kr1* расположен в длинном плече хромосомы 5B, проксимальнее гена *Ph1*, между EST-SSR-маркерами *Xw5145* и *Xw9340*. Маркеры, разработанные для гена *SKr*, применяли для контроля переноса его рецессивного аллеля *skr* в другие генотипы мягкой пшеницы, что позволило получать формы с высокой завязываемостью гибридных зерновок при скрещивании с рожью. Однако в целом использование маркеров генов *SKr* и *Kr1* в практической маркер-ориентированной селекции и молекулярном скрининге образцов *ex situ* коллекций изучено недостаточно. Большие перспективы в этом плане открывает определение полной нуклеотидной последовательности гена *Kr1* у контрастных по скрещиваемости сортов мягкой пшеницы, это дает возможность создания внутригенных аллель-специфичных маркеров. В представленном обзоре рассмотрены генетические ресурсы, созданные посредством гибридизации мягкой пшеницы с рожью; вопросы географического распространения легко скрещивающихся форм пшеницы и генетического контроля совместимости пшеницы и ржи; достижения в использовании молекулярных маркеров в картировании *Kr*-генов и контроле их передачи.
Ключевые слова: *Triticum aestivum*; рожь; *Kr*-гены; QTLs; молекулярное картирование; молекулярно-генетические карты; генетические ресурсы пшеницы.

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Molecular markers in the genetic analysis of crossability of bread wheat with rye

I.V. Porotnikov ✉, O.Yu. Antonova, O.P. Mitrofanova

Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
✉ e-mail: i.v.porotnikov@gmail.com

Abstract. Bread wheat (*Triticum aestivum* L.), the varieties of which are widely used for the grain production, is difficultly crossable with related species of Triticeae Dum. This factor limits the chance of introduction of alien genetic material into the wheat gene pool and the possibility of new varieties breeding with good adaptation to adverse environmental factors. The crossability between wheat and related species is controlled by *Kr1-Kr4* genes (Crossability with Rye, *Hordeum* and *Aegilops* spp.) and the *SKr* gene (Suppressor of crossability). *SKr* and *Kr1* have the largest influence on the trait. In the case of the recessive alleles, these genes do not function and the quantity of hybrid seeds after pollination with alien species can achieve more than 50 %. *SKr* is located on 5BS between the GBR0233 and *Xgwm234* markers, closely linked with the markers *Xcfb341*, TGlc2 and gene12. *Kr1* was mapped on 5BL, proximally to the *Ph1* gene, between the EST-SSR markers *Xw5145* and *Xw9340*. The markers of *SKr* were used to control the transfer of its recessive allele into other wheat genotypes, which made it possible to obtain highly crossable forms. However, the advantages of using the *SKr* and *Kr1* markers in marker-assisted selection and in the screening of *ex situ* collections are not sufficiently studied. The published *Kr1* sequence for varieties with different crossability offers great prospects, because it will be possible to create allele-specific markers. In this review,

the following issues are considered: genetic resources created by wheat and rye hybridization, the geographical distribution of easy-to-cross forms of wheat, genetic control of the wheat and rye compatibility, advances of the use of molecular markers in the mapping of *Kr*-genes and their transmission control.
Key words: *Triticum aestivum*; rye; *Kr*-genes; QTLs; molecular mapping; molecular-genetic maps; wheat genetic resources.

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Введение

Пшеница – одна из важнейших сельскохозяйственных культур. В 2017 г. в мире было собрано 771.3 млн тонн ее зерна. Россия среди стран-производителей занимает третье место – 85.3 млн тонн (www.fao.org). Успехи в сборе зерна мягкой пшеницы *Triticum aestivum* L. ($2n = 6x = 42$, BBAADD) и твердой пшеницы *T. durum* Desf. ($2n = 4x = 28$, BBAA) – это результат интенсивной селекции, направленной в основном на повышение урожайности и улучшение качества зерна. Для защиты новых сортов от резких перепадов погоды, происходящих изменений в климате, действия абиотических и биотических стрессоров необходимо иметь соответствующий запас генетической изменчивости у этих культур. Однако в генофондах современных селекционных сортов такого запаса изменчивости нет. К тому же с середины прошлого столетия по всему миру имеет место процесс обеднения генетического разнообразия селекционных сортов, порожденный ходом исторического развития программ селекции и методами отбора (Porceddu et al., 1988; Kahiluotoa et al., 2019). О наличии этого процесса свидетельствуют результаты анализа геномов сортов с применением SSR-маркеров во Франции (Roussel et al., 2004), Китае (Hao et al., 2006), Канаде (Fu, Somers, 2009). Чтобы защитить возделываемые пшеницы от действия различных неблагоприятных факторов, необходимо обогатить их генофонды аллелями генов, увеличивающими разнообразие адаптивных реакций. В качестве источников новых аллелей генов для пшеницы довольно широко используют другие виды родов трибы Triticeae Dum.

Одна из проблем, препятствующих успешным скрещиваниям пшеницы с представителями родов *Aegilops* L., *Hordeum* L. и *Secale* L., – наличие прогамной несовместимости (Pershina, Trubacheeva, 2017), выражающейся в замедлении или ингибировании роста пыльцевых трубок (Zeven, Van Heemert, 1970; Lange, Wojciechowska, 1976, 1977; Jalani, Moss, 1981). У мягкой пшеницы обнаружено пять генов – *Kr1*, *Kr2*, *Kr3*, *Kr4* (Crossability with Rye and *Hordeum* and *Aegilops* spp.) (McIntosh et al., 2014) и *SKr* (Suppressor of crossability) (Tixier et al., 1998), доминантные аллели которых отвечают за проявление признака несовместимости. Эти гены контролируют скрещиваемость пшеницы с видами всех названных выше родов (Snape et al., 1979; Falk, Kasha, 1981; Fedak, Jui, 1982; Sitch et al., 1985; Koba, Shimada, 1993).

Цель настоящего обзора – систематизировать сведения о генетических ресурсах, созданных с помощью межродовой гибридизации мягкой пшеницы с рожью (*Secale* L.), показать успехи в познании генетического контроля признака «легкая скрещиваемость», рассмотреть ДНК-маркеры, которые можно было бы использовать для быстрой и точной идентификации аллелей генов, ответственных за этот признак, и в контроле передачи их в другие генотипы.

Генетические ресурсы, созданные при скрещивании мягкой пшеницы с рожью, и их использование в селекции

Рожь посевную *Secale cereale* L. ($2n = 2x = 14$, геном R) вовлекали в скрещивания с мягкой пшеницей с конца XIX–начала XX веков (Backhouse, 1916). В 1930-х годах в Германии были получены первые формы мягкой пшеницы с транслокациями короткого плеча хромосомы 1R ржи в длинное плечо хромосомы 1B пшеницы (T1BL.1RS). В настоящее время в мире известно более 1050 сортов пшеницы, содержащих эту транслокацию, а также около 100 сортов с транслокацией T1AL.1RS (Rabinovich, 1998; Pershina, 2014; Schlegel, 2019). Широкое распространение обеих транслокаций обусловлено наличием в 1RS комплекса генов, контролирующих устойчивость к различным грибным патогенам, таким как стеблевая (*Sr31*), бурая (*Lr26*) и желтая (*Yr9*) ржавчины, мучнистая роса (*Pm8*) (Mago et al., 2005; Ren et al., 2009; Crespo-Herrera et al., 2017). Следует отметить, что большая часть сортов с транслокацией T1BL.1RS несет чужеродный хроматин от сорта ржи Petkus из Германии, а с T1AL.1RS и T1DL.1RS – от Insave и Imperial соответственно (Rabinovich, 1998; Schlegel, 2019).

Ограниченное разнообразие ржаного генетического материала, очевидно, не в состоянии обеспечить устойчивость пшеницы к новым расам вредных организмов, которые стали появляться уже в 90-е годы XX в. (Porter et al., 1991; Pretorius et al., 2000). Для решения этой проблемы начали создавать линии пшеницы с новыми ржаными транслокациями путем скрещивания как с теми же сортами ржи, Petkus и Insave, (Ren et al., 2009; Tang et al., 2009), так и с новыми (Ren et al., 2012, 2017; Yang et al., 2014; Li et al., 2016). Кроме того, линию пшеницы, хорошо скрещивающуюся с рожью и несущую T1BL.1RS, использовали в гибридизации с *Secale cereanum* cv. Kriszta, и за счет рекомбинации между хромосомами T1BL.1RS и 1R в пшеницу были введены новые аллели генов ржи (Molnár-Láng et al., 2010). Одна из таких рекомбинантных форм дала начало линии M9kr1-Kriszta T1BL.1RS line 179, обладающей устойчивостью к желтой и бурой ржавчинам в сочетании с высоким качеством зерна (Szakács et al., 2020). При использовании совместимых с рожью местных китайских сортов пшеницы Mianyang 11 и A42912 были также созданы линии с T1BL.1RS от разных сортов ржи, которые содержали новые эффективные аллели генов устойчивости к расам *Puccinia striiformis* f. sp. *tritici* и

Blumeria graminis f. sp. *tritici* (Ren et al., 2012, 2017; Yang et al., 2014; Li et al., 2016). Передача генетического материала хромосомы 2R ржи в пшеницу сопровождалась переносом генов устойчивости к бурой (*Lr25* и *Lr45*) и стеблевой (*Sr59*) ржавчинам, повышением адаптивности растений к засушливым условиям климата (Ehdaie et al., 2003). В хромосомах 5RL и 7R обнаружены гены, повышающие эффективность усвоения меди и цинка из почвы (Owuoche et al., 1996; Cakmak et al., 1997). Наиболее полный перечень всех известных пшенично-ржаных транслокаций и генов устойчивости приведен в обзоре (Crespo-Herrera et al., 2017). Следует отметить, что в настоящее время потенциал генетического разнообразия, заключенный в хромосомах ржи, помимо 1R, в селекции пшеницы практически остался неиспользованным (Schlegel, 2019).

Считаем нужным подчеркнуть, что в разные периоды времени на базе коллекции Всероссийского института генетических ресурсов растений им. Н.И. Вавилова (ВИР) также проводили исследования по скрещиваемости мягкой пшеницы с рожью. Признак «легкая скрещиваемость пшеницы с рожью» был целенаправленно передан в озимый сорт Приекульская 481, на его основе созданы легко скрещивающиеся линии (Суриков, Киссель, 1980), которые наряду с другими линиями использовали в гибридизации с дву- и шестирядными формами ячменя для получения различных межродовых гибридов (Суриков, Киссель, 1987). Созданы оригинальные, хорошо совместимые с разными видами ржи формы озимой мягкой пшеницы АМ 808 (с привлечением к-48253 Arthur, США) и МА 808 (к-43920 Мироновская 808, УССР), которые при опылении рожью давали жизнеспособные гибридные и частично фертильные растения F_1 (Рехметулин, 1988). Возможность получения частично фертильных гибридных растений и от них константных первичных высокопродуктивных зимостойких пшенично-ржаных линий подтверждена и в наши дни, при этом методом геномной *in situ* гибридизации у всех линий обнаружены элиминация хромосом генома D и наличие полных геномов B, A и R (Pyukkenen et al., 2019).

Факторы, влияющие на уровень скрещиваемости пшеницы с рожью

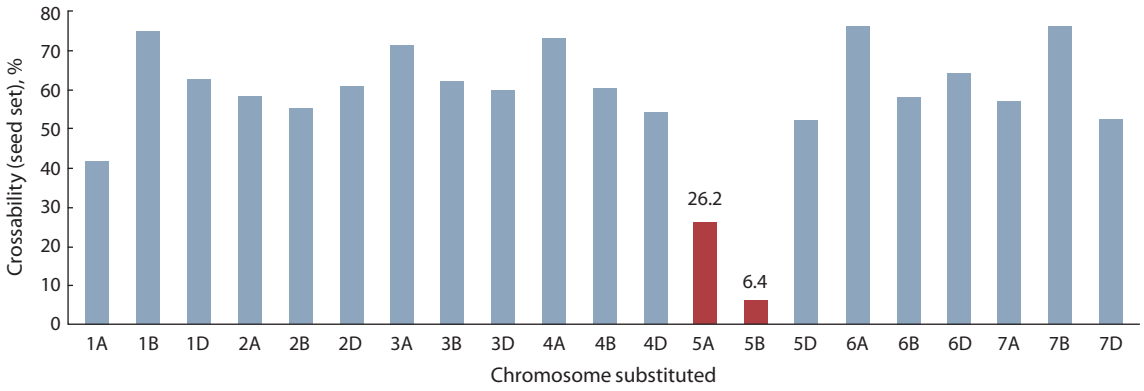
Большая работа по обобщению имеющихся в литературе сведений и результатов собственных исследований по скрещиваемости сортов/линий преимущественно мягкой пшеницы с рожью выполнена A.C. Zeven (1987). На основании собранных нами данных (см. таблицу) можно заключить, что мягкая пшеница в целом плохо скрещивается с рожью. Легко (хорошо) скрещивающиеся формы находили с частотой 5.9 %, причем в определенных географических регионах (Lein, 1943; Писарев, 1960; Ригин, 1976; Lange, Wojciechowska, 1976; Falk, Kasha, 1981; Zeven, 1987). Так, выполненная в ВИР оценка сортов мягкой пшеницы различных агроэкологических групп показала, что наиболее высокую способность завязывать гибридные зерновки (52.5 и 53.7 % соответственно) имели сорта китайского и восточносибирского экотипов (Писарев, 1960; Ригин, 1976). Считают, что частое выявление хорошо скрещивающихся форм среди восточноазиатских сортов пшеницы, возможно, связано с тем, что в процессе эволюции у них не сформировалась генетическая система, препятствующая скрещиваемости пшеницы с рожью, поскольку ареал ржи был ограничен исключительно районами Европы и Западной Азии, и с этой культурой пшеница в Юго-Восточной Азии вместе не произрастала (Lein, 1943; Riley, Chapman, 1967).

Показано, что на успех скрещиваемости пшеницы с рожью также влияет целый ряд факторов, в том числе видовая принадлежность отцовских и материнских генотипов. Наиболее высокий процент завязываемости гибридных зерновок наблюдали у гексаплоидных пшениц: *T. aestivum*, *T. spelta* L., *T. compactum* Host; средний – у тетраплоидных *T. turgidum* L., *T. durum*, и самый низкий – у диплоидных *T. monococcum* L. и *T. boeoticum* Boiss. Варьировала также успешность применения разных видов ржи в качестве опылителей: высокая скрещиваемость при опылении пыльцой диплоидных видов *S. cereale* и *S. vavilovii* Grossh., низкая – тетраплоидного вида *S. fragile* M. Bieb. (Ригин, 1965, 1976; Суриков, Киссель, 1985;

The distribution of wheat varieties by the effectiveness of hybrid seed formation in pollination with rye

Hybrid seed set, %				Total no. of varieties	References
0–10	10–30	30–50	> 50		
48	2	–	–	50	Rekhmetulin, 1987
97	14	6	9	126	Rigin, 1976
3	3	1	10	17	Surikov, Kissel, 1985
105	20	1	5	131	Zheng-Song et al., 1998
53	3	3	2	61	Singh, Sethi, 1991
29	23	11	34	97	Rui et al., 1995
577	27	4	6	614	Zeven, 1987
477	67	29	34	607	Zeven, 1987*
1389	159	55	100	1703	Total
81.6	9.3	3.2	5.9	100	Percentage in the total number of varieties

Note. The intervals for the distribution of varieties into groups are taken from the classical work of A. Lein (1943).
* Varieties from other sources cited by A.C. Zeven (1987).



Crossability of wheat intervarietal substitution lines of Chinese Spring/Hope to rye (according to Riley, Chapman, 1967).

Рехметулин, 1987; Molnár-Láng, 2015). Различные аспекты проблемы скрещиваемости пшеницы с рожью и другими видами рассмотрены в обзорной статье (Molnár-Láng, 2015).

Гибридологический анализ скрещиваемости пшеницы с рожью и локализация генов легкой скрещиваемости

Первые сведения о наследовании признака «легкая скрещиваемость мягкой пшеницы с рожью» получены немецким ученым A. Lein (1943). Выяснение характера наследования признака у реципрокных гибридов F_1–F_3 между сортом Chinese 466 из Аргентины, характеризующимся высокой (57.3 %) завязываемостью гибридных зерновок, и плохо скрещивающимися с рожью сортами Marguis (Канада), Peragis и Blausamtiger Kolben (Германия) с завязываемостью 1.9, 2.5 и 12.7 % соответственно, показало в F_1 доминирование низкой завязываемости, а в F_3 – дигибридное расщепление. Аллельные пары факторов (генов) были обозначены как *Kr1-kr1* (Kreuzbarkeitsgene) и *Kr2-kr2*, а генотипы родительских сортов – *kr1kr1kr2kr2* (Chinese 466), *Kr1Kr1Kr2Kr2* (Marquis и Peragis) и *Kr1Kr1kr2kr2* (Blausamtiger Kolben). Отмечено также, что доминантный аллель *Kr1* обладает более сильным ингибирующим эффектом роста пыльцевых трубок, чем *Kr2*, а различные генотипы характеризуются следующим процентом завязываемости гибридных зерновок: *Kr1Kr1Kr2Kr2* – до 10 %, *Kr1Kr1kr2kr2* – 10–30, *kr1kr1Kr2Kr2* – 30–50, *kr1kr1kr2kr2* – более 50 % (Lein, 1943).

Для установления хромосомной локализации генов *Kr1* и *Kr2* была использована серия из 21 линии с межсортовым замещением хромосом Chinese Spring/Hope (Sears et al., 1957; Riley, Chapman, 1967). При опылении диплоидной рожью у Chinese Spring (далее CS) наблюдали высокую (74.3 %) завязываемость гибридных зерновок, а сорт Hope таких зерновок практически не образовал. Существенное снижение уровня завязываемости зерновок обнаружено у линий с замещенными хромосомами 5A и 5B, хотя различия между линиями не имели статистического подтверждения (см. рисунок). Полученные данные навели R. Riley и V. Chapman на мысль, что замена хромосом 5B вызывает бóльшую редукцию уровня скрещиваемости, чем хромосом 5A. Ген *Kr1* был локализован в хромосоме 5B, а *Kr2* – в 5A (Riley, Chapman, 1967). Чтобы ответить на вопрос, рецессивные аллели *Kr*-генов поддерживают межродовую совместимость или, напротив, доминантные аллели ингибируют скрещиваемость, авторы процитированной работы протестировали линию CS nulli-5B-tetra-5D, у которой отсутствовали хромосомы 5B, но присутствовала в учетверенной дозе гомеологичная ей хромосома 5D. Эта линия, как и моносомик CS по хромосоме 5B, формировала зерновки при самоопылении. При опылении рожью в первом случае 116 цветков дали 67 гибридных зерновок (завязываемость 57.8 %), во втором – при опылении 261 цветка было получено 184 зерновки (70.5 %). Отсутствие хромосом 5B и, соответственно, пары рецессивных аллелей *kr1* или изменение их дозы не снижало уровень завязываемости гибридных зерновок. Из этого R. Riley и V. Chapman сделали вывод, что рецессивные аллели не активны, доминантные аллели гена *Kr1* действуют как ингибиторы скрещиваемости. Поскольку линии CS/Hope 5A и CS/Hope 5B, в отличие от сорта Hope, не теряли полностью способности образовывать гибридные зерновки при опылении рожью, эффекты ингибиторов *Kr1* и *Kr2*, по мнению этих ученых, были или аддитивными, или комплементарными.

Нужно отметить, что о возможном кумулятивном действии генов *Kr1* и *Kr2* ранее сообщил A. Lein (1943): гетерозиготные генотипы *Kr1kr1kr2kr2* и *kr1kr1Kr2kr2* имели более 10 % успешных скрещиваний с рожью, тогда как часть растений с генотипами *Kr1Kr1Kr2kr2* и *Kr1kr1Kr2Kr2* не завязала ни одной гибридной зерновки. Впоследствии это предположение подтвердили результаты работы (Falk, Kasha, 1983), было отмечено также, что рецессивные аллели *kr1* и *kr2* кумулятивным эффектом не обладали.

Для картирования гена *Kr1* в хромосоме 5B использовали гибриды, полученные от скрещивания замещенной линии CS/Hope 5B, имеющей низкую завязываемость зерновок при опылении рожью, с дителосомной линией CS DT5BL, у которой хромосома 5B была представлена парой телоцентриков по ее длинному плечу, а короткое плечо отсутствовало (Lange, Riley, 1973). По способности завязывать гибридные зерновки эта линия не отличалась от сорта CS. Средняя частота рекомбинации между *Kr1* и центромерой составила 11.45 ± 3.0 %. Локализацию гена *Kr1* в длинном плече хромосомы 5B подтвердили

L.A. Sitch с коллегами (1985), однако ген располагался на большем расстоянии от центромеры – 44.8±3.28 % рекомбинации. Причиной такого несоответствия могло быть использование другой родительской формы – сорта Highbury.

Ген *Kr2* картировали у гибридов, полученных от скрещивания CS с линией CS/*T. spelta* 5A, у которой пара хромосом 5A мягкой пшеницы замещена на пару хромосом 5A от *T. spelta* (Sitch et al., 1985). Ген *Kr2* оказался тесно сцепленным (частота рекомбинации 4.8±4.66 %) с геном *Vrn1* (Response to Vernalization), определяющим яровой тип развития растений пшеницы, и был расположен дистальнее гена *Q* (Squarehead/spelt), обусловливающего образование спельтоидного типа колоса (McIntosh et al., 2014), на расстоянии 38.1±10.60 % рекомбинации от него (Sitch et al., 1985). Примерно одинаковое положение *Kr1* и *Kr2* по отношению к центромере авторы рассматривали как доказательство в пользу гомеоаллельности этих генов. Кроме того, в данной работе статистически значимые различия по завязываемости гибридных зерновок были выявлены у эуплоидных растений сорта CS и у полученных на его основе моносомиков mono-5B и дителосомных линий DT5BL. Эуплоидные растения CS завязывали в два раза меньше зерновок, чем растения двух упомянутых линий. Этот эффект L.A. Sitch с коллегами (1985) связали с уменьшением дозы короткого плеча хромосомы 5B и предположили присутствие в нем супрессора скрещиваемости. Позднее наличие такого супрессора, получившего название *SKr* (Supressor of crossability), было доказано методами молекулярно-генетического картирования (Tixier et al., 1998).

Еще один ген, *Kr3*, был картирован у гексаплоидной пшеницы в хромосоме 5D (Krolow, 1970). У доминантного аллеля этого гена эффект ингибирования скрещиваемости был слабее, чем у *Kr1* и *Kr2.* При гибридизации с рожью влияние *Kr3* зачастую оказывалось статистически незначимым, и в ряде работ (Riley, Chapman, 1967; Falk, Kasha, 1983; Sitch et al., 1985; Zheng et al., 1992) участие хромосомы 5D в контроле завязываемости пшенично-ржаных гибридных зерновок не подтверждено. Однако действие *Kr3* как ингибитора межродовой совместимости удалось продемонстрировать при гибридизации с *Hordeum bulbosum* L. на линиях CS/Hope 5D и CS/Hope 5A, получивших от сорта-донора Hope хромосомы с доминантными аллелями генов *Kr3* и *Kr2* соответственно (Snape et al., 1979). Снижение скрещиваемости с *H. bulbosum* у этих замещенных линий было примерно одинаковым, т. е. эффект гена *Kr3* был равен эффекту гена *Kr2*.

Ген *Kr4* обнаружен моносомным анализом линии мягкой пшеницы J-11 из провинции Сычуань (Китай). Эта линия показала почти 100 % завязываемость гибридных зерновок при опылении рожью (Zheng et al., 1992). Наследование признака хорошей скрещиваемости изучали у гибридов F_1 между линией J-11 и моносомными линиями сортов Abbondanza (скрещиваемость с рожью менее 2.13 %, предположительный генотип *Kr1Kr1Kr2Kr2*) и Chinese Spring (генотип *kr1kr1kr2kr2*). В первом случае присутствие хромосом 1A, 5A и 5B от линии J-11 оказывало положительное влияние на успех скрещивания с рожью, в то время как у гибридов с моносомиками CS для тех же хромосом наблюдали снижение скрещиваемости. При этом влияние хромосомы 1A на завязываемость зерновок оказалось сильнее, чем 5A (*kr2*), но слабее, чем 5B (*kr1*). Это позволило Y.L. Zheng с коллегами (1992) сделать вывод о наличии в хромосоме 1A линии J-11 дополнительного гена *kr4*, сходного по действию с *kr1* и *kr2*. Точная хромосомная локализация *kr4* пока остается неизвестной.

Молекулярно-генетическое картирование гена *SKr* и поиск локусов, влияющих на уровень скрещиваемости пшеницы с рожью

Новые возможности для точной локализации генов легкой скрещиваемости пшеницы с рожью возникли в связи с введением в генетический анализ технологии молекулярного маркирования и созданием насыщенных молекулярно-генетических карт (Xie et al., 1993; Nelson et al., 1995; Cadalen et al., 1997; Röder et al., 1998; Somers et al., 2004; Leonova, 2013; Khlestkina, 2014). Результаты работ разных исследователей по молекулярным маркерам и картам хромосом мягкой пшеницы и других видов трибы Triticeae доступны в цифровой базе данных GrainGenes (https://wheat.pw.usda.gov/GG3/).

Основополагающая работа по определению локусов количественных признаков (QTLs), влияющих на скрещиваемость, выполнена с использованием маркеров RFLP на популяции из 187 DH-линий, производных от гибрида CS×Courtot (Tixier et al., 1998). Скрещиваемость родительских форм и всех DH-линий проверяли путем опыления пыльцой ржи сорта Dankowskie Nowe, высокий уровень скрещиваемости был подтвержден для CS (95 %, генотип *kr1kr1kr2kr2*), низкий – для Courtot (10 %, *Kr1Kr1kr2kr2*) (Gay, Bernard, 1994). Для локализации в хромосомах QTLs, оценки их аддитивных эффектов и эффектов доминирования M.H. Tixier с коллегами (1998) применяли метод логистической линейной регрессии, который приложим к линиям, полученным от F_1 путем самоопыления или бекроссирования, и DH-линиям, при условии, что имеется информация об их генотипах по RFLP, RAPD или другим типам маркеров (Kearsey, Hyne, 1994). О местоположении QTLs судили по отношению величины аддитивного эффекта к фенотипической дисперсии:

$$avp = a^2/\sigma_p^2,$$

где a – аддитивный эффект, σ_p^2 – фенотипическая дисперсия, p – вероятность успешного скрещивания.

Всего в хромосомах 5B и 7A у DH линий было обнаружено три локуса от сорта Courtot, подавляющие способность скрещиваться с рожью. При этом в хромосомах 5A (*Kr2*) и 5D (*Kr3*) таких локусов не найдено. Главный локус (*avp* = 16.8 %) находился в дистальном районе короткого плеча хромосомы 5B вблизи маркера *Xfba367*, который имел наибольший аддитивный эффект (a = 13.6) и был тесно сцеплен (2.5 сМ) с маркером *Xpsr170*. Другой локус, ассоциированный с маркером *Xtam51* (хромосома 7AL), оказывал значительный аддитивный эффект (a = 8.1) при рассмотрении его в модели вместе с *Xfba367*. Еще один QTL, влияющий на скрещиваемость и, по-видимому, относящийся к гену *Kr1*, был картирован в длинном плече хромосомы 5B и фланкирован RFLP-

маркером *Xwg583* (*a* = 6.1; *avp* = 3.3 %) и SSR-маркером *Xgwm271*. Этот QTL оказывал более слабое влияние на фенотипическое проявление признака, для расчета его эффекта понадобилось привлечь методы маркерной логистической регрессии с двумя QTL в хромосоме 5B (Tixier et al., 1998).

Ранее в процедурах по картированию *Kr*-генов изучали расщепление в потомствах гибридов от скрещивания дителосомной по длинному плечу хромосомы 5B линии CS (*kr1kr1*) с замещенной линией CS/Hope 5B или сортом Highbury, имеющими доминантные аллели гена *Kr1*, и рассчитывали частоту рекомбинации между *kr1* и центромерой. Влияние короткого плеча хромосомы 5B CS на скрещиваемость не обнаружено (Lange, Riley, 1973; Sitch et al., 1985). Однако в работе L.A. Sitch с коллегами (1985) показано, что уменьшение дозы 5BS сопровождалось повышением уровня скрещиваемости CS с *H. bulbosum*. Выявление у гибрида CS×Courtot в коротком плече 5B локуса, существенно влияющего на уровень скрещиваемости с рожью, дало основание предположить наличие у сорта Courtot еще одного доминантного гена, подавляющего межродовую гибридизацию. Ген-супрессор был обозначен как *SKr* (Supressor of crossability) (Tixier et al., 1998). Работа по его молекулярному картированию выполнена D. Lamoureux с коллегами (2002). Анализ QTLs проводили на основе тех же фенотипических данных, что были получены M.H. Tixier с коллегами (1998), но для картирования использовали преимущественно маркеры AFLP. Молекулярная карта хромосомы дистального участка 5BS была увеличена на 16.1 cM путем интегрирования маркеров E36M49-287 (после секвенирования назван *Xdl103*) и E32M61-233. Несколько маркеров были также интегрированы в хромосомную карту 5B в район центромеры. Анализ подтвердил наличие в 5BS главного QTL (*a* = 15.9 %; *avp* = 22.1 %), ингибирующего скрещиваемость с рожью, который наиболее вероятно расположен на участке размером в 5.1 cM между маркерами *Xdl103* и *Xfba367* и на расстоянии 12.7 cM от дистального конца хромосомы. Поиск областей, ортологичных гену *SKr*, был проведен посредством скрининга библиотеки BAC-клонов риса с помощью зондов *Xpsr170* и *Xdl103* (клонированный маркер AFLP E36M49-287), картированных вблизи *SKr*. В хромосомах 5 и 6 генома риса обнаружены QTLs с функциями, близкими к *SKr*, в том числе: локус f_5, ответственный за фертильность (Wang et al., 1998), локус S_5, контролирующий стерильность у межсортовых гибридов (Liu et al., 1997), а также локус S_{10}, детерминирующий мужскую стерильность (Lorieux et al., 2000).

Создание насыщенной генетической карты для района хромосомы 5BS с геном *SKr* получило продолжение в работе W. Alfares с коллегами (2009). Они изучали популяцию линий-потомков F_6 от самоопыления гибридов MP98×Courtot. Родительская линия MP98 была отобрана по признаку высокой скрещиваемости с рожью (70 ± 11.3 %) из популяции дигаплоидов гибрида CS×Courtot (Tixier et al., 1998; Lamoureux et al., 2002). Для картирования были применены новые маркеры из опубликованных баз данных: для хромосомы 5BS было взято восемь маркеров, детектирующих полиморфизм у родительских форм, включая микросателлиты *Xgwm234*, *Xgpw4098*, *Xwmc149* и *Xgwm443*, все они располагались проксимально по отношению к гену *SKr* (Alfares et al., 2009). Среди них *Xgwm234* был локализован довольно близко к *SKr* (0.2 cM). Для насыщения карты и поиска маркеров в дистальной области привлекли дополнительные маркеры с EST-карты генома ячменя (Stein et al., 2007) как наиболее родственного пшенице злака. В результате были найдены два EST-маркера, GBR0233 и GBR1541, картированные как RFLP-зонды дистально и проксимально от *SKr* соответственно.

Секвенированная последовательность маркера GBR0233 оказалась гомологична участку гена риса *Os12g44150* (хромосома 12L) (Alfares et al., 2009), который, в свою очередь, относится к группе генов *Os12g44180– Os12g44250*, идентифицированных в другой работе как гомологи гена *GSP* (Grain softness protein = белок мягкозерности зерновки) мягкой пшеницы (Chantret et al., 2004). На основе последовательности BAC клона 1793L02 (GenBank #CT009585), содержащего ген *GSP*, авторы разработали сцепленные с геном *SKr* SSR-маркеры *Xcfb306* и *Xcfb309*. Кроме того, последовательность RFLP-маркера GBR0233 проявляла гомологию с двумя EST-контигами мягкой пшеницы, один из которых, CTG_WHP_856.1-G356.103K22R011024, содержал специфичную для хромосомы 5B последовательность ATPase1-5B, для ее детекции также была разработана пара праймеров (Alfares et al., 2009).

Маркеры *Xcfb306*, *Xcfb309*, ATPase1-5B применяли для скрининга библиотеки BAC-клонов сорта CS. Были выделены BAC-клоны, 2163O14 и 317L24A, расположенные проксимально и дистально по отношению к области *SKr* соответственно. Секвенирование этих клонов дало информацию для разработки дополнительных ISBP- и SSR-маркеров. Определены 5B-специфичные SSR-маркеры *Xcfb341* и *Xcfb382*, тесно сцепленные с локусом *SKr*. Таким образом, ген *SKr* был картирован в интервале 0.1 cM с тесно сцепленными SSR-маркерами *Xcfb306*, *Xcfb382*, *Xcfb341* и фланкирован в промежутке 0.3 cM между GBR0233 (проксимальное положение) и X*gwm234* (дистальное положение) (Alfares, 2009; Alfares et al., 2009). Позднее была показана достоверная ассоциация гена *SKr* и маркеров *Xcfb341*, TGlc2 (ISBP), gene12 и gene13, последние два получены на основе ортологичных генов риса *Os12g44100-1* и *Os12g44090* соответственно (Bouguennec et al., 2018). Маркеры *Xcfb306* и *Xcfb341* использовали при картировании гена *SKr* и в других популяциях, например популяции F_2 (Renan×CS) (Alfares et al., 2009), а также для молекулярно-генетического картирования хромосомы 5B (Timonova et al., 2013).

Другая попытка картирования генов легкой скрещиваемости мягкой пшеницы с рожью предпринята в работе японских исследователей (Mishina et al., 2009). Для картирования использовали рекомбинантные по хромосоме 5B инбредные линии (CS×CS/Cheyenne5B) и 81 SSR-маркер из работы D.J. Somers с коллегами (2004) и интернет-ресурса Wheat composite 2004 map at GrainGenes 2.0 (https://wheat.pw.usda.gov/GG3/). Включение в анализ описанных ранее маркеров *Xcfb341* и *Xgwm234* (Tixier et al., 1998; Lamoureux et al., 2002; Alfares et al., 2009) не дало положительных результатов из-за отсутствия полиморфизма у родительских форм. В этой популяции основной по силе

QTL (*SKr*) был тесно сцеплен с SSR-маркером *Xgwm443* хромосомы 5BS и ограничен генетическими локусами *Xcfd5* и *Xbarc216* (интервал 50 сМ), расположенными дистально и проксимально соответственно (Mishina et al., 2009). Список молекулярных маркеров, сцепленных с геном *SKr*, приведен в Приложении[1].

Возможность практического использования маркеров гена *SKr* в маркер-ориентированной селекции (marker assisted selection, MAS) продемонстрирована в работах (Alfares et al., 2009; Bouguennec et al., 2018). Так, на этапах получения гибридов от скрещивания линии Ct(FK5B), у которой пара хромосом 5B Courtot была замещена на пару гомологов от японского сорта Fukuho Komugi, имеющего хорошую скрещиваемость, с шестью сортами мягкой пшеницы с плохой скрещиваемостью и проведения беккроссов BC_2F_2 наличие/отсутствие рецессивных аллелей *skr* контролировали с помощью маркеров *Xcfb306* и *Xcfb341*. Практически все гибридные линии показали полное соответствие между наличием диагностических фрагментов маркеров *Xcfb306* и *Xcfb341* и ожидаемым уровнем скрещиваемости с рожью (Alfares et al., 2009).

В работе A. Bouguennec с коллегами (2018) для контроля передачи рецессивных аллелей *skr* от двух линий, полученных W. Alfares с коллегами (2009), в сорт пшеницы Barok использовали как уже известные SSR-маркеры *Xcfb306*, *Xcfb341* и *Xgwm234*, так и в дополнение к ним новые разработанные маркеры TGlc2 (ISBP), gene12 и gene13. Маркеры TGlc2, *Xcfb341* и gene12 косегрегировали с *SKr*. На каждом этапе скрещиваний (до BC_3F_2) гибридные формы тестировали на наличие молекулярных маркеров, благодаря чему подтвердили возможность их использования в контроле передачи рецессивного аллеля *skr* в другие сорта. Кроме того, диагностическая эффективность маркеров *Xcfb341*, TGlc2 gene12 и gene13 была проверена при генотипировании 15 сортов и линий мягкой пшеницы различного географического происхождения. По результатам молекулярного скрининга, 11 из них (12 по маркеру gene13) имели правильную ассоциацию «маркер-признак», а для четырех сортов с плохой скрещиваемостью (трех в случае gene13) соотнести признак и диагностические фрагменты маркеров не удалось – продукты амплификации у них по размеру соответствовали аллелю «легкой скрещиваемости» (Bouguennec et al., 2018).

Молекулярно-генетическое картирование гена *Kr1*

Сначала местоположение гена *Kr1* было определено на расстоянии 20 сМ от гена-супрессора гомеологичного спаривания хромосом *Ph1*, расположенного в проксимальной области хромосомы 5BL (Snape et al., 1995). По результатам последующих исследований (Lamoureux et al., 2002) *Kr1* был локализован на расстоянии 90.3 сМ от дистального конца 5BL между маркерами E33M60-233 (AFLP) и *Xgwm271* (SSR), при этом наблюдали независимое расщепление потомства по генам *Kr1* и *SKr*. Эффект QTL, который был соотнесен с *Kr1*, оказался намного слабее (a = 7.9 %; avp = 5.5 %) эффекта *SKr* (a = 15.9 %; avp = 22.1 %).

Для уточнения локализации гена *Kr1* I. Bertin с коллегами (2009) использовали популяцию рекомбинантных линий, полученных от скрещивания сорта Hobbit sib, несущего реципрокно-транслоцированные плечи хромосом 5BL-7BL и 5BS-7BS, с замещенной линией Hobbit sib (CS 5BL, 7BL), у которой длинные плечи хромосом 5B и 7B были замещены на гомологи от CS. Показано, что *Kr1* расположен в области 13 сМ между SSR-маркерами *Xgwm213* и *Xgwm371*. С помощью этих маркеров у мутантной линии *ph1b* подтверждена локализация вблизи *Ph1* гена *Kr1*. Сведения о локализации *Ph1* и установленная синтения содержащего его участка по отношению к хромосомам 9 риса и Bd4 коротконожки *Brachypodium distachyon* L. позволили привлечь новые маркеры для тонкого картирования *Kr1* и выявить два локуса, влияющих на скрещиваемость с рожью. Один из них расположен проксимально к гену *Ph1* на расстоянии не более 2 сМ и фланкирован EST-SSR-маркерами *Xw5145* (дистальное положение) и *Xw9340* (проксимальное положение). Этот район также включает SSR-маркер *Xgwm213*, ранее примененный для картирования *Kr1*. Другой участок – *Crossability region 2*, расположенный дистально от *Ph1*, оказался более важным для тонкого картирования, поскольку имел выше уровень рекомбинации, а также синтению с участком хромосомы Bd4 у *B. distachyon* размером 2.5 Мб. Участок *Crossability region 2* ограничен в интервале 14 сМ EST-SSR-маркерами 1275L15_cg3 и Os09g38060 и включает SSR-маркер *Xgwm371*. Линии с рекомбинацией по этому участку могли скрещиваться с рожью даже при наличии доминантных аллелей гена *Kr1*. Нужно отметить, что первоначальное картирование *Kr1* на участке в 13 сМ между SSR-маркерами *Xgwm213* и *Xgwm371* не позволило доказать присутствие второго локуса. Перечень молекулярных маркеров, сцепленных с локусами *Kr1* и *Crossability region 2*, представлен в Приложении.

В настоящее время удалось определить нуклеотидную последовательность доминантного (от сорта Mazhamai) и рецессивного (от CS) аллелей гена *Kr1*. Основой для этого послужили работы японских исследователей (Manickavelu et al., 2009a, b), изучивших экспрессию генов в тканях пестиков у рекомбинантных инбредных линий, различающихся по скрещиваемости с рожью, при этом впервые получены дифференциально экспрессированные фрагменты кДНК (differentially expressed fragment, DEF), среди которых, возможно, были фрагменты гена *Kr1*. Путем сравнения полученных DEF-последовательностей с последовательностями баз данных генбанков DDB Blastx и NCBI Blast обнаружены по меньшей мере две нуклеотидные последовательности, относящиеся к гену *Kr1*. Фрагмент DEF1 (#AB289691) был идентифицирован как гомолог гена *ZmPti1a*, который входит в группу PTI1-подобных киназ кукурузы (Pti1-like kinases) и участвует в стимулировании прорастания пыльцевых трубок. Последовательность DEF2 (#AB379558.1) была гомологична гену кальций-зависимой протеинкиназы риса – CDPK (Calcium-dependent protein kinase) – белку, участвующему в передаче различных стресс-чувствительных сигналов при развитии цветка (Manickavelu et al., 2009a, b).

На основе опубликованной последовательности кДНК гена *Kr1* (#AB379558.1) китайские ученые с помощью

[1] Приложение см. по адресу:
http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx7.pdf

специфичных праймеров амплифицировали фрагменты этого гена у трех сортов пшеницы (Cai et al., 2012). Полученные последовательности на 85 % были гомологичны гену *SRK* (S-locus receptor kinase) (Stein et al., 1991), который экспрессируется в тканях пестика и отвечает за распознавание пыльцы у вида *Brassica oleracea* L. Наряду с другими генами *S*-локуса, *SRK* участвует в контроле совместимости рыльца пестика с чужеродной пыльцой.

На базе той же частичной последовательности гена *Kr1* (#AB379558.1) были разработаны наборы праймеров, позволившие провести полное секвенирование этого гена с помощью методов прогулки по геному (Genome walking) и быстрой амплификации концов кДНК (Rapid amplification of cDNA ends) (Cai et al., 2016). У плохо скрещивающегося с рожью сорта Mazhamai (*Kr1*/–) последовательность гена имеет длину 4006 п. н. и содержит три интрона, четыре экзона общей длиной 1671 п. н., которые кодируют 557 аминокислот. У сорта CS (*kr1/kr1*) последовательность гена короче (3945 п. н.) и только на 56.24 % гомологична доминантной аллели. Основные отличия рецессивного аллеля от функционального отмечены в третьем и четвертом экзонах, которые у *kr1* содержат большое количество стоп-кодонов, что указывает на невозможность его экспрессии.

Другие гены скрещиваемости пшеницы с рожью

Все описанные выше гены и QTLs, ответственные за скрещиваемость с рожью, характерны для мягкой пшеницы. Синтетическая гексаплоидная пшеница имеет тот же геномный состав, что и мягкая, но обладает куда более широким генетическим разнообразием, поскольку для ее получения в качестве родительских форм привлекают различных представителей тетраплоидных пшениц ($2n = 4x = 28$, BBAA) и вида *Ae. tauschii* (Хакимова и др., 2019). Это позволяет предположить, что у синтетической пшеницы могут присутствовать другие локусы, эффект которых будет схож с генами семейства *Kr*.

Действительно, такие гены обнаружены у синтетической гексаплоидной пшеницы Am3, полученной путем гибридизации пшеницы карталинской *T. carthlicum* Nevski = *T. persicum* Vav. с *Ae. tauschii* (Zhang et al., 2011). После скрещивания Am3 с китайским сортом пшеницы Laizhou953 при последующем многократном беккроссировании и самоопылении гибридов популяция из интрогрессивных линий BC_5F_6 была подвергнута молекулярному скринингу с использованием 1256 SSR-маркеров. Всего было найдено 13 QTL, для 5 из них, *QCa.caas.1A*, *QCa.caas.2D*, *QCa.caas.4B*, *QCa.caas.5B* и *QCa.caas.6A*, влияние на скрещиваемость установлено в обеих провинциях. Наиболее сильный локус *QCa.caas.5B* в хромосоме 5BS был сцеплен с SSR-маркером *Xwmc149* (унаследован от *T. carthlicum*), что позволило ассоциировать его с геном *SKr*. Дополнительные локусы отмечены в хромосомах 1A, 2D, 4B и 6A. Локус *QCa.caas.1A*, полученный также от пшеницы карталинской и фланкированный SSR-маркерами *Xbarc213* (проксимальный) и *Xbarc287* (дистальный) в интервале 10 сМ в коротком плече хромосомы 1A, интересен тем, что он может относиться к *Kr4*. Как утверждают L. Zhang с коллегами (2011), три других локуса – *QCa.caas.2D* (унаследован от *Ae. tauschii*), *QCa.caas.4B* и *QCa.caas.6A* (унаследованы от Laizhou953) – были открыты впервые.

Заключение

Насущная необходимость обогащения генофонда пшеницы новыми аллелями генов, прежде всего контролирующими различные адаптивные реакции растений, стимулирует проведение исследований по поиску новых форм, легко скрещивающихся с представителями родов *Secale*, *Aegilops* и *Hordeum*, а также по изучению генетики межродовой совместимости. Полученные новые знания о генетической природе признака «легкая скрещиваемость» напрямую связаны с введением в генетический анализ разных типов молекулярных маркеров, построением насыщенных генетических карт хромосом пшеницы, молекулярно-генетическим картированием *Kr*-генов и идентификацией QTLs, влияющих на успех межродовой гибридизации, созданием доступного для изучения исходного материала и формированием общего информационного банка данных.

В настоящее время на молекулярно-генетических картах локализованы гены *SKr* и *Kr1* и разработано значительное число ассоциированных с ними маркеров. Однако примеры применения этих маркеров в маркер-ориентированной селекции пока немногочисленны. Как сообщается в литературе, с определенной долей успеха эффективны тесно сцепленные и косегрегирующие с геном *SKr* маркеры TGlc2, *Xcfb341* и gene12. В качестве дополнительных маркеров могут выступать gene13, *Xcfb306* и *Xgwm234*. С их помощью удалось отследить интрогрессию участка хромосомы, ассоциированного с легкой скрещиваемостью, в отдельные сорта пшеницы, что во многом ускорило процесс создания желаемых форм. Эффективность маркеров, сцепленных с геном *Kr1* и районом *Crossability region 2*, в контроле такой передачи, а также для скрининга *ex situ* коллекций пшеницы только предстоит проверить. Большие перспективы открываются в связи с опубликованной недавно полной нуклеотидной последовательностью гена *Kr1*, что дает возможность разработки внутригенных аллель-специфичных маркеров.

Поиск генов легкой скрещиваемости с рожью продолжается. Примером может послужить синтетическая гексаплоидная пшеница Am3, где с помощью молекулярных маркеров обнаружено три новых QTL. Созданные путем гибридизации пшеницы с рожью разного типа генетические линии, сорта, содержащие транслокации и интрогрессии с чужеродными аллелями генов, формируют ценный ресурс для проведения научных исследований и селекции.

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ORCID ID

I.V. Porotnikov orcid.org/0000-0001-5841-8803
O.Yu. Antonova orcid.org/0000-0001-8334-8069

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Изучение генетических факторов, определяющих признак «тетраостость» мягкой пшеницы

О.Б. Добровольская[1, 2] ✉, А.Е. Дресвянникова[1], Е.Д. Бадаева[3], К.И. Попова[4], М. Травничкова[5], П. Мартинек[6]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Российский университет дружбы народов (РУДН), Аграрно-технологический институт, Москва, Россия
[3] Институт общей генетики им. Н.И. Вавилова Российской академии наук, Москва, Россия
[4] Новосибирский государственный аграрный университет, Новосибирск, Россия
[5] Научно-исследовательский институт растениеводства, Прага, Чешская Республика
[6] Агротест Фито Лтд., Кромержиж, Чешская Республика
✉ e-mail: oxanad@bionet.nsc.ru

Аннотация. Ости – тонкие заостренные отростки, сформированные в дистальной части чешуй колоска соцветия некоторых видов злаков, включая такие экономически значимые культуры, как пшеница мягкая (*Triticum aestivum* L.) и твердая (*T. durum* Desf.), ячмень (*Hordeum vulgare* L.), рис (*Oryza sativa* L.), рожь (*Secale cereal* L.). Наличие длинных остей на колосковых чешуях характерно для одного вида пшеницы – *T. carthlicum* Nevski, киль колосковой чешуи которого переходит в длинный остевидный отросток или ость, равную по длине ости цветковой чешуи. Колос *T. carthlicum* имеет удвоенное число остей, а сам признак получил название «тетраостость» или персикоидность. Ости на месте килевого зубца колосковых чешуй могут формироваться у пшениц *T. aestivum* и *T. aethiopicum*, однако такие формы встречаются редко. Особенности развития признака тетраостости и его генетические детерминанты изучены мало. В настоящем исследовании рассмотрены особенности развития и наследования признака «тетраостость» линии CD 1167-8 мягкой пшеницы *T. aestivum* с применением классического генетического анализа, молекулярно-генетического картирования и сканирующей электронной микроскопии. Показано, что признак наследуется как рецессивный моногенный. Ген, контролирующий тетраостость линии CD 1167-8, картирован в длинном плече хромосомы 5A с использованием 15K-SNP-микрочипа, содержащего 15 000 ассоциированных с генами SNP пшеницы (Illumina Infinium 15K Wheat Array, TraitGenetics GmbH). Результаты теста на аллелизм продемонстрировали, что изучаемый ген аллелен *b1*, рецессивному аллелю гена-ингибитора остистости *B1* (5AL). Таким образом, ген, контролирующий формирование остей на колосковых чешуях мягкой пшеницы, является рецессивным аллелем гена ингибитора остистости *B1*. Новый аллель обозначен *b1.ag* (*b1. awned glume*). Анализ развивающегося соцветия линии CD 1167-8 с помощью сканирующей электронной микроскопии выявил, что зачатки остей колосковых чешуй формируются по мере развития и роста колосковых чешуй одновременно с развитием остей на цветковых чешуях, различий в развитии остей на цветковых и колосковых чешуях не обнаружено.
Ключевые слова: пшеница; *Triticum aestivum* L.; колос; остистость; тетраостость; молекулярно-генетическое картирование; SEM; ингибитор остистости *B1*.

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The study of genetic factors that determine the awned glume trait in bread wheat

O.B. Dobrovolskaya[1, 2] ✉, A.E. Dresvyannikova[1], E.D. Badaeva[3], K.I. Popova[4], M. Trávníčková[5], P. Martinek[6]

[1] Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] RUDN University, Agrarian and Technological Institute, Moscow, Russia
[3] Vavilov Institute of General Genetics of the Russian Academy of Sciences, Moscow, Russia
[4] Novosibirsk State Agricultural University, Novosibirsk, Russia
[5] Crop Research Institute, Prague, Czech Republic
[6] Agrotest Fyto, Ltd., Kroměříž, Czech Republic
✉ e-mail: oxanad@bionet.nsc.ru

Abstract. Awns are bristle-like structures, typically extending from the tip end of the lemmas in the florets of cereal species, including such economically important crops as wheat (*Triticum aestivum* L., *T. durum* Desf.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), and rye (*Secale cereale* L.). The presence of long awns adhered at tip end of glumes is a characteristic feature of "Persian wheat" *T. carthlicum* Nevski spike. Glume outgrowth of *T. carthlicum* Nevski spike passes into a long awn, equal in length to the lemma awn. Awned glumes can be formed in *T. aestivum* and *T. aethiopicum* wheats, however, such forms are rare. Features of the awned glume development and the genetic determinants

of this trait have been little studied. In this paper, we described the features of the development and inheritance of the tetra-awness (awned glume) trait of the bread wheat *T. aestivum* line CD 1167-8, using classical genetic analysis, molecular genetic mapping, and scanning electron microscopy. It was shown that the trait is inherited as a recessive monogenic. The gene for the awned glume trait of CD 1167-8 was mapped in the long arm of chromosome 5A, using the Illumina Infinium 15K Wheat Array (TraitGenetics GmbH), containing 15,000 SNPs associated with wheat genes. Results of allelism test and molecular-genetic mapping suggest that the gene for awned glumes in bread wheat is a recessive allele of the *B1* awn suppressor. This new allele was designated the *b1.ag* (*b1. awned glume*). Analysis of the CD 1167-8 inflorescence development, using scanning electron microscopy, showed that awns had grown from the top of the lemmas and glumes simultaneously, and no differences in patterns of their development were found.
Key words: wheat; *Triticum aestivum* L.; spike; awnedness; awned glume; molecular-genetic mapping; SEM; *B1* awn suppressor.

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Введение

Ости представляют собой тонкие заостренные отростки, сформированные в дистальной части чешуй соцветий некоторых видов злаков, в том числе экономически значимых сельскохозяйственных культур – пшеницы (*Triticum aestivum*, *T. durum*), ячменя (*Hordeum vulgare*), риса (*Oryza sativa*) и ржи (*Secale cereale*).

У дикорастущих злаков основная функция остей – распространение плодов (зерновок). Кроме того, они выполняют защитную роль и препятствуют поеданию плодов животными и птицами. В процессе доместикации эти функции утратили свое значение. Доместикация сопровождалась редукцией органов, способствующих распространению семян – остей, волосков, щетинок (Fuller, Allaby, 2018). У многих культивируемых сортов пшеницы и ячменя ости сохранились, однако, по сравнению с дикорастущими предками, стали короче, тоньше и легче (Peleg et al., 2010; Haas et al., 2019). Ости пшеницы, ячменя и ржи содержат хлоренхиму и являются фотосинтезирующими органами. Показано, что доля вклада остей в фотосинтезе колоса ячменя составляет 70–90 %. При этом 30 % всех сухих веществ зерна, в том числе 50 % крахмала, создается остями (Ионова, 2005). Наличие остей может способствовать увеличению урожайности в определенных климатических условиях (засушливость, повышенные температуры). Остистые формы преобладают среди сортов пшениц Австралии, Южной и Центральной Америки, США (Rebetzke et al., 2016). В условиях Северной и Центральной Европы остистость не обеспечивает адаптивные преимущества и среди сортов пшеницы преобладают безостые формы (Börner et al., 2005; Rebetzke et al., 2016).

Наружная цветковая чешуя у мягкой пшеницы несет ость или острый зубец (изредка вершина тупая, без зубца), на колосковых чешуях этого вида могут встречаться острые длинные (до 5 см) килевые зубцы (Дорофеев и др., 1979). У тетраплоидной пшеницы *T. carthlicum* Nevski (классификация В.Ф. Дорофеева (1979), синоним «Персидская пшеница» *T. persicum* Vav. – наименование вида, данное Н.И. Вавиловым) киль колосковой чешуи переходит в длинный остевидный отросток или ость длиной до 12 см. Длина ости колосковой чешуи равна длине цветковой чешуи, и весь колос имеет удвоенное число остей. Наличие длинных остей на колосковых чешуях *T. carthlicum* – одна из основных характерных черт этого вида. Ости вместо килевого зубца колосковых чешуй могут формироваться у некоторых рас *T. aestivum*, однако у других видов пшениц встречаются крайне редко (Вавилов, Якушкина, 1925). В.Ф. Дорофеев с коллегами (1979) отмечали наличие тетраостых колосьев у *T. aethiopicum*.

У мягкой пшеницы безостость доминирует над остистостью (Гончаров, 2012). Известны три доминантных неаллельных гена, ингибирующих развитие остей пшеницы, *B1*, *B2* и *Hd*, локализованные в хромосомах 5AL, 6BL и 4BS соответственно (Watkins, Ellerton, 1940; Sears, 1954, 1966; Kato et al., 1998; Sourdille et al., 2002; Yoshioka et al., 2017; Huang et al., 2020). Наиболее распространен аллель *B1*, он ингибирует развитие остей у гексаплоидных и тетраплоидных пшениц (Гончаров, 2002; Le Couviour et al., 2011; Mackay et al., 2014; Yoshioka et al., 2017). Для всех трех генов определена локализация в хромосомах с помощью молекулярно-генетического картирования (Yoshioka et al., 2017). Недавно D. Huang с коллегами (2020) показали, что ген *B1* кодирует транскрипционный фактор с мотивом «цинковые пальцы» C2H2-типа. Анализ гаплотипов *B1* показал, что доминантный аллель этого гена – наиболее распространенный ингибитор остей у пшеницы (Huang et al., 2020).

Признак «тетраостость» пшеницы изучен в меньшей степени. Н.И. Вавилов и О.В. Якушкина (1925) исследовали характер наследования ряда признаков, характерных для «Персидской пшеницы» *T. carthlicum*, среди которых был признак «длина остевидных придатков колосковых чешуй». Они провели масштабный гибридологический анализ, скрещивая *T. carthlicum* с различными видами ди-, тетра- и гексаплодных пшениц, а также эгилопсами и рожью (всего 64 комбинации скрещиваний), и изучили закономерности наследования признаков. Была отмечена сложная генетическая природа признака, показано, что он контролируется несколькими генами. В других работах был обнаружен рецессивный характер наследования признака тетраостости у *T. carthlicum* (Мигушова, Жуковский, 1969). По данным П.А. Гандиляна (1973), наличие тетраостости (персикоидности) связано со специфическим геном *T*. Р.В. Рожков с коллегами (2014) показали, что признак «тетраостость» *T. carthlicum* и *T. petropavlovskyi* при скрещивании с сортами твердой (*T. durum*) и мягкой (*T. aestivum*) пшениц соответственно, наследуется как рецессивный. При этом, если при скрещивании *T. petropavlovskyi* с мягкой пшеницей за формирование тетраостости

отвечает один ген, то в комбинациях с *T. persicum* влияние оказывают несколько генов.

Настоящее исследование посвящено изучению генетического контроля и особенностей формирования признака «тетраостость» мягкой пшеницы. С использованием молекулярно-генетического картирования была определена локализация главного гена, контролирующего тетраостый фенотип, в хромосоме 5AL мягкой пшеницы. Локализация гена и результаты теста на аллелизм предполагают, что этот ген является рецессивным аллелем ингибитора остистости *B1*.

Материалы и методы

Растительный материал. Исследовали линию мягкой пшеницы *T. aestivum* CD 1167-8 с тетраостым фенотипом, полученную д-ром П. Мартинеком (Агротест Фито Лтд., Кромержиж, Чешская Республика).

Изучение особенностей наследования признака «тетраостость» проводили на гибридах F_1, F_2 и F_3 от скрещивания CD 1167-8 и безостой линии мягкой пшеницы сорта Новосибирская 67 (Н67) и последующих самоопылений. Линия CD 1167-8 использована в скрещиваниях и как материнское, и как отцовское растение.

Тест на аллелизм проведен при скрещивании CD 1167-8 с остистой линией мягкой пшеницы Ruc 204, полученной д-ром П. Мартинеком. Эта линия была ранее детально охарактеризована с применением методов молекулярной генетики и цитогенетики (Добровольская, 2018). Все используемые в работе линии мягкой пшеницы имеют яровой тип развития.

Растения выращивали в полевых условиях на базе селекционно-генетического комплекса Института цитологии и генетики СО РАН (г. Новосибирск, 2015–2016 гг.), а также в полевых условиях в г. Кромержиж (Чешская Республика, 2016 г.). Оценку фенотипов колоса (наличие/отсутствие остей колосковых и цветковых чешуй) проводили после полного созревания растений. Соответствие фактического расщепления теоретически ожидаемому в популяциях гибридов оценивали по критерию χ^2 (Рокицкий, 1973).

Молекулярно-генетическое картирование. Образцы суммарной ДНК выделяли из листьев 90 индивидуальных растений популяции F_2 CD 1167-8 × Н67 (далее – картирующей популяции) и родительских линий CD 1167-8 и Н67, согласно методу J. Plaschke с коллегами (1995).

Генотипирование растений картирующей популяции выполняли с использованием 15K-SNP-микрочипа, содержащего 15 000 SNP, ассоциированных с генами пшеницы (Illumina Infinium 15K Wheat Array, TraitGenetics GmbH, Гатерслебен, Германия). Анализ полученных данных, построение молекулярно-генетической карты осуществляли при помощи программы MultiPoint версии UltraDense (Ronin et al., 2010, 2015), как описано ранее (Dresvyannikova et al., 2019). Графическое изображение молекулярно-генетической карты выполнено с помощью программы MapChart 2.2 (Voorrips, 2002).

Сканирующая электронная микроскопия. Развивающиеся соцветия линии CD 1167-8 вычленяли из вторичных побегов растений с использованием бинокулярного микроскопа Альтами ПС0745 («Альтами», Санкт-Петербург, Россия). Особенности строения соцветия изучали при помощи сканирующего электронного микроскопа Hitachi TM-1000 (Hitachi, Ltd., Япония) при постоянном ускоряющем напряжении 15 кВ и степени разряжения в камере для образца 30–50 Па. Растительный материал для сканирующей электронной микроскопии не подвергали предварительной обработке. Для получения и обработки изображений использовали программное обеспечение для Hitachi TM-1000.

Изучение кариотипа. Кариотип тетраостой линии CD 1167-8 изучен с применением С-дифференциального окрашивания, проведенном по раннее опубликованной методике (Badaeva et al., 1994). Препараты анализировали при помощи микроскопа Leitz Wetzlar (Leika microsystems, Германия). Для получения изображений использовали цифровую камеру CCD Leica DFC 280 (Leika microsystems). Хромосомы классифицировали в соответствии со стандартной номенклатурой (Gill et al., 1991).

Результаты и обсуждение

Линия мягкой пшеницы CD 1167-8 характеризуется наличием остей как на цветковых, так и на колосковых чешуях (рис. 1, *а*, *б*). Ости колосковых чешуй этой линии (2.5 ± 0.1 см) короче и тоньше остей цветковых чешуй. Признак проявлялся при выращивании растений линии в полевых условиях г. Новосибирска (2015–2016 гг.) и г. Кромержижа Чешской Республики (2016 г.), а также в условиях тепличного комплекса ИЦиГ СО РАН (2009–2017 гг.). Признак стабильно наследовался при самоопылении линии. Таким образом, наличие тетраостости изучаемой линии стабильно наследуется и проявляется в различных условиях выращивания.

Изучение ранних этапов развития соцветия линии CD 1167-8 мягкой пшеницы с использованием сканирующей электронной микроскопии показало, что зачатки остей колосковых чешуй формируются по мере развития и роста колосковых чешуй одновременно с развитием остей на цветковых чешуях (рис. 2). Особенностей, отличающих развитие остей колосковых чешуй от развития остей цветковых чешуй, не обнаружено.

Для выявления генетических детерминант тетраостости мягкой пшеницы были получены популяции гибридов от скрещиваний CD 1167-8 и безостой линии пшеницы Новосибирская 67 (Н67), в которых растение тетраостой линии CD 1167-8 использовано и как материнское (CD1167-8 × Н67), и как отцовское (Н67 × CD1167-8). Гибриды F_1 были безостыми, а в поколении гибридов F_2 наблюдали расщепление на безостые и остистые (тетраостые) формы с преобладанием безостых. Все остистые растения F_2 были тетраостыми.

Из 117 растений гибридов F_2 от скрещивания Н67 × CD 1167-8 34 были остистыми, а остальные 83 – безостыми, что соответствует моногенному рецессивному типу наследования признака «тетраостость» (χ^2 = 1.028, p = 0.05). Аналогичные результаты получены и в скрещивании CD 1167-8 × Н67 – соотношение остистых растений (41) к безостым (119) соответствует 1 : 3 (χ^2 = 0.033, p = 0.05), что означает моногенное рецессивное наследование признака. Различий в реципрокных скрещиваниях не обнаружено.

Fig. 1. A four-awned glume spike of the bread wheat line CD 1167-8.

Gray and black arrows designate awns of (l) lemma and (g) glume, respectively. G, grain; p, palea.

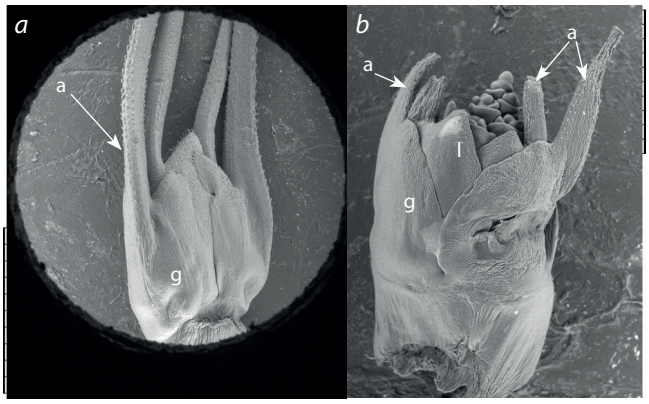

Fig. 2. SEM analysis of a spikelet of a developing bread wheat inflorescence in line CD 1167-8.

a – awns (a) on glumes (g) of a spikelet, scale bar 2 mm; *b* – the initial growth stage of glume and lemma (l) primordia, scale bar 1 mm.

При изучении наследования ряда характерных для «Персидской пшеницы» признаков Н.И. Вавилов и О.В. Якушкина (1925) отмечали, что развитие остевидных придатков на колосковых чешуях – хорошо наследуемый, мало зависящий от внешних условий признак. Обобщая результаты 62 комбинаций скрещиваний с различными видами пшениц, они сделали вывод о полимерном наследовании признака. Вместе с тем отмечено, что при скрещивании с безостыми формами безостость доминирует и в поколении гибридов F_2 выщепляются остистые формы, составляющие 1/4 часть от всех гибридных растений. Остистые формы остаются константными в F_3 (Вавилов, Якушкина, 1925). Рецессивный тип наследования подтвержден и другими учеными (Мигушова, Жуковский, 1969; Рожков и др., 2014). Кроме того, был обнаружен моногенный рецессивный тип наследования признака при скрещивании гексаплоидных видов пшениц (Рожков и др., 2014).

Результаты генетического анализа показали, что признак тетраостости у мягкой пшеницы также стабильно наследуется и находится под моногенным рецессивным контролем. Обращающей на себя внимание особенностью стало совместное наследование остистости колосковых и цветковых чешуй.

Локализация гена, контролирующего признак «тетраостость» у мягкой пшеницы, была определена с применением молекулярно-генетического картирования на субпопуляции F_2 CD 1167-8 × H67, включающей 90 растений. Из 90 растений картирующей популяции F_2 28 были остистыми (тетраостыми), остальные – безостыми. При самоопылении гибридов F_2 получены 90 семей гибридов F_3, анализ которых проводили в полевых условиях г. Новосибирска (1127 растений) и г. Кромержижа (954 растения). Анализ гибридов F_3 показал, что 28 семей от самоопыления тетраостых гибридов F_2 проявляли признак «тетраостость», а в семьях от безостых гибридов наблюдалось либо расщепление на остистые и безостые формы (43 семьи), либо потомки были безостыми (19 семей). Расщепление семей F_3 подтверждает моногенный рецессивный характер наследования признака 1 : 2 : 1 ($\chi^2 = 1.97$, $p < 0.05$). Признак «тетраостость» несколько варьировал в своем проявлении, но ни одного растения с парой остей, расположенных исключительно на цветковых или только на колосковых чешуях, не обнаружено.

Для молекулярно-генетического картирования использовали данные высокопроизводительного генотипирования. Всего проанализировано 5160 информативных SNP-локусов. Показано сцепление генетического локуса, контролирующего формирование тетраостого фенотипа, с маркерами хромосомы 5A (рис. 3, см. Приложение)[1]. Изучаемый ген расположен дистально по отношению к SNP-маркеру BS00023138-51 (на расстоянии 3 сМ).

Известно, что хромосома пшеницы 5AL несет доминантный ген-ингибитор развития остей – *B1*. Локализация гена, определяющего тетраостость, и гена *B1* в дистальном районе хромосомы 5AL совпадает (Yoshioka et al., 2017). На основании этого можно предположить, что тетраостость определяется рецессивным аллелем гена *B1*, который отличается от рецессивного аллеля, распространенного у сортов мягкой и твердой пшениц и приводящего к развитию пары остей на цветковых чешуях.

Далее был выполнен тест на аллелизм, линия CD 1167-8 с тетраостым колосом была скрещена с линией Ruc 204 с типичным для *T. aestivum* остистым колосом (ости развиваются только на цветковых чешуях). Анализ фенотипа гибридов F_1 показал, что все они были остистыми и имели ости как на цветковых, так и на колосковых чешуях. Таким образом, наличие ости на колосковой чешуе доминировало над отсутствием, однако длина ости у гибридов (1.4 ± 0.1 см) была короче, чем у тетраостого родителя (2.5 ± 0.1). Среди гибридов поколения F_2 совершенно безостых растений не обнаружено, все растения (176) имели ости на цветковых чешуях; на колосковой чешуе ости длиной 1–4 см обнаружены у 93 растений, у 83 растений

[1] Приложение см. по адресу:
http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx8.pdf

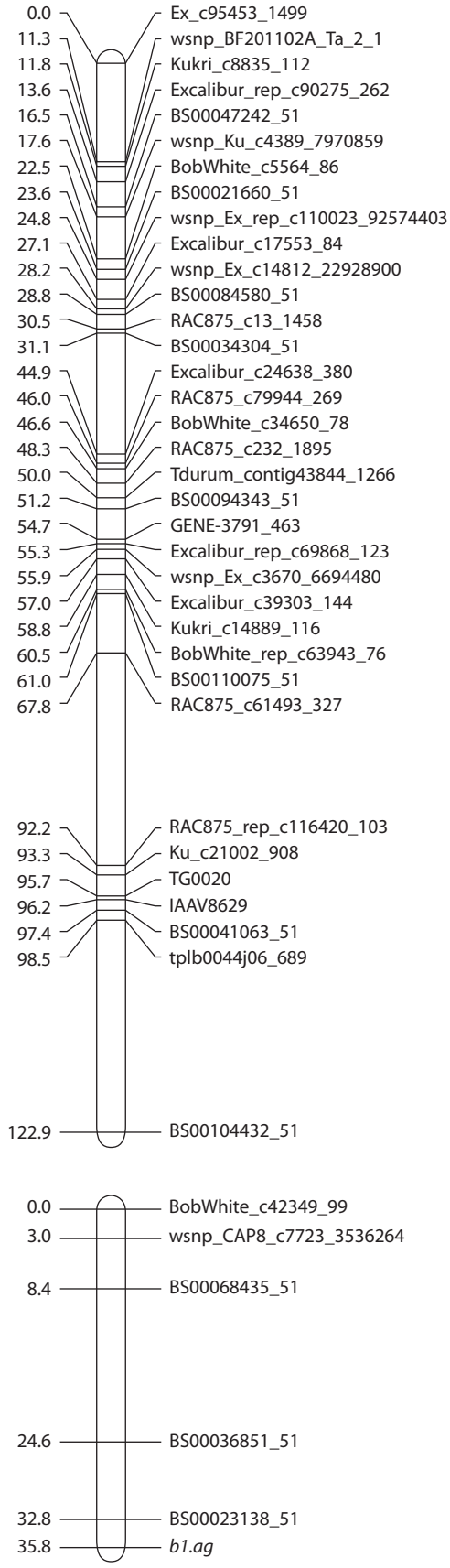

Fig. 3. A molecular map of chromosome 5A involving the *b1.ag* gene for the awned glume of bread wheat.

Distances in cM are shown on the left, and SNP markers are shown on the right (only skeleton markers representing groups of cosegregating markers are shown).

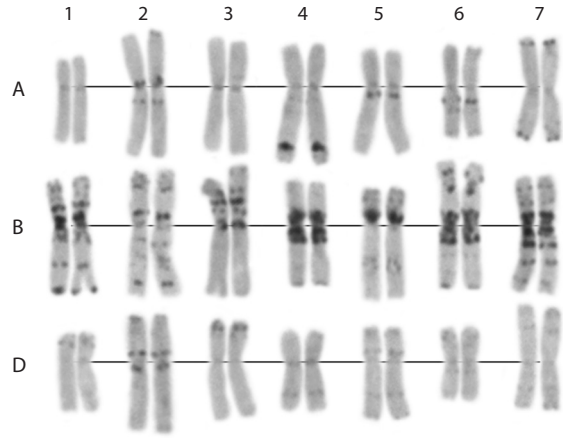

Fig. 4. Karyotype of the CD 1167-8 line (C-banding).

ости на колосковой чешуе не достигали длины 0.5 см, у 4 растений ости на колосковых чешуях полностью отсутствовали, как у линии Ruc 204.

Полученные результаты указывают на то, что ген, контролирующий тетраостый фенотип мягкой пшеницы, аллелен гену-ингибитору остистости *B1* и является его рецессивным аллелем. Этот аллель мягкой пшеницы описан нами впервые и обозначен как *b1.ag* (*b1. awned glume*).

Выявлено, что отсутствие остей под контролем гена-ингибитора остистости *B1* доминирует над их наличием (*b1* и *b1.ag*), при этом наличие остей на колосковой чешуе под контролем аллеля *b1.ag* доминирует над отсутствием под контролем аллеля *b1*. При этом характер расщепления признака «остистость колосковых чешуй», обнаруженный при скрещивании тетраостой CD 1167-8 и остистой Ruc 204 линий пшеницы, более соответствует количественному наследованию признака, следовательно, в контроле изучаемого признака наряду с *b1.ag* принимают участие и другие гены.

Н.И. Вавилов и О.В. Якушкина (1925) показали, что на особенности наследования остистости колосовых чешуй в комбинациях скрещиваний «Персидской пшеницы» с другими видами пшениц могут оказывать влияние не только наличие или отсутствие коротких (до 2 мм) остей у второго родителя, но и форма, ширина и другие особенности колосковых чешуй, что предполагает участие и других генов в контроле изучаемого признака.

Тетраостость не распространена широко среди образцов мягких пшениц. Н.И. Вавилов и О.В. Якушкина (1925) отмечали, что тетраостый фенотип, сходный с фенотипом «Персидской пшеницы», встречается среди туркестанских, персидских и бухарских мягких пшениц. Вместе с тем характерным (систематическим) признаком «тетраостость» является только для одного вида пшениц – *T. carthlicum*.

Кариотип линии CD 1167-8 изучен с использованием дифференциального С-окрашивания хромосом с целью выявления возможных сегментов интрогрессии от других видов пшеницы. Показано, что линия CD 1167 имеет кариотип, характерный для мягкой пшеницы (см. рис. 4). Вероятно, *b1.ag* – это редкий аллель гена *B1*, встречающийся у мягкой пшеницы.

Заключение

Тетраостость, или развитие остей на колосковых чешуях у мягкой пшеницы, наследуется как рецессивный моногенный признак и находится под контролем гена, локализованного в хромосоме 5AL. Обобщая результаты проведенного нами генетического анализа и молекулярно-генетического картирования, можно предположить, что ген тетраостости мягкой пшеницы является рецессивным аллелем ранее изученного гена-ингибитора остистости *B1*. Этот аллель описан нами впервые и обозначен как *b1.ag* (*b1.awned glume*). Показано, что отсутствие остей под контролем гена-ингибитора остистости *B1* доминирует над их наличием (*b1* и *b1.ag*), при этом наличие остей на колосковой чешуе под контролем аллеля гена *b1.ag* доминирует над отсутствием под контролем аллеля *b1*. Различий в развитии остей на колосковых и цветковых чешуях не обнаружено.

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Somatic embryogenesis in *Larix*: the state of art and perspectives

V.N. Shmakov[1], Yu.M. Konstantinov[1, 2] ✉

[1] Siberian Institute of Plant Physiology and Biochemistry of Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia
[2] Irkutsk State University, Irkutsk, Russia
✉ e-mail: yukon48@mail.ru

Abstract. Clonal propagation of conifers using somatic embryogenesis is essential for the selection of tree species, and for the implementation of afforestation and reforestation. In combination with cryopreservation, somatic embryogenesis creates the basis for the development of economically valuable lines of clones and elite genotypes. The industrial use of such genetically verified clone lines in forestry can significantly increase forest productivity compared to any conventional methods for improving tree crops that are available. Larch is considered as one of the main conifer candidates for large-scale reforestation, not only due to the vastness of its habitat, but also due to the unique quality of its wood, rapid growth and high ecological plasticity. However, the vast majority of larch species are characterized by uneven yields and extremely low seed quality. In this regard, obtaining planting material for reforestation from larch seeds on seed plantations is not advisable, but can be successfully implemented in afforestation programs using somatic embryogenesis technologies. Research on the somatic embryogenesis of larch has been conducted for over 30 years, which allowed considerable experience in this field to be accumulated. To date, the conditions for the initiation and maintenance of embryogenic cultures, as well as for the formation and development of somatic embryos have been determined. Significant progress has been made in the study of both the factors affecting these processes and the molecular mechanisms that underlie the various stages of embryogenesis. Nevertheless, despite the successes achieved, knowledge available today on the somatic embryogenesis of representatives of the genus *Larix* is still not enough to develop technologies for producing valuable plant-breeding material *in vitro*. This review analyzes the current state of research on the problem of somatic embryogenesis of representatives of the genus *Larix*. Particular attention is paid to the choice of explants for somatic embryogenesis, the composition of the media for cultivation, the dependence of the potential of somatic embryogenesis on the duration of cultivation, and the genetic control of somatic embryogenesis.
Key words: *Larix*; somatic embryogenesis; genetic control.

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Соматический эмбриогенез представителей рода *Larix*: состояние и перспективы

В.Н. Шмаков[1], Ю.М. Константинов[1, 2] ✉

[1] Сибирский институт физиологии и биохимии растений Сибирского отделения Российской академии наук, Иркутск, Россия
[2] Иркутский государственный университет, Иркутск, Россия
✉ e-mail: yukon48@mail.ru

Аннотация. Клональное размножение хвойных с использованием соматического эмбриогенеза имеет существенное значение для селекции древесных видов, реализации программ лесоразведения и лесовосстановления. В сочетании с криоконсервацией соматический эмбриогенез создает основу для получения хозяйственно ценных линий клонов и элитных генотипов. Использование в промышленных масштабах в лесном хозяйстве таких генетически проверенных линий клонов может значительно увеличить продуктивность лесов по сравнению с любыми доступными традиционными методами улучшения древесных культур. Лиственница считается одним из основных кандидатов для широкомасштабного лесовосстановления не только за счет обширности занимаемых ареалов, но и благодаря уникальному качеству ее древесины, быстрому росту и высокой экологической пластичности. Однако большинство видов лиственницы характеризуется неравномерностью урожаев и чрезвычайно низким качеством семян. В связи с этим получение посадочного материала для лесовосстановления из семян лиственниц на семенных плантациях нецелесообразно, но может быть успешно реализовано в программах по лесоразведению с применением технологий соматического эмбриогенеза. Исследования по соматическому эмбриогенезу лиственницы проводятся уже более тридцати лет, что позволило накопить значительный опыт в данной области. К настоящему времени изучены условия инициации и поддержания эмбриогенных культур, формирования и развития соматических зародышей. Достигнут значительный прогресс в изучении

как факторов, влияющих на эти процессы, так и молекулярных механизмов, лежащих в основе различных этапов эмбриогенеза. Однако имеющихся на сегодняшний день знаний о соматическом эмбриогенезе представителей рода *Larix* все еще недостаточно для разработки технологий получения селекционно-ценного растительного материала *in vitro*. В обзоре проведен анализ современного состояния исследований по проблеме соматического эмбриогенеза представителей рода *Larix*. Особое внимание уделено вопросам выбора эксплантов для соматического эмбриогенеза, составу сред для культивирования, зависимости потенциала соматического эмбриогенеза от продолжительности культивирования, генетическому контролю соматического эмбриогенеза.
Ключевые слова: *Larix*; соматический эмбриогенез; генетический контроль.

Introduction

Representatives of the genus *Larix* are widespread in the cool-temperate and cold (subarctic and subalpine) regions of the planet (Gowere, Richards, 1990; Kim, 2015). The genus *Larix* includes 10 to 25 species, native to the northern hemisphere of three continents: North America, Europe and Asia (Dylis, 1981; Koropachinsky, Milyutin, 2013; Pâques et al., 2013). In the forest fund of our country, larch forests are uppermost both in area (about 37 %, 264 million hectares) and in wood stock (31 %, 25.2 billion m^3); these markers surpass other species significantly (Efremov, Milyutin, 2010; Rysin, 2010). The issue of the exact number of larch species is to some extent controversial due to the ease of crossing *in vivo* and the production of hybrids, which, in turn, continue to hybridize (Wei, Wang, 2003; Koropachinsky, Milyutin, 2013).

Larch is considered one of the main candidates for extensive reforestation not only due to the vastness of its habitat, but due to the unique quality of its wood, rapid growth, and high ecological plasticity, too (Gowere, Richards, 1990; Bailian, Wyckoff, 1994). Larch is the only widespread polytypic deciduous conifer genus. It is a unique characteristic of this woody plant. Some way due to this quality, many larch trees can withstand extreme winter temperatures and low humidity levels (Bonga et al., 1995). The practical use of representatives of the genus *Larix* for reforestation is very effortful due to the low production and quality of seeds (Lelu et al., 1994a; Zhang Y. et al., 2012; Tretiakova et al., 2015). Therefore, it is not practical to obtain planting stock for reforestation from larch seeds in seed orchards. This problem can be solved using the methods of clonal propagation of embryos or seedlings derived from a limited number of seeds (Munoz-Concha, 2017). With *Larix* this has been done by grafting and rooting of stems taken from young seedlings and grown in a greenhouse. An alternative to this method is the use of the somatic embryo cultivation system, which makes it possible to derive an unlimited number of seedlings with the same genetic composition since they are derived from the same seed (Attree, Fowke, 1993; Isah, 2016).

Somatic embryogenesis can be much more efficient than traditional grafting. Cell lines produced by somatic embryos can be maintained in a juvenile state during indefinitely long time by their cryopreservation. Cryopreservation allows much longer field testings of cloned lines, while some of these lines are maintained in a physiological juvenile state until field testings show which of these lines are most preferable for mass propagation. That makes selection within families possible, which is not applicable to rooting by cuttings (Park, Bonga, 1992; Bonga, 2016). Moreover, the technology of using somatic embryogenesis can accelerate traditional reforestation programs by reducing the time required to obtain genetically improved trees (Kim, 2015). For conifers, unfortunately, the extensive use of somatic embryogenesis for practical propagation is often limited to only a few selected genotypes, and, in general, this process is still effortful and expensive. It is necessary to solve many problems for its universal use (Bonga, 2016; Klimaszewska et al., 2016).

This review is devoted to the analysis of the current status of research on the problem of somatic embryogenesis in representatives of the genus *Larix*. Hopefully, the acceleration of scientific progress in this area (due to the use of genomics, transcriptomics, proteomics, and metabolomics) will allow the development of new, more effective protocols of somatic embryogenesis for implementation in breeding, afforestation, and reforestation.

Choice of the explant type for somatic embryogenesis in larch

For the first time, the method of somatic embryogenesis in *Larix* was successfully applied in 1985 for *Larix decidua* (Nagmani, Bonga, 1985). Since that time, great progress has been made in this field for most larch species and its hybrids. Table 1 shows *Larix* species in which somatic embryogenesis was obtained and the first references. As noted in numerous studies, the formation of embryogenic cultures depends on the type and stage of development of the explant.

Initial work was mainly carried out using megagametophytes *L. decidua*, *L. leptolepis* (=*L. kaempferi*) and their reciprocal hybrids *L.* × *eurolepis* and *L.* × *leptoeuropaea* as explants (von Aderkas et al., 1987, 1990; von Aderkas, Bonga, 1988; Rohr et al., 1989). Using the megagametophytes made it possible to obtain haploid embryogenic cultures and, on their basis, somatic embryos. The plants formed from the latter were most often mixoploids with a predominance of diploid cells (von Aderkas, Anderson, 1993; von Aderkas, Bonga, 1993; von Aderkas et al., 2002). In the same years, successful attempts were made to initiate somatic embryos from protoplasts of *L. decidua* and a hybrid of *L.* × *eurolepis* (*L. decidua* × *L. leptolepis*) (Klimaszewska, 1989a; von Aderkas, 1992; Korlach, Zoglauer, 1995; Pattanavibool et al., 1998). However, according to the research carried out in subsequent years, somatic embryogenesis is initiated most efficiently in immature zygotic embryos. This fact was determined both for species of the genus *Larix* and for other representatives of conifers (Chen et al., 2010; Bonga, 2016;

Table 1. *Larix* species, explant type and basal cultural medium used to initiate somatic embryogenesis

Species	Explant type	Basal medium	Induction frequency	Reference
L. decidua	IPCZE	LM	N. a.	Cornu, Geoffrion, 1990
	ICZE	MSG	20	von Aderkas et al., 1990
	MZE	MSG	5	Lelu et al., 1994c
	Megagametophyte	MS, 1/2 MS, LM, 1/2 LM	0.3	Nagmani, Bonga, 1985
L. leptolepis	ICZE	MSG	15	von Aderkas et al., 1990
	IPCZE	LM, LP, MS	59–67	Kim et al., 1998
	MZE	LP, LM	22–38	Kim, 2015
	Megagametophyte	MSG	2	von Aderkas et al., 1990
L. × *eurolepis*	IPCZE	MSG, DCR, LM	6–12	Klimaszewska, 1989b
	ICZE	MSG	2	Lelu et al., 1994c
L. × *leptoeuropaea*	ICZE	MSG	26	Lelu et al., 1994c
	IPCZE	MSG	62	
	MZE	MSG	8	
	SE, cotyledons	MSG	12–98	Saly et al., 2002
	SE, needles	MSG	3	Lelu et al., 1994c
L. occidentalis	ICZE	1/2 LM	70	Thompson, von Aderkas, 1992
	IPCZE	1/2 LM	30	
	MZE	1/2 LM	1	von Aderkas et al., 1995
L. sibirica	IPCZE	MSG	67–98	Belorussova, Tret'yakova, 2008
L. gmelinii	IPCZE	1/2 MS, MSG	50–81	Tretyakova, Barsukova, 2012
L. sukaczewii	IPCZE	1/2 MS, MSG	98	
L. laricina	IPCZE	MSG	44	Klimaszewska et al., 1997
L. olgensis	ICZE	MS, S	N. a.	Song et al., 2016
L. principis-rupprechtii	ICZE	S, B	N. a.	Qi et al., 2000

Note. Explant type: MZE – mature zygotic embryo, IPCZE – immature precotyledonary zygotic embryo, ICZE – immature cotyledonary zygotic embryo, SE – somatic embryo.

Sarmast, 2018; Shuklina, Tret'yakova, 2019). In this case, pre-cotyledonary and cotyledonary zygotic embryos are most often used (Lelu et al., 1994a; Ogita et al., 1999a; Lu et al., 2005; Lelu-Walter, Pâques, 2009), although positive results were obtained when using zygotic embryos of earlier or later stages of development (Wang et al., 2007; Belorussova, Tret'yakova, 2008; Wang, Yang, 2010). There is little progress in initiating somatic embryogenesis in mature embryos. Thus, for the *L.* × *leptoeuropaea* hybrid (*L. leptolepis* × *L. decidua*), needles of somatic seedlings produced embryonic masses with a lower frequency (3 %) than mature somatic embryos of the same genotype (83 %) (Lelu et al., 1994c). Much more data on the successful initiation of somatic embryogenesis in mature embryos have been obtained to date for representatives of other taxonomic groups of conifers. Thus, embryogenic cultures were derived from mature zygotic embryos of *Pinus geradiana*, *P. kesya*, *P. koraiensis*, *Abies alba*, *A. nordmanniana*, *A. balsamea*, *A. fraseri*, *Picea abies*, *P. glauca, P. morisonicola*, *P. likiangensis*, *P. omorika*, and others (Yeung, Thorpe, 2005; Vooková, Kormuťák, 2007; Chen et al., 2010; Shuklina, Tret'yakova, 2019).

A significant disadvantage of using juvenile material (zygotic embryos) as explants for clonal propagation, including using somatic embryogenesis, is the impossibility of using the material from plants whose genetic potential has already appeared phenotypically (adult trees with strictly defined characteristics) (Bonga, 2017). Therefore, the use of vegetative parts of plants (segments of shoots and mature needles) as a primary explant is of greatest interest. Thus, there are a number of studies indicating the possibility of obtaining somatic embryogenesis from plant material from adult coniferous plants: primordial meristems of 2- and 10-year-old somatic plants *Picea glauca* (Klimaszewska et al., 2011; Klimaszewska, Rutledge, 2016), needles of 2-month- to 3-year-old somatic plants *Picea abies* (Harvengt et al., 2001), vegetative apexes of shoots, adventive buds and needles of adult trees *Pinus*

kestiya, *P. patula*, *P. roxburghii*, *P. sylvestris*, *P. wallichiana*, *P. pinea*, *P. radiata*, *P. pinaster* (Trontin et al., 2016a; Shuklina, Tret'yakova, 2019).

Difficulties in using plant material from adult trees are associated with the fact that significant physiological, biochemical, genetic and other changes in plant tissues occur at the vegetative and reproductive stages of development of adult trees. That significantly reduces the likelihood and frequency of embryogenesis from explants of adult trees (Klimaszewska et al., 2011). While the explant maturity increases, the genetic program for the induction of embryogenic tissue is gradually repressed; after the formation of the apical meristem, the potential for obtaining embryogenic tissue is completely disrupted (Bonga et al., 2010).

Other negative factors include the following: due to the presence of many compounds formed and released into nutrient media by plants, cell division and growth are inhibited, breakages of embryogenic response and normal expression of genes involved in the induction of somatic embryogenesis are observed (Isah, 2016; Sarmast, 2018). One of the important reasons for the above breakages is a change in the genomic DNA methylation pattern (von Aderkas, Bonga, 2000). To overcome the inability of the material of adult plants to form an embryogenic culture, the procedure of tissue rejuvenation is used in some cases. For this purpose, explants are placed in various kinds of stressful conditions: fasting, cold treatment at low positive temperatures, or using heavy metals as part of culture media (Bonga, 1996, 1997; Wendling et al., 2014). Rejuvenation is stimulated by a change in culture medium pH, use of enzymes of cell wall degradation, and decrease in the level of endogenous antioxidants (glutathione, ascorbic acid, vitamin E) by changing the content of exogenous auxins (von Arnold, 1987; Earnshaw, Johnson, 1987; Mo et al., 1996). The effect of auxin has been shown to increase the level of DNA methylation, which, in turn, leads to stimulation of cell division and their dedifferentiation; thus, it can stimulate the initiation of somatic embryogenesis (von Aderkas, Bonga, 2000).

In addition, tissues of adult coniferous trees contain many surface microorganisms reducing the tissue's ability to regenerate (Tretiakova et al., 2014). Unfortunately, to date, no effective methods have been developed to combat such bacterial contamination. Only a few procedures are known to reduce but not completely eliminate such contamination in plant tissue culture systems (Sarmast, 2018).

Despite decades of research efforts (Chalupa, 1989; Bonga, Pond, 1991; Bonga, 1996, 2004; Ewald, 1998), somatic embryogenesis from adult larch trees has not been derived down to recent times; it remains an urgent task for future research (von Aderkas, Bonga, 2000; Klimaszewska et al., 2016).

Effect of the parent plant genotype on the somatic embryogenesis in larch

The genotype of parental trees is much more likely the main factor (in addition to the explant type) determining whether somatic embryos will form (Tret'yakova, Barsukova, 2010; Bonga et al., 2010). In such a case, the initiation of somatic embryogenesis in many conifers is influenced by additive genetic variability, which provides an opportunity for selection to enhance the initiation pattern of somatic embryogenesis (Park, 2002). Somatic embryogenesis can be obtained only from certain genotypes; that significantly complicates the use of this technology for practical extensive propagation of conifers.

Experiments to study the genetic control of the initiation of somatic embryogenesis were carried out on representatives of several conifer genera, including the genus *Larix* (Klimaszewska et al., 2016). In Tretyakova et al. (Tretyakova et al., 2015), 200 *L. sibirica* trees were used. However, only one genotype initiated a stably maintained embryogenic culture. Further, the analysis of the results obtained indicates a stronger maternal than paternal effect on the culture initiation (Tretyakova et al., 2015; Klimaszewska et al., 2016). The maternal effect at the initiation stage can be explained by both the genotype and the stage of development or physiological state of the maternal tree, as well as inherited maternal alleles of the zygotic embryo (Niskanen et al., 2004).

Composition of culture media for embryogenic masses and somatic embryos of larch

The growth and development of the embryogenic culture of conifers, including representatives of the genus *Larix*, is strongly influenced by the nutrient medium composition. The choice of salts (micro- and macroelements) and organic components, as well as correction of their balance and concentrations play an important role both in the induction of callusogenesis and in the further maintenance of the resulting culture *in vitro* (Pâques et al., 2013). Moreover, the choice of the nutrient media composition often depends on the plant species and the type of material used as an explant (Isah, 2016).

The following basal media are used in studies on the somatic embryogenesis in larch, depending on the tasks to be solved and the initial plant material (see Table 1): LM (full or half strength (1/2 LM)) (Litvay et al., 1985), MS (full or half strength (1/2 MS)) (Murashige, Skoog, 1962), MSG (Becwar et al., 1990), LP (Quoirin, Lepoivre, 1977), S (Ewald et al., 1995), B (Ewald et al., 1997), AI (Tret'yakova et al., 2012), DCR (Gupta, Durzan, 1985), WPM (Lloyd, McCown, 1980). During all the stages of cultivation, the basal medium is supplemented with such organic compounds as: L-glutamine (0.05–1.5 g/L); myo-inositol (0.1–1.0 g/L); casein hydrolyzate (0.5–1.0 g/L); ascorbic acid (0.4 g/L) (Cornu, Geoffrion, 1990; von Aderkas et al., 1990; Lelu et al., 1994c; Klimaszewska et al., 1997; Kim, 2015; Tretyakova et al., 2015).

Phytohormones are key components of the nutrient medium that control the entire process of somatic embryogenesis (von Aderkas et al., 2001; Vondráková et al., 2016). Moreover, their composition and ratios depend on the development stage of somatic embryos. During the induction of embryogenic masses, the presence of endogenous auxins in combination with cytokinins is necessarily in the medium. The exception is species *Abies*, in which only cytokinins are most often required for the induction of embryogenesis (Pullman, Frampton, 2018). The use of 2,4-dichlorophenoxyacetic acid (1.0–2.0 mg/L) in conjunction with 6-benzylaminopurine (0.5–1.0 mg/L) is shown in the overwhelming majority of studies on embryogenesis in representatives of the genus *Larix* (Klimaszewska, 1989a, b; Korlach, Zoglaue, 1995; Lelu-Walter, Pâques, 2009;

Tretyakova et al., 2019). In a number of studies, naphthylacetic acid, picloram, or 4-chlorophenoxyacetic acid at 1.0 mg/L (Qi et al., 2004; Kim, 2015) and kinetin (0.1–5.0 mg/L) as a representative of cytokinins (Cornu, Geoffrion, 1990; Qi et al., 2000; Song et al., 2016) are mentioned as auxins. Indoleacetic acid, as shown for *L. leptolepis*, plays an important role in controlling the germination of somatic embryos (Li Z. et al., 2017a, b).

At the maturation of somatic embryos, abscisic acid becomes the most important component of the nutrient medium (Lelu et al., 1994b, 1995). The optimal content of this phytohormone (0.01–32.0 mg/L) and the cultivation time of somatic embryos in its presence (1–4 weeks) vary significantly in different larch species (Label, Lelu, 1994, 2000; von Aderkas et al., 1995, 2002, 2015; Gutmann et al., 1996; Klimaszewska et al., 1997; Ogita et al., 1999b; Kim, Moon, 2007; Tret'yakova et al., 2012; Song et al., 2018). Sometimes, indolebutyric acid at 1.0 mg/L (Tret'yakova et al., 2012), 5.0 mg/L of auxin transport inhibitor 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB), 5.0 mg/L phloroglucinol (auxin synergist) (Kim, Moon, 2009) or silver nitrate (2.0–5.5 mg/L) (Saly et al., 2002; Song et al., 2018) are used together with abscisic acid to improve the process of maturation of somatic embryos. Improving the quality of somatic embryos, their germination and the formation of full-fledged plants is achieved by combining abscisic acid with activated carbon (0.5–10 g/L), which is introduced into the nutrient medium during the pre-maturation of somatic embryos (Harry et al., 1991; Qi et al., 2004; Umehara et al., 2004; Klimaszewska et al., 2016; Tretyakova et al., 2016). Considering that maturing somatic embryos should be exposed to water stress, similar to developing zygotic embryos *in vivo*, substances such as polyethylene glycol 3000–4000 at 4–10 %, sucrose at an increased concentration (3–8 %) or maltose (3 %), and gelling agents gelright or phytagel (0.3–0.4 %) are introduced into the nutrient medium in order to reduce the available water (Klimaszewska et al., 1997; Ma et al., 1998; Qi et al., 2004; Lu et al., 2005; Teyssier et al., 2011; Tret'yakova, Barsukova, 2012; Tret'yakova et al., 2012; Song et al., 2018). At the initial stages of the induction of the embryogenic culture development, the concentration of sucrose used is 1–3 % (von Aderkas et al., 1987; Lelu et al., 1994c; Kim, 2015); agar at 0.7 % is most often used as a gelling agent (Klimaszewska, 1989b; von Aderkas et al., 1990; Belorussova, Tret'yakova, 2008).

In addition to studying the positive effect of certain compounds making up the culture media on various stages of somatic embryogenesis, we also studied substances whose presence in the medium negatively affects the culture *in vitro*. For *L.* × *leptoeuropaea*, it was shown that the atmosphere enrichment with ethylene or the addition of 2-chloroethylphosphonic acid (5.0 and 10.0 mg/L) or 1.0–10.0 mg/L of 1-aminocyclopropane-1-carboxylate to the culture medium greatly reduced the induction of secondary somatic embryogenesis (Saly et al., 2002). In *L. leptolepis*, vanillyl benzyl ether and 4-[(phenylmethoxy)methyl] phenol inhibited the early development of somatic embryos, namely, the differentiation of suspensors (Umehara et al., 2005, 2007). These substances were shown to be present in sufficient for inhibition amounts in a high cell density suspension culture, while they were at significantly lower, non-deleterious concentrations in a low cell density culture (Umehara et al., 2004).

To date, extensive experience has been accumulated in the field under study. However, due to the still low efficiency of somatic embryogenesis of representatives of the genus *Larix*, work on optimizing the nutrient medium composition, including specific sugars, vitamins, organic acids and modifiers of redox potential, etc. continues.

Dependence of somatic embryogenesis potential of larch trees on the culture age *in vitro*

In works on the induction and maintenance of an embryogenic culture, a serious attention is paid to the efficiency of obtaining somatic embryos in culture *in vitro* during long periods of time. The issue is of both fundamental and applied importance for reforestation programs requiring long-term regenerated trees testing from seperate cell lines to their extensive use. Consequently, tissue culture lines should be maintained in a functionally unchanged form until the elite characteristics of the regenerants derived from them are experimentally confirmed (Charest, Klimaszewska, 1995). Although, the age of the embryogenic culture, i. e., the number of subcultures, can undermine their ability to regenerate full-fledged somatic embryos (Pâques et al., 2013). That may be primarily connected with an increase in the rate and accumulation of a large number of mutations and general genetic instability of cultures kept *in vitro* for a long term as a result of somaclonal variability (Krutovsky et al., 2014; Klimaszewska et al., 2016).

Somaclonal variability can appear on morphological, cytological (number and structure of chromosomes), biochemical (metabolic disorders) and molecular genetic (nucleus and organelle genomes) levels (Cyr, Klimaszewska, 2002). Partially differentiated cultures, such as *in vitro* embryonic masses, were found to show less variability than true callus-type cultures (Cyr, 1999). Moreover, embryogenic coniferous cultures are considered genetically more stable as opposed to angiosperms (Isabel et al., 1996). A number of studies assessing the level of somaclonal variability in embryogenic cultures of conifers showed the absence of any somaclonal changes in embryogenic tissues and in somatic embryos of *Picea abies*, *Picea glauca* × *P. engelmannii*, *Pinus pinea*, *Picea mariana* (Heinze, Schmidt, 1995; Isabel et al., 1996; Cuesta et al., 2008; Krutovsky et al., 2014). In general, it is seen that embryogenic cultures of representatives of the genus *Larix* have a relatively high stability (Klimaszewska et al., 2016). Thus, in *L.* × *eurolepis*, the embryogenic line was stable after 4 years of subculturing (Pâques et al., 2013), and in *L. leptolepis* it was stable for 9 years (Wang et al., 2007; Lelu-Walter, Pâques, 2009). In the latter case, the embryogenic cultures became non-embryogenic over the course of time (Li W. et al., 2013). The embryogenicity of *L. decidua* cultures derived from haploid material (megagametophytes) was not lost for 9 years (Pattanavibool et al., 1995). In such case, almost all lines doubled ($2n = 24$) their number of chromosomes during the observation period, but both haploid and dihaploid lines remained embryogenic. Further studies (17-year-old culture) showed that none of the lines retained constant embryogenicity

during the course of the entire cultivation period (von Aderkas et al., 2003). In several lines, the embryogenic potential was completely lost, while in others the loss was temporary since there was an embryogenesis periodic restoration. The proliferative activity of 15 embryogenic cell lines of *L. sibirica* persisted for 2–8 years (Pak et al., 2016; Tretyakova, Pak, 2018). The chromosome ploidy of the cells of these lines did not change until two years of cultivation (Tretyakova et al., 2017). Further, a scatter of chromosome numbers from 24 to 30 and a large number of mitosis and micronuclei cells pathologies were revealed (Goryachkina et al., 2017). However, there were separate cell lines, in which the cultures genetic stability was preserved for up to 7 years. According to microsatellite analysis, embryogenic lines were characterized by weak allelomorphic variability (Tretyakova et al., 2017). In general, nonetheless, the capacity of somatic embryos from long-term maintained lines for maturation and germination decreased sooner or later (Tretyakova et al., 2016).

Embryogenic masses long-term cultivation, leading to the formation of somatic embryos and plants, can lead to rare phenotypic anomalies in *Picea glauca* and *P. mariana* (Isabel et al., 1996; Tremblay et al., 1999) or to genetic instability in *Pinus sylvestris* and *P. pinaster* (Burg et al., 2007; Marum et al., 2009). The changes in the relative content of mitochondrial DNA were observed in embryogenic tissues in *Larix leptolepsis*, *L. decidua*, and their reciprocal hybrids (DeVerno et al., 1994). Thus, despite the relatively high stability of embryogenic cultures of representatives of the genus *Larix*, the quality and quantity of somatic embryos changes over time. In this respect, long-term maintenance of these cultures with the method of regular subcultivation does not make sense.

Embryogenic lines cryopreservation

Aside from the main issue, which is the decrease or loss of the embryogenic culture potential against the background of possible genetic changes in the course of the long-term maintenance *in vitro*, it is also worth considering high labor costs if regular subcultivation is necessary; there is also an increasing risk of material loss caused by the pollution, human errors or technical failures.

The embryogenic cultures periodic re-initiation can be the solution to these problems. At the same time, this rather time-consuming and expensive procedure cannot be used for the species, including conifers, for which the most suitable explants for the embryogenic callus induction are available only during limited time of year (Ozudogru, Lambardi, 2016).

Another approach to overcome the forenamed difficulties is to use the technique decreasing the growth rate and increasing the subculture intervals by the way of incubation at a low temperature (4–5 °C) and low light intensity (for example, 10 μmol/m^2/s), change of the conservation medium osmotic potential, reduction of the inorganic nutrients intake, addition of growth retardants to the culture medium (Hassan, 2017). Preservation within the minimal growth conditions is a very simple method to keep the culture *in vitro* during the periods from 6 to 12 months, but no more than 3 years, depending on the plant species (Ozudogru et al., 2010). Longer crops seasoning under such conditions leads to a sharp drop in the plant regeneration frequency and an increase in the genetic changes number.

For a long-term stable preservation of embryogenic cultures, cryopreservation is an ideal method ensuring their safety and stability (Charest, Klimaszewska, 1995). Embryogenic cultures can be kept in liquid nitrogen at –196 °C or at –150 °C in the nitrogen vapor phase without any time limitations and the juvenility loss (Park et al., 1998). This method provides long-term preservation of various types of tissues and organs, including shoot tips, somatic and zygotic embryos, whole seeds, pollen, anthers and buds (Vendrame, 2018).

There are different types of cryopreservation methods. Traditional methods are based on the freeze-induced dehydration. Among various cryopreservation methods available for embryogenic cultures, the most common approach is slow material cooling. In the last few years this approach has allowed developing some effective protocols for the material preservation without loss or with a slight loss of regenerative capacity for a long time (up to 20 years) for various species such as broadleaf (*Citrus* ssp., *Hevea brasiliensis*, *Fraxinus excelsior*, *Quercus suber*, *Q. robur* et al.) and conifers (*Abies cephalonica*, *Picea abies*, *P. glauca*, *P. sitchensis*, *Pinus caribaea*, *P. nigra*, *P. patula* et al.) (Ozudogru, Lambardi, 2016).

Cryopreservation methods are developed and successfully applied to hybrids of *L.* × *eurolepis* and *L.* × *leptoeuropaea* (Klimaszewska et al., 1992; Pâques et al., 2013). The use of these methods made possible the achieving of the growth resumption of all tested lines after their thawing. In addition, cryopreservation and its duration (at least 18 years) did not influence noticeably the somatic embryos productivity (Lelu-Walter, Pâques, 2009). Hybrid larch trees cryopreserved lines have been routinely used in experiments for many years. However, until now there is no data on the cryopreservation methods use for long-term preservation of embryogenic lines of the main part of larch species, except for their two hybrid forms. At the same time, the successful experience of the cryopreservation methods use for representatives of other conifer genera creates promising prospects for wider application of this technology for long-term preservation of larch species embryogenic cultures.

Genetic control of larch trees somatic embryogenesis

At present, in order to explore the molecular mechanisms of the somatic embryogenesis process, a lot of attention is paid to the study of the entire genome profiling on the basis of transcriptomics, proteomics, and metabolomics (Trontin et al., 2016b). Thus, it was shown that during *Larix* species cell cultures somatic embryogenesis *in vitro* changes in the mitochondrial genome organization and the relative representation of some genomic regions occur (DeVerno et al., 1994). 454 libraries containing cDNA sequences were created under the study of various stages of somatic embryogenesis of *L. leptolepis* using the method of RNA sequencing (Zhang Y. et al., 2012). It's shown that 25773 identified transcripts are connected with 160 biochemical pathways of primary and secondary metabolism. 78 % of genes connected with embryo-

genesis were completely homologous to those of *Arabidopsis thaliana.* The genes of the transcription factors *LaMYB33* and *LaSCL6* are important for the preservation of competence and maintenance of the state of embryogenicity in *L. leptolepis* as a part of the gene expression regulation epigenetic complex (Li S. et al., 2013; Li W. et al., 2014). During early embryogenesis, the genes *LdLEC1* and *LdWOX2* (*L. decidua*) (Rupps et al., 2016), *LaSERK1* (*L. leptolepis*) (Li L. et al., 2013) play an important role. *LaNFYA1*, *LaNFYA2*, *LaNFYA3*, and *LaNFYA4* play an important role during early stages of determination and at the beginning of somatic embryo maturation (*L. leptolepis*) (Zhang L. et al., 2014). At the precotyledonary stage of somatic embryo development, the expression of antioxidant defense genes (*SOD*, *CAT*, and *APX* (*L. leptolepis*)) is required (Zhang S. et al., 2010a). At the stages of initiation and late maturation of somatic embryos, the expression of genes connected with the auxins synthesis or transport increases: *LaHDZ31*, *32*, *33* and *34* (*L. leptolepis*) (Li S. et al., 2013; Li Z. et al., 2017b), *LaNIT* (*L. leptolepis*) (Li Z. et al., 2018). *LmAP2L1 (L. × leptoeuropaea*) (Guillaumot et al., 2008), *LkBBM* (*L. leptolepis* × *L. olgensis*) (Li K. et al., 2014), *LdBBM* and *LdSERK* (*L. decidua*) (Rupps et al., 2016) are the most active at the stage of somatic embryo germination. Herewith, the *LmAP2L2* gene of the transcription factor is constitutively expressed at all the embryogenesis stages (*L. × leptoeuropaea*) (Guillaumot et al., 2008).

The highly-productive sequencing strategy was used to identify miRNAs involved in the corresponding target genes regulation at certain stages of somatic embryogenesis in *L. leptolepis* (Zhang J. et al., 2012). More than 100 target genes have been identified for 60 miRNAs. Differential expression of different miRNAs (miR156, miR159, miR160, miR162, miR165, miR166, miR167, miR168, miR169, miR171, miR172, miR397, miR398) was found in embryogenic and non-embryogenic *L. leptolepis* cultures (Zhang J. et al., 2012; Zhang L. et al., 2014; Li S. et al., 2013; Li W. et al., 2013, 2014). In conifers, miRNAs regulate the activity of most genes of transcription factors, including genes involved in the embryogenesis process. The transcription factor *MYB* (*LaMYB33*) was identified as a target gene for miR159 (Li W. et al., 2013). In its turn, *LaHDZ31*, *LaHDZ32*, *LaHDZ33*, and *LaHDZ34* are regulated by miR165/166 (Li Z. et al., 2016). MiR169 targets are *LaNFYA1*, *LaNFYA2*, *LaNFYA3* and *LaNFYA4* (Zhang L. et al., 2014), and the homologue *Larix SCARECROW-LIKE 6* (*LaSCL6*) is a target for miR171 (Zang et al., 2019). These genes post-transcriptional regulation with miRNA can be involved in maintaining the developmental potential, as described above, at various stages of somatic embryogenesis in representatives of the genus *Larix*. The genes identified in representatives of the genus *Larix*, playing an important role in somatic embryogenesis at different stages, are summarized in Table 2.

Table 2. Genes involved in somatic embryogenesis in representatives of the genus *Larix*

Species	Embryogenesis stage	Gene identified	Reference
L. kaempferi	Early embryogenesis	*miR159*, *169*, *171*, *172*	Zhang S. et al., 2010b
	Competence determination and maintenance of embryogenic state	MYB-like (*MYB33*), *miR159*	Li S. et al., 2013; Li W. et al., 2013
		Scarecrow-like (*SCL6*), *miR171*	Li W. et al., 2014; Zang et al., 2019
	Precotyledonary stages of somatic embryo development	Superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*)	Zhang S. et al., 2010a
	Precotyledonary and cotyledonary stages of somatic embryo development	*TAS3/miR390*, *laccase/miR397*, *plastocyanin/miR398*, *ARF/miR160*, *miRNA167*, *class III HD-ZIP/miR166*, *miRNA156*	Zhang J. et al., 2012
	All stages of embryogenesis	*miRNA162*, *miRNA168*	
	Early embryogenesis	*LaSERK1*	Li L. et al., 2013
	Early stages of determination and beginning of somatic embryo maturation	*LaNFYA1*, *LaNFYA2*, *LaNFYA3*, *LaNFYA4*, *miR169*	Zhang L. et al., 2014
	Stages of induction and somatic embryo late maturation	*LaHDZ31*, *LaHDZ32*, *LaHDZ33*, *LaHDZ34*, *miR165/166*	Li Z. et al., 2016, 2017b
		LaNIT	Li Z. et al., 2018
L. × marschlinsii	Stages of somatic embryo maturation and their germination	Germin-like protein 1 (*LmGER1*)	Mathieu et al., 2006
	Stages of somatic embryo germination	Apetala 2-like (*LmAP2L1*)	Guillaumot et al., 2008
	All stages of embryogenesis	Apetala 2-like (*LmAP2L2*)	
L. decidua	Early embryogenesis	*LdLEC1*, *LdWOX2*	Rupps et al., 2016
	Late embryogenesis, stages of somatic embryo germination	*LdBBM*, *LdSERK*	
L. leptolepis × *L. olgensis*	Stages of somatic embryo germination	*LkBBM*	Li K. et al., 2014

Considering the future prospects of genomic studies of *Larix* species cell cultures somatic embryogenesis, it is possible to note the fundamental importance of clarifying such important theoretical and applicative issues as (1) structural and functional changes in the mitochondrial genome of larches occurring in culture *in vitro*, and the possibility of their reversion; and (2) genetic regulation of the interaction of the nucleus, mitochondria and chloroplasts genomes during somatic embryogenesis. It is obvious that the success of the development of this technology for cell culture *in vitro* will largely depend on the resolution of the issues mentioned above.

Work to determine changes in the proteome and composition of fatty acids at different development stages of embryogenic masses and somatic embryos of a number of larch species was carried out. Comparative proteomic analysis of embryogenic and non-embryogenic *L. principis-rupprechtii* calli revealed 503 proteins, 71 of which were differentially regulated (Zhao et al., 2015b). In addition, proteins were analyzed at three stages of somatic embryo development of the same larch species: stages of proembryogenic mass, globular and cotyledonary embryos. 96 proteins differentially expressed at different stages of development were identified. Functional analysis showed that the content of proteins involved in the primary metabolism, phosphorylation, and maintenance of the cellular redox potential, increases during the development of somatic embryos. The study of the general profile of *L.* × *eurolepis* proteins showed significant differences in their content at certain stages of somatic embryo maturation (Teyssier et al., 2014). It was found that the 147 proteins found in the work are mainly involved in the primary metabolism and stabilization of the resulting metabolites. Thus, storage proteins identified as legumin- and vicilin-like appeared at the precotyledonary stage of development.

When studying the fatty acid composition of the lipids of embryogenic and non-embryogenic *L. sibirica* calli, a high content of oleic acid in total lipids of the embryogenic cell culture was found against the background of a lower content of linoleic acid compared to the non-embryogenic callus (Makarenko et al., 2016). The review proposes to use the concentration of these fatty acids as a marker of embryogenic potential in the selection of promising cell lines of Siberian larch during early embryogenesis. Significant differences in the composition and content of neutral lipids in tissues of embryogenic and non-embryogenic *L. sibirica* cell lines were also revealed (Semenova et al., 2020). Glycerides were found to be dominant lipids of two types of lines. Triglycerides and 1,2-diglycerides accumulated more actively in embryogenic cell lines, while the content of sterol esters in these lines was reduced.

Nevertheless, despite significant advances in understanding the molecular genetic mechanisms underlying somatic embryogenesis in conifers, in particular representatives of the genus *Larix*, there is currently an urgent need to expand complex research in this field of study in order to get new knowledge necessary for the development of methods and approaches to obtaining plant material *in vitro* and using it in reforestation and afforestation programs.

Use of the method of somatic embryogenesis in representatives of the genus *Larix* in reforestation breeding programs

The method of individual conifer species microclonal propagation by somatic embryogenesis has already begun to be widely used in various countries of the world, primarily in France, Canada, Germany, Great Britain, Ireland, Scandinavian countries, China, in plantation forestry and in the implementation of MVF (Multi Variety Forest) programs (Park et al., 2016). MVF is defined as the use of a range of genetically tested woody species in a production forest seed orchard (Weng et al., 2011). Back in the mid-90s of the last century, INRA (National Institute for Agricultural Research) in France organized studies on somatic embryogenesis in hybrid larch species (Pâques et al., 2013). This led to the development of an improved procedure leading to the routine production of regenerant plants from somatic embryos. The new protocol was applied for the hybrid *L.* × *leptoeuropaea* cultivar REVE-VERT propagation (Lelu-Walter, Pâques, 2009). In the same years, a program of breeding hybrid *L.* × *eurolepis* was launched at the state enterprise "Staatsbetrieb Sachsenforst" (Germany), based on the achievements in the field of combining the method of clonal propagation and the original plant material having an excellent genetic background (controlled crosses) (Kraft, Kadolsky, 2018). Breeding programs, including biotechnological approaches of somatic embryogenesis and genetic engineering, were launched in China and aimed at improving the existing gene pool of the local species *L. principis-rupprechtii* (Zhao et al., 2015a). Genetic engineering methods in conjunction with somatic embryogenesis of larch trees were successfully tested in laboratory conditions a long time ago. At the same time, both agrobacterial and bioballistic transformation were used to introduce genes into cells of embryogenic culture and directly somatic embryos of *L. laricina*, *L. leptolepis*, *L.* × *eurolepis*, *L. principis-rupprechtii* (Klimaszewska et al., 1997; Levée et al., 1997; Qi et al., 2000; Li Z. et al., 2016). In the next while, for the genetic improvement of conifers, it is planned to use the rapidly developing and promising genomic editing technologies based on the CRISPR-Cas system along with traditional methods of transformation. These technologies have already been successfully applied to broad-leaved tree species, such as grapefruit (*Cītrus paradīsi*), orange (*Cītrus* × *sinēnsis*), apple (*Malus domestica*, *M. prunifolia* × *M. pumila*), poplar (*Populus tomentosa*, *P. tremula* × *alba*, *P. tremula* × *tremuloides*), and others (Sarmast, 2016; Chang et al., 2018).

The technology of genomic selection has been successfully used in recent decades in order to increase the efficiency of tree selection, along with the methods of somatic embryogenesis and cryopreservation (Park, 2002). This technology, based on a set of mapping of quantitative traits loci, makes possible the prediction of the phenotype of an individual (Goddard, Hayes, 2007). Thus, genomic selection makes it possible to identify elite genotypes at a very early stage of development without phenotyping through field testing and, thereby, to reduce significantly the duration of variety testing within the framework of afforestation programs (Park et al., 2016).

In our country, thanks to intensive studies of the conifers somatic embryogenesis, including Siberian larch species, conducted for more than 10 years by the staff of the Sukachev Institute of Forest SB RAS (Krasnoyarsk) under the leadership of I.N. Tretyakova, significant achievements have been made in this area, starting from the conditions for the embryogenic culture induction up to the carrying out of field testing of plants grown from somatic embryos – regenerants (Tretyakova et al., 2018). The results obtained along with the use of progressive methods of cryopreservation, creation and selection of elite genotypes based on genetic engineering and genomic selection can significantly increase the effectiveness of traditional reforestation breeding programs carried out in Russia. The international project "Larch" with the participation of Sweden, Norway, Finland, Iceland, Canada, China, Japan, and the United States can be considered as the most ambitious of these projects (Abaimov et al., 2002; Martinsson, 2002). The aim of this project, launched in 1992, is to create a collection of seeds of four larch species from Russia (*Larix sukaczewii*, *L. sibirica*, *L. gmelini* and *L. cajanderi*), study the genetics of these species, carry on research on the breeding and modification of populations created on plantations in different parts of the Northern hemisphere, and select promising forms and populations.

Conclusion

Somatic embryogenesis is increasingly considered as the most promising method of clonal propagation of conifers (in particular, larches) since it has a number of advantages over traditional afforestation technologies. For more than 30 years since the first successful somatic embryogenesis in representatives of the genus *Larix*, considerable experience has been accumulated in this research area. The conditions of induction, maintenance of embryogenic cultures, maturation and germination of somatic embryos, regeneration of full-fledged plants and their growth in field conditions have been studied. Based on cryopreservation methods, protocols for maintaining embryogenic cultures in a functional juvenile state for long periods of time have been developed. Research is being actively carried out to elucidate the molecular genetic mechanisms underlying the implementation of individual stages of somatic embryo development.

Despite the existing successes, today it is required to intensify comprehensive research in the field of somatic embryogenesis of representatives of the genus *Larix*. In the nearest future, the research will provide the creation of scientific foundations for the development of new approaches and methods for obtaining high-quality plant material *in vitro* and its use in extensive industrial reforestation and afforestation programs. Hopefully, integrating efforts and closer cooperation of individual research teams successfully working in the Russian Federation on the problem of somatic embryogenesis of species of the genus *Larix* and other conifer species, as well as the already begun use of an integrated approach based on the use of genomics, transcriptomics, proteomics, metabolomics, cryopreservation, and genomic selection in these studies will accelerate the resolution of this planetary biological problem.

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ORCID ID

V.N. Shmakov orcid.org/0000-0003-2965-1758
Yu.M. Konstantinov orcid.org/0000-0002-0601-2788

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Identification of duplicate accessions in the sweet maize collection by means of zein electrophoresis

V.V. Sidorova, Yu.A. Kerv ✉, A.V. Konarev

Federal Research Centre the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
✉ e-mail: kerv@mail.ru

Abstract. Of all the subspecies of *Zea mays* L. cultivated in the world, sweet maize is the most important for the global economy. The leading seed-growing companies and research institutions around the world are engaged in breeding this crop. To meet the increasing demands of the industry to grain quality, it is important to select appropriate local varieties and lines for hybridization. Local (usually heterogeneous) varieties are a valuable source material for creating self-pollinated lines that contribute to a significant broadening of the genetic base of parental forms used in breeding. The advantages of sweet maize varieties and the interest of the food industry in them make it possible to consider accessions from the maize collection of the N.I. Vavilov Institute (VIR) as a potentially valuable source material for breeding. The present research concentrated on 19 local sweet maize varieties with different grain colors from the VIR collection, that is, 9 varieties with the blue color of ripe grain, 4 with white (colorless) grain, 3 with yellow, and 3 with red. The research included an analysis of zein electrophoretic patterns (protein markers); a study of their biotype composition and the nature of genetic polymorphism, as well as the creation of a protein pattern database for each accession. For a series of accessions with the same varietal name, but different catalog numbers, the degree of their identity was determined from their biotype composition in order to exclude duplication. Zein electrophoresis was carried out in vertical plates of 10 % polyacrylamide gel according to the standard ISTA technique developed with the participation of the Biochemistry and Molecular Biology Department of VIR. Zein patterns were used for the first time to electrophoretically study sweet maize varieties with different grain colors. Unique zein patterns were established for all the accessions studied, which makes possible their identification by specific marker components. The results of this work characterize zein electrophoresis as a useful tool for the identification and registration of duplicate accessions in the VIR collection of sweet maize varieties.

Key words: sweet maize; duplicate accessions; zein electrophoresis; protein markers.

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Выявление дублетных образцов в коллекции сахарной кукурузы с использованием электрофореза зеина

В.В. Сидорова, Ю.А. Керв ✉, А.В. Конарев

Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
✉ e-mail: kerv@mail.ru

Аннотация. Из всех подвидов *Zea mays* L., возделываемых на земном шаре, самый значимый для мировой экономики – сахарная кукуруза. Ее селекцией занимаются ведущие семеноводческие фирмы и научные учреждения мира. Для удовлетворения возрастающих запросов производства к качеству зерна важное значение имеет правильный подбор местных сортов и линий для гибридизации. Местные (как правило, гетерогенные) сорта – ценный исходный материал для создания самоопыленных линий, что способствует существенному расширению генетической базы используемых в селекции родительских форм. Достоинство сортов сахарной кукурузы и интерес к ним пищевой промышленности позволяют рассматривать генетические ресурсы коллекции Всероссийского института генетических ресурсов растений им. Н.И. Вавилова (ВИР) в качестве потенциально ценного исходного материала для селекции. Целью нашей работы было выявление дублетных образцов в коллекции сахарной кукурузы ВИР. В задачи исследования входило: проведение скрининга 19 местных сортов подвида сахарной кукурузы из коллекции ВИР с разным цветом зерна (9 стародавних местных с синей окраской зрелого зерна, 4 – с белой (бесцветной), 3 – с желтой и 3 – с красной окраской) по электрофоретическим спектрам зеина (белковым маркерам); изучение их биотипного состава и характера генетического полиморфизма, создание паспортной базы данных каж-

дого изученного образца по белковым спектрам; для некоторых сортов, имеющих одинаковое сортовое название, но разные каталожные номера, установление степени идентичности их по биотипному составу с целью исключения дублетов. Электрофорез зеина проводили по стандартной методике ISTA, разработанной с участием отдела биохимии и молекулярной биологии ВИР, в вертикальных пластинах 10 % полиакриламидного геля. Методом электрофореза по спектрам зеина впервые исследованы сорта подвида сахарной кукурузы с разной окраской зерна. Для всех изученных образцов установлена уникальность спектров зеина, что позволяет идентифицировать их по специфичным для них маркерным компонентам. Результаты настоящей работы свидетельствуют о перспективности использования электрофореза зеина для выявления, идентификации и регистрации дублетных образцов в коллекции сортов подвида сахарной кукурузы ВИР.
Ключевые слова: сахарная кукуруза; дублетные образцы; электрофорез зеина; белковые маркеры.

Introduction

According to many researchers, *Zea mays* L. is the only species of the genus *Zea* L. unknown in the wild (Shmaraev, 1999; Matsuoka et al., 2002). The primary focus of primitive maize formation was the territory of Mexico (Piperno, Flannery, 2001; Wu, Messing, 2014), and the secondary one was the highlands of Peru (Zhukovsky, 1971). According to the taxonomy of the genus developed at VIR, sweet maize has been separated into the subspecies *Zea mays* L. subsp. *saccharata* (Sturt.) Zhuk. The *su1* gene found in regular sweet maize ensures a high free sugar content at the expense of a reduced proportion of starch in the endosperm.

Supersweet maize has the *sh2* gene in its genome, which is located on the third chromosome in the recessive state. Both *su1* and *sh2* genes affect the synthesis of carbohydrates in grain: *su1* blocks the conversion of sugars into starch, and *sh2* blocks the synthesis of starch during the conversion of sugars into dextrins. When *su1* and *sh2* are combined in the same genotype, the sugar content increases up to 21–35 %, while mature kernels look feeble and wrinkled (Suprunov et al., 2017). Currently, the world's leading breeding and seed-producing companies are working on the creation of varieties of supersweet maize, which is not a genetically modified product; all its hybrids are produced by crossing plants and breeding for high *sh2* values (Tracy, 1997).

In the Russian Federation, sweet maize breeding is carried out at the All-Russian Research Institute of Maize, the Kabardino-Balkarian Research Institute of Agriculture (Nalchik city), the Krasnodar Research Institute of Agriculture, the KOS MAIS Scientific and Production Association, and the Research Institute of Agriculture of the South-East. According to the State Commission of the Russian Federation for Testing and Protection of Breeding Achievements, 121 varieties of sweet maize have been registered and admitted for use in the Russian Federation (State Register for Selection Achievements, 2019).

At the stage of milky wax (technical) ripeness, sweet maize grain has a very tender pericarp, which is especially valuable for canning (Tanaboon, 1995). In terms of basic nutrients content, sweet maize keeps abreast of such nutritionally valuable vegetable legumes as green peas and green beans, and in terms of carbohydrate content it is significantly superior to them (Hooda, Kawatra, 2013). It is very important for the organization of a healthy diet that maize protein is much less allergenic than wheat protein (Holding, 2014). Besides, unlike other vegetable crops, sweet maize does not accumulate nitrates in kernels, and leaf wrapped around the cobs protect the grain from airborne pollution with various substances, including radionuclides. Sweet maize is also used for medicinal purposes. Extracts from maize flower parts (stigmas) are used in official and folk medicine for the treatment of inflammatory diseases of the liver and gall bladder (Kumar, Jhariya, 2013).

To meet the growing demands of the industry to grain quality, the proper selection of local varieties and lines for hybridization is important. Old (usually heterogeneous) varieties are a valuable source material for creating self-pollinated lines, which contributes to a significant broadening of the genetic base of parental forms used in breeding. The ripe kernels of various local varieties of sweet maize can have different colors, e. g., white (no color), yellow, brown, red, violet, blue, etc. Breeding for grain color is a result of the development of a new trend, that is, the aesthetic breeding (Novoselov, 2007).

The advantages of sweet maize varieties and the interest of the food industry in them make it possible to consider the germplasm available in the maize collection of the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) as a potentially valuable source material for breeding. The collection acquires a higher significance with the increasing completeness of information about each accession conserved in it. In this regard, the identification of duplicate accessions gains importance as it helps to avoid expenses associated with studying the accessions identified as duplicates, as well as with their maintenance and storage.

At present, molecular (DNA and protein) markers are used along with morphological characters in order to control the genetic integrity (authenticity) of accessions, identify duplicates and reveal errors that can occur in the course of regeneration (Pyukkenen et al., 2005; Konarev, 2006; Potokina, 2009; Strelchenko, Kovaleva, 2009). Storage proteins should be recognized as more reliable for the purposes of seed control and solving a number of breeding problems. They are numerous, most polymorphic, and localized in morphogenetically homogeneous tissues, i. e., in the mature

seed endosperm (Konarev, 1983). Protein markers make it possible to control the biotypic (genotypic) composition of a variety's population – for example, to reveal a decrease in the population heterogeneity that leads to a deterioration of the adaptive properties of the variety (Konarev et al., 2000; Konarev, 2006). The analysis of grain storage protein polymorphism is the basis of the international and domestic standard methods for identification of lines and varieties (Cooke, 1978; Konarev et al., 1987). The present work employed zeins, the maize storage proteins, whose electrophoretic patterns are reliable markers for the varietal identification and maize genepool registration. Maize is a cross-pollinated plant, therefore zeins are characterized by a rather high polymorphism and are widely used in the study of maize genetic resources (Sidorova et al., 2012, 2015, 2018). When assessing the specificity of a variety from protein bands, the analysis of individual grains is required. The electrophoretic pattern of zein of a single grain marks the corresponding biotype (genotype).

The objective of the present study was to identify duplicate accessions in the sweet maize collection at VIR. The tasks set were as follows: to use electrophoretic patterns of zein to determine the biotype composition and character of polymorphism of differently colored sweet maize local varieties from the VIR collection; carry out their certification on the basis of protein bands; to use the biotype composition for establishing the degree of identity of some accessions with the same name but different catalog numbers in order to eliminate duplicates.

Materials and methods

The work was carried out in the Department of Biochemistry and Molecular Biology of VIR. The material used for the study were the ripe kernels of 19 local sweet maize varieties with different grain colors (50 kernels per each accession), regenerated at the Volgograd Experiment Station of VIR (see Table).

Zein electrophoresis was carried out in vertical PAAG plates without cooling for 4.5 h at a voltage of 500–580 V, according to the standard ISTA method developed with the participation of the Department of Biochemistry and Molecular Biology of VIR. The gel plates contained 10 % acrylamide and 8 M urea. Zein was isolated from single grains with a solution containing 6 M urea and 0.01 M dithiothreitol. The stained and dried gels with electrophoretic patterns were scanned. The registration of electrophoretic zein patterns was carried out using a standard, the self-pollinated F2 line from France. The numbering of protein components corresponds to the magnitude of their electrophoretic mobility (Kerv, Sidorova, 2018).

List of accessions used in the research

VIR catalog No.	Variety	Grain color	Origin, year of entry to the collection	Year of regeneration
k-30	Black Mexican	Blue	USA, New York, 1921	2013
k-95	Black Mexican		USA, Los Angeles, 1921	
k-898	Black Mexican		USA, California, 1921	2001
k-13817	Black Mexican		Canada, Montreal, 1959	2005
k-1578	Local blue		China, Manchuria, 1924	2006
k-4655	Burpee's Early Earliest Catawba 302	Purple-blue	USA, Philadelphia, 1927	2005
k-893	Pickaninny	Blue	Canada, Ottawa, 1922	
k-10999	Pickaninny	Gray-blue	USA, Wisconsin, 1947	
k-20870	Local	Blue	Hungary, Budapest, 1983	1999
k-83	Early June	White	USA, Sacramento, 1921	2013
k-115	White Ever green		USA, New York, 1921	
k-127	Oregon Evergreen		USA, Los Angeles, 1921	
k-143	Early Mayflower		USA, Connecticut, 1921	
k-29	Golden Bantam	Yellow	USA, New York, 1921	
k-69	Golden Bantam		USA, New York, 1921	
k-146	Golden Bantam		Canada, Manitoba, 1921	
k-5811	Marshall's Earliest	Orange-red	USA, New York, 1930	2002
k-5842	Early Dawn	Dark-red	USA, Connecticut, 1930	2005
k-10998	Nuetta	Red	USA, Wisconsin, 1947	

Results and discussion

Figure 1 shows zein electrophoretic patterns of four accessions of the sweet maize variety 'Black Mexican' with blue grain, registered in the collection in different years under different catalog numbers. The patterns of biotypes 3, 4 and 5 (with a frequency of occurrence of 15 %) lack the combination of components 52–67. Between themselves, they differ by the presence/absence of components 40, 47, and 63. Biotype 6 is rare (10 %); its patterns lack combinations of components 38–57 and 52–67.

Five biotypes were identified by zein patterns in the variety with the same name and the catalog number k-95. It does not have a basic pattern type. Biotype 1 and the frequency of occurrence (35 %) make accessions k-30 and k-95 identical. Biotype 2 of accession k-95 differs from biotype 2 of k-30 only by the absence of component 37 in its patterns, and by a higher frequency of occurrence (25 %). Biotypes 3 and 4 (k-95) have patterns that are different in composition and are not found in k-30. Biotype 5 is rare (10 %); it is identical in composition and frequency of occurrence to biotype 6 of k-30. On the basis of the foregoing, accessions k-30 and k-95 can be regarded as genetically close (due to the presence of the frequent biotype 1); however, they are not duplicates.

Unlike accessions k-30 and k-95, accessions k-13817 and k-898 have different compositions of zein patterns. They exhibit low intra-varietal polymorphism (four and two biotypes, respectively). Their patterns lack the combination of components 52–67, which obligatorily occurs in the patterns of the frequently encountered biotypes in accessions k-30 and k-95. Biotypes 2, 3, and 4 of k-13817 (with the total frequency of occurrence of 55 %) and biotype 1 in k-898 (60 %) do not have a combination of components 38–57 in the patterns. Such a biotype as the one in accessions k-30 and k-95 occurs rarely (10 %). The patterns of the frequently encountered biotype 1 in k-13817 (45 %) and biotype 2 in k-898 contain a combination of components 38–57 (40 %). However, these biotypes are not identical, since the intensity of the combination of components 38–57 is higher in k-13817 than in k-898. Also, the patterns of the accessions considered contain additional components. These types of patterns do not occur in k-30 and k-95.

All the accessions with the same varietal name have zein patterns that differ in component composition, which indicates that these accessions should be given different catalog numbers and stored separately.

Figure 2 shows zein electrophoretic patterns of two accessions of the sweet maize variety 'Pickaninny' with blue grain, registered in the collection under different catalog numbers (k-10999 and k-893). Accession k-10999 is characterized by significant intra-varietal polymorphism. Six types of zein patterns with different frequencies of occurrence have been revealed. Biotype 1 occurs more often than the others (30 %). Its patterns have no combinations of components 38–57 and 52–67. Biotype 2 is rare (10 %), it is identical to biotype 1 in terms of the presence of intense components 46, 50, and 55 in the patterns, and differs from it by the absence of components 40 and 63, as well as by an additional component, 70. The patterns of the remaining biotypes (3–6) have a combination of components 38–57. Of these, only biotype 3 differs from the others by the presence of a combination of components 52–67 (20 %) in its patterns. The patterns of the remaining biotypes (4–6) are characterized by the presence/absence of a number of components with weak intensity. Accession k-893 of the old variety 'Pickaninny' differs from k-10999 by low intra-varietal polymorphism. Only three biotypes have been identified in it. The frequency of occurrence of the main biotype 1 is 80 %. The zein pattern makes it identical to biotype 1 of k-10999 (30 %). Its patterns lack a combination of components 38–57. Biotype 2 is less common (15 %); in terms of the presence of intense components in the patterns, it is identical to biotype 1, but differs from it by the presence of additional components with low intensity. The main biotypes 1 and 2 in k-893 are identical concerning the basic types of patterns 1 and 2 of k-10999. Biotype 3 is extremely rare (5 %). This type of pattern does not occur in k-10999.

The accessions with the same varietal name have zein patterns with different compositions and, therefore, they cannot be regarded as one and the same accession. Figure 3 shows zein patterns of three accessions of the sweet maize variety 'Golden Bantam' with yellow grain. The old accession k-146 showed significant intra-varietal polymorphism. Six pattern types have been identified in it. Patterns of all biotypes are characterized by the presence of a combination of components 52–67 with a varying degree of intensity. Four of them (1–4) also have a combination of components 38–57. Biotypes 5 and 6 are rare. Unlike the frequently encountered biotypes, they lack a combination of components 38–57 in their patterns. Biotypes 5 and 6 are rare. They differ from the frequently encountered biotypes by the absence of a combination of components 38–57 in their patterns. The frequently occurring biotypes 1 and 2 are distinguished by the patterns without components 40 and 63, which are quite intense in the patterns of rare biotypes 5 and 6. Biotypes 1–4 are characterized by different combinations of components 47, 48 and 50. Component 50 is absent in the patterns of biotypes 1 and 2.

Accession k-69 is characterized by low intra-varietal polymorphism. Three biotypes with the frequency of occurrence of 28–36 % have been identified in it. According to the pattern types, k-69 is close to biotypes 1, 2 and 3 of accession k-146. Biotypes 4, 5, and 6 in k-146 have compositionally different patterns, which are not found in k-69.

The third accession from the 'Golden Bantam' k-29 group is characterized by high intra-varietal polymorphism, and the number of the biotypes identified is six. The most common is biotype 1 (30 %); the frequency of occurrence of the remaining ones is approximately the same and equals

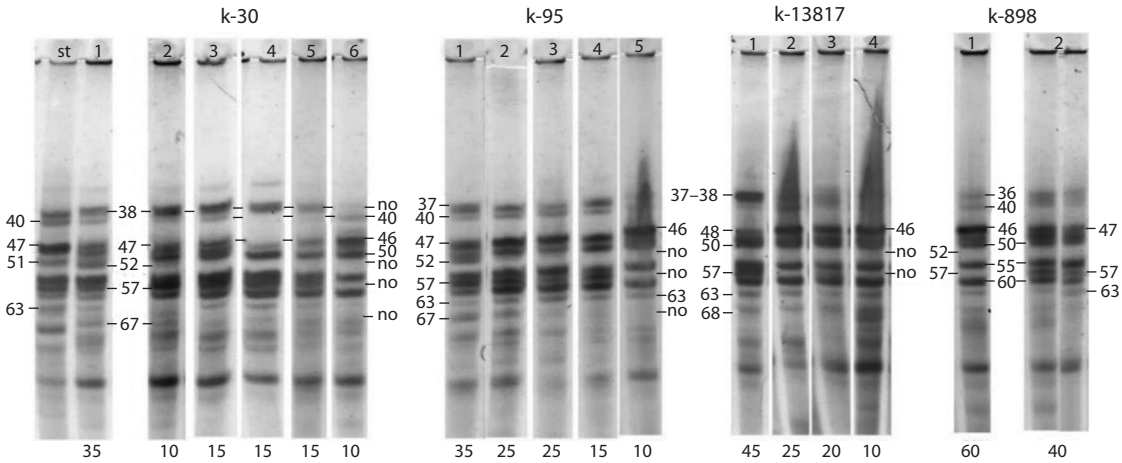

Fig. 1. Zein electrophoregrams for the accessions of the sweet maize variety 'Black Mexican' with blue grain: k-30, k-95, k-13817 and k-898.

Here and also in Fig. 2–6: the figures above the pattern indicate the biotype number, and those along the pattern indicate the numbers of polypeptides in the pattern. The figures under the patterns indicate the frequency of occurrence of each biotype.

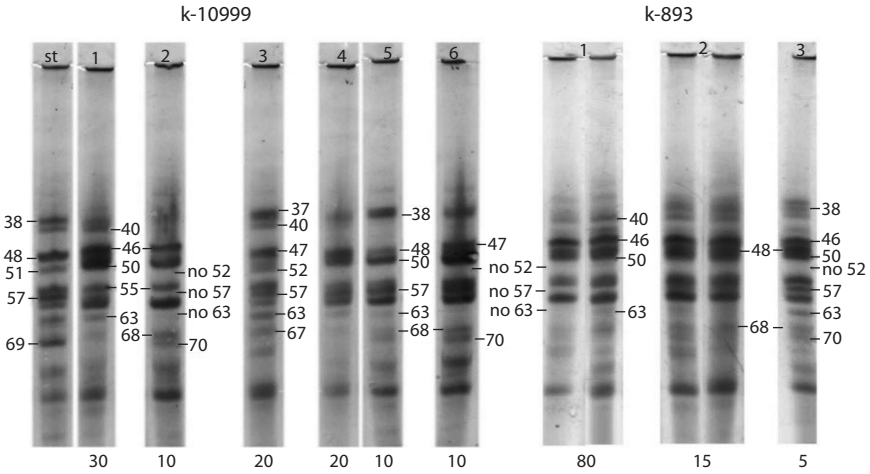

Fig. 2. Zein electrophoregrams for the accessions of the sweet maize variety 'Pickaninny' with blue grain: k-10999 and k-893.

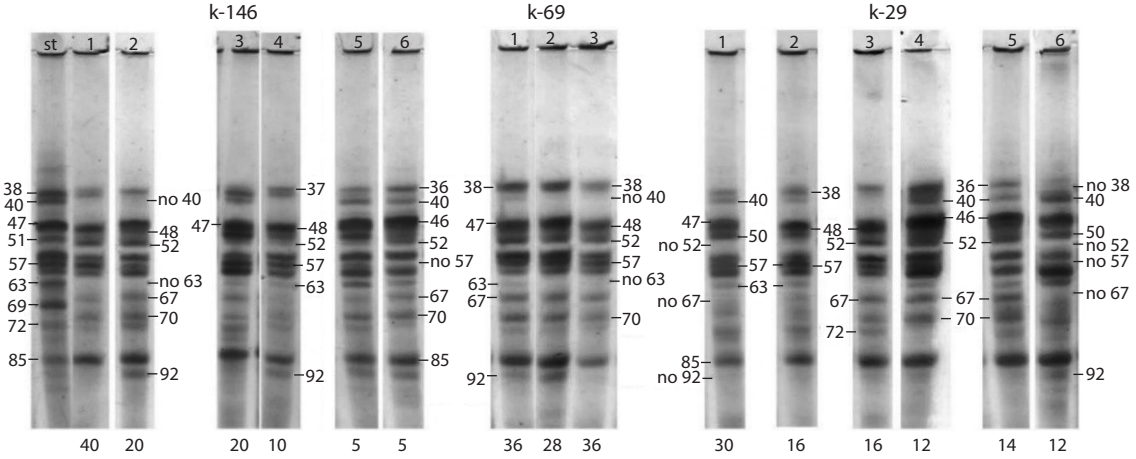

Fig. 3. Zein electrophoregrams for the accessions of the sweet maize variety 'Golden Bantam' with yellow grain: k-146, k-69 and k-29.

12–16 %. Only two biotypes (3 and 4) have patterns that are identical to those of biotypes 2 and 3 of accession k-146. Biotypes 1, 2, 5 and 6 from k-29 are absent in 'Golden Bantam' accessions k-146 and k-69, since their patterns do not have a combination of components 52–67, which is typical of all types of patterns of k-146 and k-69.

The old accessions with the same varietal name have zein patterns with different compositions, therefore, they are not duplicates.

Figure 4 presents the electrophoretic zein patterns of three local sweet maize varieties with red grain. The accessions studied have individual zein patterns specific for each variety.

Accession k-5842 was found to have the main pattern type (biotype 1) with the frequency of occurrence of 75 %, and two rare ones (biotypes 3 and 4). Biotype 2 (15 %) differs from biotype 1 by the absence of component 40 in the patterns. Specific for this variety was the presence of the intense component 63 and a combination of components 38–57, which are present in the patterns of all biotypes. Also, the absence of a combination of components 52–67 in its patterns is specific to it.

Accession k-10998 is noted for high intra-varietal polymorphism. No main type of zein pattern was revealed for it. Biotype 1 with the 35 % frequency of occurrence, biotype 2 (25 %) and biotype 3 (22 %) are more common than the others. Specific to this variety is the absence of component 63 in the patterns of all biotypes. This distinguishes it from accessions k-5842 and k-5811 and increases the likelihood of obtaining a good hybrid combination with red grain.

Accession k-5811 is characterized by low intra-varietal polymorphism. The main pattern type (biotype 1) with the 75 % frequency of occurrence and three biotypes with a low frequency of occurrence (from 5 to 10 %) were revealed. The absence of the combinations of components 38–57 and 52–67 in the patterns of the main biotype turned out to be specific for it, as well as the presence of components 40 and 63 in the patterns of all biotypes.

It was found that all studied varieties with red grain color have specific components by which they can be identified, new hybrids can be created, and new lines can be selected on their basis.

Figure 5 demonstrates zein patterns of four sweet maize varieties with white grain. The accessions studied have individual specific patterns. Two accessions, k-143 and k-115, exhibit significant intra-varietal polymorphism. They have five types of patterns with different frequencies of occurrence. The broader the polymorphism of the varieties, the more difficult it is to identify their main pattern type. However, biotype 1 (35 %) and biotype 2 (35 %) are more common in k-143 than the others. They have identical pattern types and differ from each other in the intensity of manifestation of individual components. Biotypes 1 and 2 can be considered as the main patterns for k-143. Biotype 3 (20 %) differs from the first two types by the absence of component 40 in the patterns. Biotypes 4 and 5, which are rare, have compositionally different zein patterns. The combination of components 52–67 is intense in the biotype 4 patterns, while it is absent in patterns of the remaining biotypes. The biotype 5 patterns have no combination of components 38–57, which is specific for all biotypes of this variety.

Biotype 1 (35 %) occurs more frequently than the others in accession k-115. The type of this biotype pattern is unique for this accession, since it is not identical in component composition to other frequently occurring biotypes 2 and 3 (25 % each), moreover, to rare biotypes 4 and 5 (10 and 5 %, respectively). A low intensity of the zone of manifestation of components 36–40 is specific for this variety.

Biotype 1 (40 %) and biotype 2 (30 %) occur more frequently in accession k-83 than in the others. A combination of components 52–67 is manifested in the patterns of biotypes 1 and 2, though in biotype 2 it has low intensity. A combination of components 38–57 is intense in the biotype 2 patterns, whereas it is absent in the patterns of biotypes 1 and 3. Component 63, which is specific for the patterns of this variety, is absent in the patterns of rare biotype 4.

Accession k-127 is characterized by low polymorphism. The main biotype 1 with a 65 % frequency of occurrence, as well as biotypes 2 (25 %) and biotype 3 (10 %) were identified. This accession is unique among all the sweet maize varieties studied. The specific component 64 is present in its patterns. The absence of a combination of components 52–67 in the patterns of all biotypes was also specific to the variety. Therefore, there is a high degree of probability that this variety can be successfully used for creating new improved hybrids.

Figure 6 presents the electrophoretic patterns of zein of three sweet maize varieties with blue grain. All accessions have different names and catalog numbers, and there are no low polymorphic varieties among them. No main type of pattern, the frequency of occurrence of which would be above 50 %, has been identified in them. However, the most common biotype amounts to 50 % in accessions k-20870, k-1578 and k-4655. The varieties studied have specific pattern types. Accession k-1578 is characterized by the presence of combinations of components 38–57 and 52–67 in the biotype 1 pattern, as well as of components 40 and 63. In contrast to biotype 1, the combination of components 52–67 and component 40 have a low intensity in biotype 2. A distinctive feature of biotype 2 is the presence of intense components 37 and 50 in the patterns. A combination of components 52–67, as well as components 37 and 47, are absent in the biotype 3 patterns. This biotype occurs less frequently than the others (20 %). The combination of components 38–57, as well as components 40 and 63, are specific to this variety.

Three biotypes have been revealed in accession k-20870. Biotype 1 (50 %) occurs more frequently than the others. The patterns of this biotype contain an intense combination

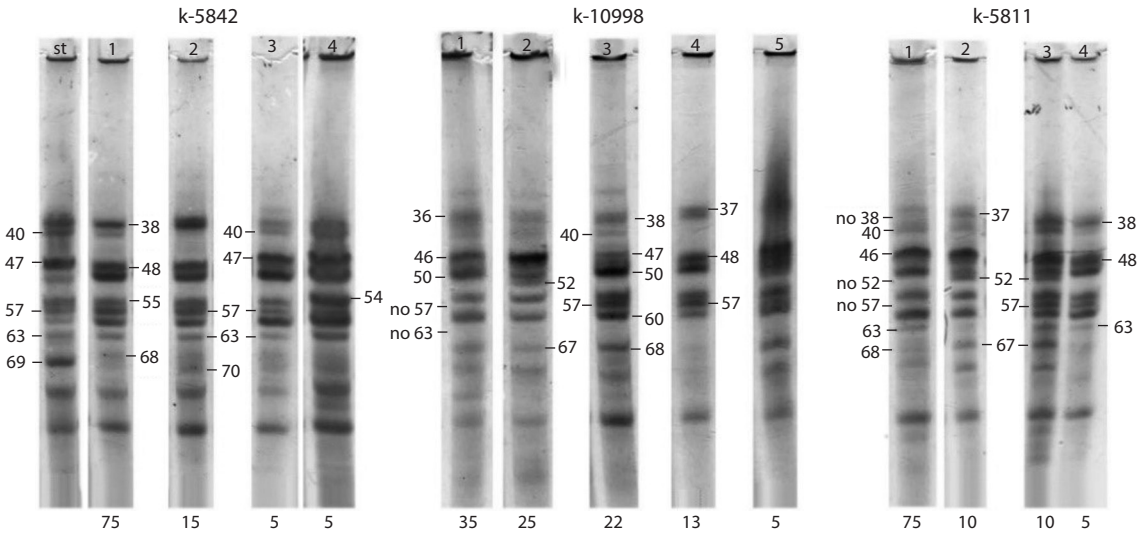

Fig. 4. Zein electrophoregrams for the accessions of sweet maize varieties with red grain: k-5842, k-10998 and k-5811.

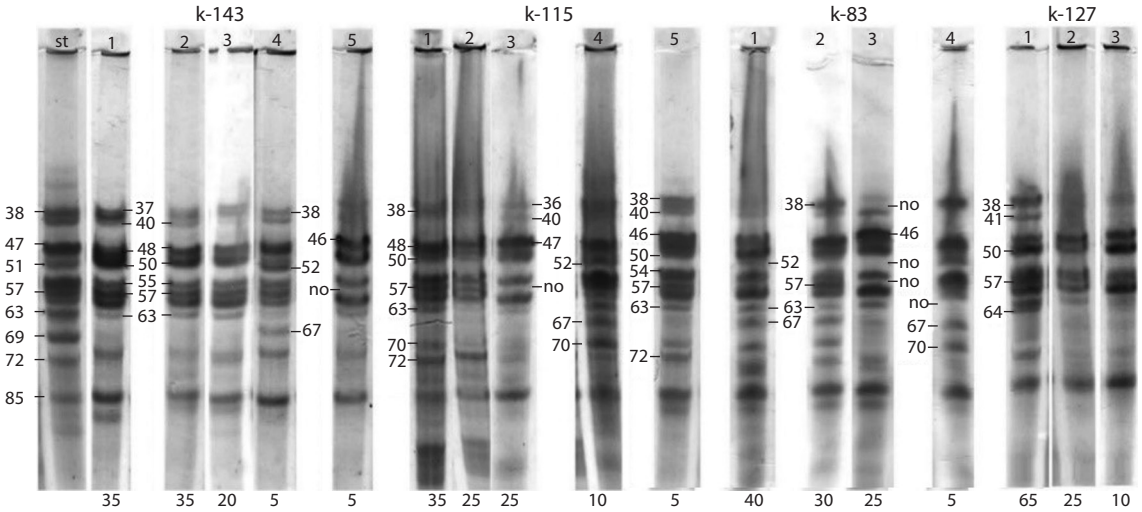

Fig. 5. Zein electrophoregrams for the accessions of sweet maize varieties with white grain: k-143, k-115, k-83 and k-127.

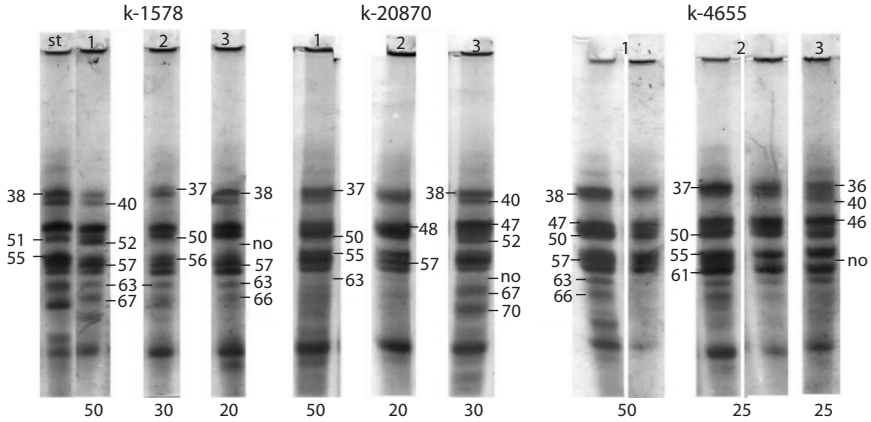

Fig. 6. Zein electrophoregrams for the accessions of sweet maize varieties with blue grain: k-1578, k-20870 and k-4655.

of components 38–57, as well as intense components 37 and 47. Components 40 and 63 are characterized by low intensity. Biotype 2 with its 20 % frequency of occurrence differs from biotype 1 only by intense component 48 and a weak intensity of components 37, 40, and 63, as well as by the absence of component 47. In contrast to biotypes 1 and 2, the combination of components 52–67, as well as components 40 and 47, are intense in the patterns of biotype 3 with a 30 % frequency of occurrence. The presence of an intense combination of components 38–57 is specific to this variety, as well as the absence or a very weak intensity of component 63 in the patterns of all biotypes.

Three biotypes have been revealed in accession k-4655. Biotype 1 occurs most often and has a 50 % frequency of occurrence. A combination of components 38–57 is well manifested in zein patterns of biotype 1. In some patterns, this combination, as well as component 63, may have a low intensity. Biotype 2 differs from biotype 1 by a lower frequency of occurrence (25 %). The patterns of this biotype contain intense components 37 and 46, which are absent in the patterns of biotype 1. The combination of components 38–57 has a weak intensity in the patterns of biotype 2.

Unlike biotypes 1 and 2, biotype 3 (25 %) has different pattern compositions. A combination of components 38–57 is absent in the patterns of biotype 3. In contrast to biotypes 1 and 2, intense components 36 and 40 are present in the patterns. The absence of a combination of components 52–67 is specific to the variety, which is characteristic of other sweet maize varieties.

Conclusion

Based on the above, it can be concluded that among the accessions with blue grain and the same varietal name of 'Black Mexican', two accessions, k-30 and k-95, can be regarded as genetically close varieties, though not as duplicates. Unlike k-30 and k-95, two other accessions, k-13817 and k-898, have low polymorphism and compositionally different pattern types. Two accessions with blue grain and the same varietal name of 'Pickaninny' have different VIR catalog numbers, k-10999 and k-893, and are not duplicates either. Accession k-10999 is characterized by significant polymorphism and has six biotypes. Accession k-893 has a low intra-varietal polymorphism (three biotypes) and demonstrates the absence of a significant number of biotypes that are characteristic of k-10999. The varieties with the same name of 'Golden Bantam' and yellow grain color (k-146, k-69 and k-29) were also found to contain no duplicates. Accessions k-146 and k-29 are characterized by high intra-varietal polymorphism and have different pattern types. Accession k-69 has low intra-varietal polymorphism. The three biotypes found in k-146 and k-29 are not present in k-69. The accessions with the same varietal name have zein patterns with different compositions, which is an evidence of a significant difference between them and the impossibility to merge them.

Three local varieties of sweet maize with red grain were studied and specific components determined for each variety. Two sweet maize accessions, k-143 and k-115, with white grain exhibit significant intra-varietal polymorphism. Specific components have been identified for them. Accession k-127 is characterized by low polymorphism. It is unique due to the presence of component 64 in its patterns. Three sweet maize varieties with blue grain, k-20870, k-1578 and k-4655, are highly polymorphic and have pattern types specific to each variety.

The results of the work performed show that it is quite promising to use zein electrophoresis for the identification, registration, and revealing of duplicate accessions in the collection of sweet maize varieties with different kernel colors.

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ORCID ID

V.V. Sidorova orcid.org/0000-0002-5233-8949
Yu.A. Kerv orcid.org/0000-0002-3728-6968
A.V. Konarev orcid.org/0000-0003-2938-1014

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Сравнительный анализ экспрессии генов у чайного растения (*Camellia sinensis* (L.) Kuntze) при низкотемпературном стрессе

Л.С. Самарина ✉, А.О. Мацькив, Н.Г. Конинская, Т.А. Симонян, В.И. Маляровская, Л.С. Малюкова

Федеральный исследовательский центр «Субтропический научный центр Российской академии наук», Сочи, Россия
✉ e-mail: q11111w2006@yandex.ru

Аннотация. Низкотемпературный стресс – один из главных факторов, ограничивающих распространение и снижающих урожайность многих субтропических культур, в том числе и чая. Для эффективной селекции чая на устойчивость к морозу необходимо выявить генетические особенности ответа на холод у устойчивых генотипов и найти маркеры для определения доноров устойчивости в коллекциях. В настоящей работе проведен сравнительный анализ экспрессии 18 генов (*ICE1*, *CBF1*, *DHN1*, *DHN2*, *DHN3*, *NAC17*, *NAC26*, *NAC30*, *bHLH7*, *bHLH43*, *P5CS*, *WRKY2*, *LOX1*, *LOX6*, *LOX7*, *SnRK1.1*, *SnRK1.2*, *SnRK1.3*), вовлеченных в абиотический стрессовый ответ у двух контрастных по устойчивости генотипов чая в условиях холода и мороза. Низкотемпературный стресс индуцировали путем помещения растений в холодильные камеры и снижением температуры до 0…+2 °C на семь дней (холодовой стресс) с последующим снижением температуры до –4…–6 °C на пять дней (промораживание). Кондуктометрическим методом измеряли электропроводность тканей листа, в результате чего были подтверждены различия по признаку устойчивости у двух исследуемых генотипов чая: холодовое воздействие не приводило к изменению электропроводности тканей листа, но после промораживания этот показатель возрастал в большей степени у неустойчивого генотипа. Методом qRT-PCR анализировали относительный уровень экспрессии генов на фоне референсного гена актина. При индукции стресса показана повышенная экспрессия всех исследуемых генов. У устойчивого генотипа чая выявлен ряд генов, более активно экспрессирующихся по сравнению с неустойчивым генотипом: *ICE1*, *CBF1*, *DHN2*, *NAC17*, *NAC26*, *bHLH43*, *WRKY2*, *P5CS*, *LOX6*, *SnRK1.1*, *SnRK1.3*. Эти гены могут быть маркерами устойчивости для поиска доноров в коллекциях геноресурсов. Показано, что у устойчивого генотипа чая экспрессия генов холодового ответа начинается уже на стадии акклиматизации. Для дальнейших исследований комплексной устойчивости растений к низкотемпературному стрессу актуальным является изучение экспрессии этих генов в других органах чайного растения (побегах, корнях) при разной силе низкотемпературного воздействия.
Ключевые слова: *Camellia sinensis*; экспрессия генов; морозоустойчивость; транскрипционные факторы; протеинкиназы; липоксигеназы.

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Comparative analysis of gene expression in tea plant (*Camellia sinensis* (L.) Kuntze) under low-temperature stress

L.S. Samarina ✉, A.O. Matskiv, N.G. Koninskaya, T.A. Simonyan, V.I. Malyarovskaya, L.S. Malyukova

Federal Research Centre the Subtropical Scientific Centre of the Russian Academy of Sciences, Sochi, Russia
✉ e-mail: q11111w2006@yandex.ru

Abstract. Low-temperature stress is one of the main factors limiting the distribution and reducing the yield of many subtropical crops, including the tea crop. Efficient breeding to develop frost-tolerant cultivars requires a reliable set of genetic markers for identifying resistance donors, and that is why it is necessary to reveal the specific genetic response in frost-tolerant genotypes in comparison with frost- susceptible ones. In this work, we performed a comparative analysis of the expression of 18 tea genes (*ICE1*, *CBF1*, *DHN1*, *DHN2*, *DHN3*, *NAC17*, *NAC26*, *NAC30*, *bHLH7*, *bHLH43*, *P5CS*, *WRKY2*, *LOX1*, *LOX6*, *LOX7*, *SnRK1.1*, *SnRK1.2*, *SnRK1.3*) under cold and frost conditions in two tea genotypes, tolerant and susceptible. Low-temperature stress was induced by placing the potted plants in cold chambers and lowering the temperature to 0…+2 °C for 7 days (cold stress), followed by a decrease in temperature to –4…–6 °C for 5 days (frost stress). Relative electrical conductivity of leaf was measured in response to the stress treatments, and a significant difference in the frost tolerance of the two tea genotypes was confirmed. Cold exposure did not lead to a change in the electrical conductivity of leaf tissue. On the other hand, frost treatment resulted in increased REC in both genotypes and to a greater extent in the susceptible genotype. Increased expression of all the genes was shown during cold and frost. The genes that were strongly expressed in the tolerant tea genotype were revealed: *ICE1*, *CBF1*, *DHN2*, *NAC17*, *NAC26*, *bHLH43*, *WRKY2*, *P5CS*, *LOX6*, *SnRK1.1*, *SnRK1.3*. These genes can be proposed as markers for the selection of frost-tolerance donors in tea germplasm collections. Additionally, it was

shown that the tolerant genotype is characterized by an earlier response to stress at the stage of cold acclimation. The study of the expression of the identified genes in different organs of tea plants and in different exposures to low temperature is relevant for further investigations.
Key words: *Camellia sinensis*; gene expression; frost tolerance; transcription factors; protein kinases; lipoxygenases.

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Введение

Низкотемпературный стресс, мороз (< 0 °C) и холод (0…+10 °C), ограничивает ареал распространения многих теплолюбивых культур, в том числе и чая. Перестройка метаболизма в ответ на этот абиотический стресс, включающая накопление антиоксидантов и осмолитов для повышения устойчивости, происходит путем запуска сложных сигнальных путей (Dubouzet et al., 2003). Регуляция этих путей осуществляется основными регуляторными генами: *COR* (cold-responsive). К ним относят гены-регуляторы холодового ответа *ICE* (inducer of *CBF* expression), *CBF* (C-repeat-binding factor) и их мишени: *KIN* (cold-induced), *LTI* (low temperature induced) и *RD* (responsive to dehydration) (Morsy et al., 2005). Продукты этих генов классифицируют на две группы: первая включает гены метаболизма семейств белков, *LEA* (late embryogenesis abundant proteins), *HSP* (heat shock proteins), антифриз-белков, белков метаболизма липидов (*LOX*), дегидринов (*DHN*) и осморегуляторов – сахаров, свободных стеролов, раффинозы, глюкозидов, аминокислот; вторая – транскрипционные факторы, регулирующие передачу сигналов и экспрессию генов ответа на холод (Megha et al., 2018).

Различные по устойчивости к этому фактору культуры характеризуются разнообразными механизмами устойчивости, а у древесных культур, таких как чай (*Camellia sinensis* (L.) Kuntze), ответ на стресс более сложный и комплексный (Hao et al., 2018). Для чайного растения, основная зона произрастания которого – тропические и субтропические регионы, актуальным является создание морозоустойчивых сортов, позволяющих значительно расширить его ареал и увеличить площади возделывания. Северо-Западный Кавказ – один из самых северных регионов промышленного выращивания чая в мире. Доместикация чайного растения в этом регионе длилась около 150 лет, в течение которых плантации распространились из района Озургетти в Грузии (41°55′27″ N, 41°59′24″ E) на север до Майкопа (44°36′40″ N, 40°06′40″ E). Поэтому коллекции гермоплазмы чая на Кавказе могут быть источником генотипов с повышенной морозоустойчивостью.

Ранее было показано, что у чайного растения гены *CsICE1* и *CsCBF1* – главные *COR*-гены ответа и адаптации к низкотемпературному стрессу (Wang et al., 2012; Yuan et al., 2013). Были сообщения также, что экспрессия *COR*-генов регулируется как *CBF*-опосредованным АБК-независимым путем, так и *bZIP*-опосредованным АБК-зависимым путем (Ban et al., 2017). Множество транскрипционных факторов (*DHN*, *WRKY*, *HD-Zip*, *NAC*, *bHLH* и др.) и генов синтеза метаболитов запускаются в ответ на холод (Yue et al., 2015; Wang et al., 2016a, b; Chen et al., 2018; Cui et al., 2018; Shen et al., 2018; Zhu et al., 2018). Об этих и других генах нами был сделан детальный обзор последних публикаций (Самарина и др., 2019). Цель настоящей работы – провести сравнительный анализ экспрессии генов, вовлеченных в абиотический стрессовый ответ у чая, в условиях холода и мороза у кавказских генотипов, произрастающих в одном из самых северных регионов промышленного выращивания чая в мире.

Материалы и методы

Растительный материал и условия эксперимента. Объектом исследования были трехлетние вегетативно размноженные горшечные растения чая двух контрастных по устойчивости генотипов (коллекции чая Федерального исследовательского центра «Субтропический научный центр Российской академии наук» (ФИЦ СНЦ РАН)): устойчивой к морозу формы А-2016, произрастающей в полевой коллекции Майкопского филиала ФИЦ СНЦ РАН и неустойчивого к морозу сорта Колхида полевой коллекции ФИЦ СНЦ РАН (Сочи) (Туов, Рындин, 2011; Гвасалия, 2015). Горшечные растения (рис. 1) в количестве 10 шт. каждого генотипа выращивали в объеме 2 л бурой кислой лесной почвы (pH_{H_2O} = 5.0). До индукции низкотемпературного воздействия растения в течение месяца находились в лабораторных условиях при температуре 20 ± 2 °C с оптимальным режимом полива и освещением лампами дневного света, фотопериодом 16/8 с интенсивностью освещения 3000 лк. После этого у них брали листья для анализа (контрольная группа). Затем эти растения помещали в холодовые камеры. Индукцию стресса проводили воздействием температурой до 0…+2 °C в течение семи дней (холодовой стресс) с последующим снижением температуры до –4…–6 °C на пять дней (промораживание), световой режим сохраняли прежним.

Для лабораторных анализов на всех этапах исследования (контроль, холод, промораживание) использовали третий (для выделения РНК) и четвертый (для физиологических анализов) листья сверху. Для физиологических анализов и выделения РНК каждая из трех повторностей представляла собой смешанную пробу из листьев от трех растений.

Фенотипирование устойчивости к холоду. Кондуктометрическим методом определяли относительную электропроводность тканей (%), стабильность клеточных мембран и повреждение тканей листа (%) – с помощью портативного кондуктометра ST300C (Ohaus). Навеску 200 мг свежего листа погружали в 150 мл деионизированной воды, определяли электропроводность сразу после погружения (L0), затем через 2 ч (L1), далее кипятили на водяной бане 60 мин при 100 °C и определяли электропро-

Fig. 1. Tea plants included in the experiments (left, susceptible genotype Kolkhida; right, resistant genotype A-2016).

водность после остывания раствора (L2). Относительную электропроводность рассчитывали по формуле:

$$REC\ (\%) = L0/L1 \times 100.$$

Стабильность клеточных мембран (CMI, %) вычисляли по формуле:

$$CMI = (1-(L1/L2))/(1-(C1/C2)) \times 100,$$

где C1 и C2 – средняя электропроводность контроля до и после кипячения соответственно (Bajji et al., 2002). Степень повреждения тканей оценивали как 100 – CMI. Статистическую обработку полученных данных выполняли методом однофакторного дисперсионного анализа.

Анализ экспрессии генов. Выделение РНК из свежих листьев проводили с использованием наборов реагентов Лира (http://biolabmix.ru/). Качество РНК оценивали методом электрофореза в агарозном геле, ее концентрацию определяли на приборе BioDrop μLite (Serva). Разведенную РНК обрабатывали ДНКазой и подтверждали отсутствие примесей геномной ДНК методом qRT-PCR. Обратную транскрипцию осуществляли набором реагентов M-MuLV-RH (http://biolabmix.ru/). Количественный анализ экспрессии генов методом ПЦР в реальном времени делали на приборе LightCycler96 (Roche). Смесь ПЦР готовили на основе наборов реагентов БиоМастер HS-qPCR SYBR Blue(2×) (http://biolabmix.ru/). Объем смеси – 12.5 мкл, в нее входили по 0.5 мкл каждого праймера (из раствора 10 ммоль) (см. таблицу), 6.25 мкл буфера, 1 мкл кДНК (500 нг/мкл) и вода. Использовали стандартные условия для ПЦР с двухшаговой амплификацией (35 циклов), температурой отжига праймеров 60 °C. В качестве референсного гена был *Actin*, анализ экспрессии выполняли в трех биологических повторностях, данные обрабатывали с помощью программного обеспечения LightCycler96. Относительный уровень экспрессии гена рассчитывали по алгоритму:

$$2^{-\Delta\Delta Cq},$$

где $\Delta\Delta Cq = (Cq_{gene\ of\ interest} - Cq_{internal\ control})_{treatment} - (Cq_{gene\ of\ interest} - Cq_{internal\ control})_{control}$.

Genes and primers included in the study

Gene	Primer sequence, 5′–3′	References
Actin	F: CCATCACCAGAATCCAAGAC R: GAACCCGAAGGCGAATAGG	Hao et al., 2014
ICE1	F: ATGTTTTGTAGCCGCAGAC R: GCTTTGATTTGGTCAGGATG	Ban et al., 2017
CBF1	F: AGAAATCGGATGGCTTGTGT R: TTGTCGTCTCAGTCGCAGTT	
DHN1	F: ACACCGATGAGGTGGAGGTA R: AATCCTCGAACTTGGGCTCT	
DHN2	F: ACTTATGGCACCGGCACTAC R: CTTCCTCCTCCCTCCTTGAC	
DHN3	F: TCCACATCGGAGGCCAAAAG R: AACCCTCCTTCCTTGTGCTC	
NAC17	F: CCAAAGAACAGAGCCACG R: TGGGTATGAAGGAGTTGGG	Wang et al., 2016a
NAC26	F: ACAAACTACGCCACAATGC R: AGGGAGGGTTCTTTTCAGG	
NAC30	F: ATTTCAGGGGTTTCAAGCA R: CAGAGAATTCATTCGCGG	
bHLH7	F: TCAACGATCAACGGACTT R: TCCTCCTCTTCTTCCTCAT	Cui et al., 2018
bHLH43	F: TCTCTGTGCTGCGAAGAC R: CCTCCGAGTGTTGCCATT	
P5CS	F: AGGCTCATTGGACTTGTGACT R: CATCAGCATGACCCAGAACAG	Ban et al., 2017
WRKY2	F: GAGACAGAAATGAGCAGGGAAAA R: TGTATCGGTGTCAGTTGGGTAGA	Wang et al., 2016b
LOX1	F: TCTTGATTAATGCCGATGG R: AAATGCCTCCAATGGTTC	Zhu et al., 2018
LOX6	F: GACCCAAGCCTCACAAATAG R: GCTTCATTTATGCTACTCACAC	
LOX7	F: ATTTCTCTTCTCTCACTCTCAC R: GAACACCTCTCCATCACACT	
SnRK1.1	F: GTTCAAAACTCATCTTCCTCGCT R: ATGGTTCTTGTCCAATCCCATCT	Yue et al., 2015
SnRK1.2	F: TCTGCTGCTTTAGCTGTGGG R: GCTCGAGACTGTAGGCCAAG	
SnRK1.3	F: TTGGAGTTGCGGTGTCACTT R: CGGGCACCATGAGACAACT	

Результаты

Исследования показали, что холодовое воздействие (0…+2 °C) не приводило к существенным изменениям показателей электропроводности тканей листа. При промораживании (–4…–6 °C) значительно возрастал относительный уровень электропроводности, а стабильность клеточных мембран снижалась, что свидетельствует о повышении выхода электролитов из тканей. При этом растения более устойчивого генотипа в меньшей степени повреждались морозом, чем растения неустойчивого генотипа (рис. 2).

Относительный уровень экспрессии всех генов, включенных в эксперимент, повышался при индукции низкотемпературного стресса (рис. 3). При сравнении ответа

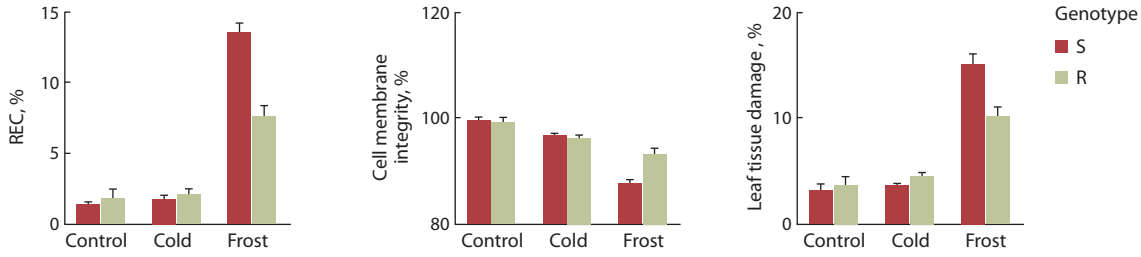

Fig. 2. Relative electrical conductivity (REC), cell membranes integrity, tissue damage rate of tea leaves during cold (0…+2 °C) and frost (–4…–6 °C) treatment: S, susceptible genotype; R, resistant genotype.

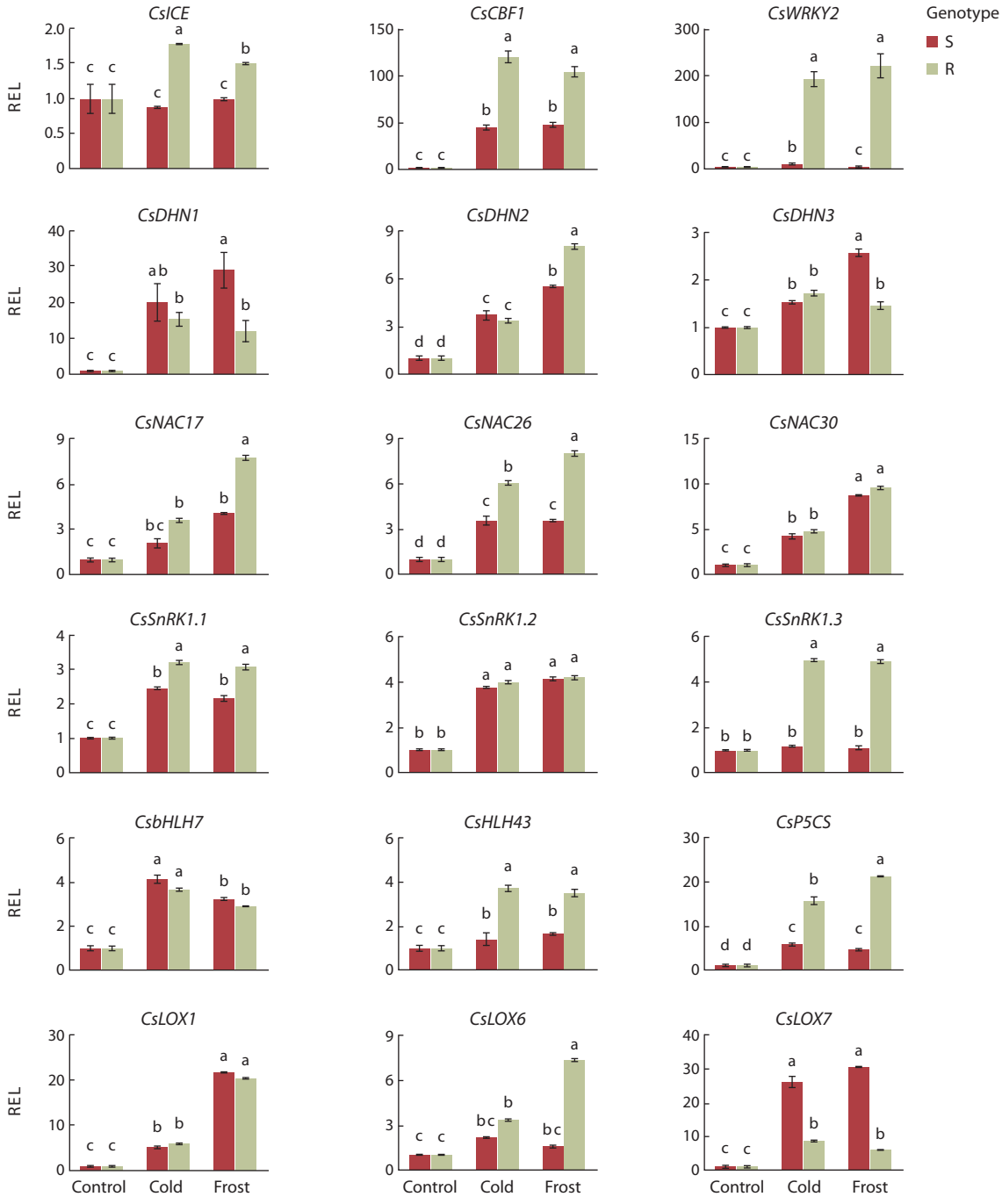

Fig. 3. Relative expression level (REL) of cold stress genes in tea leaves (S, susceptible genotype; R, resistant genotype) exposed to cold (0…+2 °C) and frost (–4…–6 °C).

Letters above bars indicate significance of differences at $p < 0.05$.

холод/мороз достоверные различия наблюдались по экспрессии генов *ICE1*, *WRKY2*, *DHN*, *NAC*, *bHLH7*, *LOX1*, *LOX6*, *P5CS*. По другим генам различия в ответе холод/мороз несущественны.

При сравнении ответа устойчивый/неустойчивый генотип по большинству генов выявлены значимые различия. Из 18 генов 11 проявили более высокий уровень экспрессии у устойчивого генотипа.

Экспрессия гена-регулятора холодового ответа *ICE1* у устойчивого генотипа достоверно повышалась в 1.5–1.8 раза при индукции стресса. У неустойчивого генотипа она оставалась на уровне контроля. Транскрипты *CBF1* накапливались у устойчивого генотипа в 3 раза сильнее, чем у неустойчивого, и составили 120 и 45 единиц соответственно. Апрегуляция гена *WRKY2* усиливалась при холоде и промораживании в большей степени у устойчивого генотипа – в 200 раз. Транскрипты генов дегидринов *DHN* усиленно накапливались у обоих генотипов при низкотемпературном стрессе. Достоверные различия между генотипами отмечены только по экспрессии *DHN2*. Оверэкспрессия трех генов семейства *NAC* наблюдалась при низкотемпературном стрессе у чая, которая в среднем составила 2–9 единиц. При этом устойчивый генотип отличался более высокой экспрессией генов *NAC26* и *NAC17* в сравнении с неустойчивым. Гены *HLH7* и *HLH43* также усиленно экпрессировались при холоде и промораживании в 3–4 раза, при этом *HLH43* активнее проявился у устойчивого генотипа. По гену *HLH7* различий между двумя генотипами не отмечено.

Экспрессия генов семейства *LOX* также повышалась в 5–26 раз при индукции холода и мороза. У устойчивого генотипа более активно проявился ген *LOX6* – в 3 и 7 раз – при холоде и морозе соответственно. Ген *LOX7* в большей степени активен при холоде у неустойчивого генотипа, а ген *LOX1* экспрессировался одинаково у двух генотипов. Три гена семейства *SnRK* апрегулировались у обоих сортов, ~2–4 раз при холоде и морозе, при этом у устойчивого генотипа отмечен более высокий уровень экспрессии *SnRK1.1* и *SnRK1.3*, по сравнению с неустойчивым сортом.

В целом сравнительный анализ экспрессии генов у двух генотипов показал, что устойчивый генотип раньше реагирует на стресс, транскрипты ряда генов у него сильнее накапливаются уже на этапе холодовой акклиматизации. Гены *ICE1*, *CBF1*, *WRKY2*, *DHN2*, *NAC17*, *NAC26*, *SnRK1.1*, *SnRK1.3*, *bHLH43*, *P5CS*, *LOX6*, проявились в большей степени у устойчивого генотипа чая при низкотемпературном воздействии и могут быть маркерами для отбора доноров устойчивости к холоду.

Обсуждение

Проведен сравнительный анализ экспрессии генов у двух контрастных по устойчивости генотипов чая в условиях индукции холода и промораживания для выявления различий в их ответных реакциях.

В анализ был включен ряд генов, которые, как ранее сообщалось, играют или могут играть важную роль в ответе на холодовой стресс. В опубликованных транскриптомных исследованиях показано, что ответ на холод и мороз у чайного растения различается (Li et al., 2019), поэтому мы сравнивали относительный уровень экспрессии генов в условиях этих двух стрессовых воздействий. В результате обнаружен ряд генов, которые экспрессируются поразному в условиях холода и мороза; установлены гены, различающиеся по уровню экспрессии у устойчивого и восприимчивого к морозу генотипов. Сравнение уровня экспрессии у двух различных по устойчивости генотипов может помочь предположить, какие из этих генов могут быть маркерами для поиска доноров устойчивости в селекции чая.

Низкотемпературный стресс приводит к нарушению стабильности клеточных мембран, изменению липидного, белкового и ферментного баланса клетки, вызванному окислительными процессами (Somerville, 1995; Thomashow et al., 1999). Наши результаты подтвердили, что А-2016 более морозоустойчив, чем сорт Колхида, так как у него в большей степени сохранялась целостность клеточных мембран при индукции заморозков.

Обнаружено, что у устойчивого генотипа более активно экспрессировались гены *ICE1*, *CBF1*, *DHN2*, *NAC17*, *NAC26*, *bHLH43*, *WRKY2*, *P5CS*, *LOX6*, *SnRK1.1*, *SnRK1.3* и их экспрессия была существенно выше уже на стадии холодовой акклиматизации, т. е. на первом этапе индукции стресса. Эти результаты согласуются с данными опубликованных исследований, в которых повышенная устойчивость растений чая обусловлена более скорым ответом на холодовой стресс (Ban et al., 2017; Li et al., 2019).

Известно, что *DHNs* – группа генов, кодирующих белки-дегидрины, которые действуют как криопротекторы, молекулярные шапероны, а также антиоксиданты, играя базовую роль в ответе растений на абиотические стрессы, входят в семейство транскрипционных факторов *LEA* II (Late Embryogenesis Abundant) (Hanin et al., 2011). Из трех генов *DHN*, ген *DHN2* может служить маркером холодоустойчивых сортов чая, что согласуется с ранее опубликованными данными по китайским генотипам чая (Ban et al., 2017).

Семейство транскрипционных факторов *NAC* кодирует белки, играющие важную роль в передаче ауксинового сигнала и развитии меристем, латеральных корней, клеточной стенки, биосинтезе флавоноидов и др. По данным других исследователей, экспрессия этих генов индуцируется засухой, засолением, холодом и повышением содержания абсцизовой кислоты (АБК) (Wang et al., 2016a). Наши результаты также подтверждают повышение уровня экспрессии этих генов у обоих генотипов как при холоде, так и при промораживании.

Гены семейства *WRKY* участвуют в АБК-зависимом пути ответа на абиотический стресс, а также в регуляции роста и развития растений. У чайного растения был выделен новый ген этого семейства, *CsWRKY2*, экспрессия которого повышалась при холодовом стрессе (Wang et al., 2016b). Полученные нами результаты согласуются с этими данными, к тому же у устойчивого генотипа *CsWRKY2* проявился более активно и при холоде, и при морозе, поэтому мы предполагаем, что он также может быть одним из маркеров устойчивости.

Гены *SnRK1.1*, *SnRK1.2*, *SnRK1.3* кодируют ферменты протеинкиназы, регулирующие катаболизм углеводов,

экспрессию генов метаболизма сахарозы, в частности генов *SUS*. В ответ на холод у чайного растения экспрессия этих генов существенно возрастала, что отмечено и у китайских сортов чая (Yue et al., 2015). Кроме того, по нашим данным, устойчивый генотип характеризовался более активной экспрессией *SnRK1.1* и *SnRK1.3*, следовательно, углеводный метаболизм у него протекал более активно.

К семейству транскрипционных факторов *bHLH*, участвующих в широком спектре биологических процессов, относятся: вторичный метаболизм брассиностероидов, жасмоновой кислоты, синтез антоцианов, модуляция роста и развития растений, контроль ветвления побегов и др. Кроме того, *bHLH* играют важную роль в передаче сигнала АБК и ответе растений на абиотические стрессы. У чайного растения обнаружено 39 генов *CsbHLH*, экспрессия которых повышалась в условиях засухи (Cui et al., 2018). Мы анализировали экспрессию двух генов из этого семейства, и оба из них апрегулировались при низкотемпературном стрессе, а ген *bHLH43* активнее экспрессировался у устойчивого генотипа.

Гены семейства липоксигеназ *LOX* вовлечены в катаболизм липидов, синтез оксилипина, жасмоновой кислоты и С6-альдегидов (Li et al., 2017). У чайного растения обнаружено, что гены *CsLOX1*, *CsLOX6* и *CsLOX7* могут играть важную роль в ответе на стрессы (холод, засуху, биотический стресс) в АБК-независимом пути ответа (Zhu et al., 2018), поэтому мы включили эти гены в эксперимент. В наших результатах показано, что эти три гена экспрессировались сильнее при холоде и промораживании, в сравнении с контролем, однако у устойчивого генотипа только ген *LOX6* более активно экспрессировался при стрессе, в сравнении с неустойчивым.

Ген *P5CS* – один из генов, вовлеченных в синтез пролина (Szekely et al., 2008). Более высокий уровень его экспрессии наблюдался при холоде у устойчивого генотипа чая. Достоверных различий экспрессии этого гена между устойчивыми и неустойчивыми сортами чая не отмечено (Ban et al., 2017). Проведение дополнительных исследований с разными сортами поможет верифицировать полученные данные.

Заключение

Таким образом, показана повышенная экспрессия всех изучаемых генов: *DHN1*, *DHN2*, *DHN3*, *NAC17*, *NAC26*, *NAC30*, *bHLH7*, *bHLH43*, *WRKY2*, *LOX1*, *LOX6*, *LOX7*, *SnRK1.1*, *SnRK1.2*, *SnRK1.3*. Выявлен ряд генов, более активно экспрессирующихся у устойчивого генотипа чая: *ICE1*, *CBF1*, *DHN2*, *NAC17*, *NAC26*, *bHLH43*, *WRKY2*, *P5CS*, *LOX6*, *SnRK1.1*, *SnRK1.3*. Обнаружены гены, различающиеся по экспрессии в холоде и в морозе: *ICE1*, *WRKY2*, *DHN*, *NAC*, *bHLH7*, *LOX1*, *LOX6*, *P5CS.* В целом устойчивый генотип характеризуется более ранним ответом на стресс. Однако в нашей работе проанализировано только два генотипа чая, поэтому для дальнейшей верификации маркеров устойчивости к холоду необходимо привлечение большего количества генотипов. Для дальнейших исследований актуально изучение экспрессии этих генов в других органах растений чая при разной силе низкотемпературного воздействия.

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ORCID ID

L.S. Samarina orcid.org/0000-0002-0500-1198
V.I. Malyarovskaya orcid.org/0000-0003-4213-8705

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Phenotypic and genetic variability of a tetraploid wheat collection grown in Kazakhstan

A. Zatybekov[1], S. Anuarbek[1, 2], S. Abugalieva[1], Y. Turuspekov[1, 2] ✉

[1] Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan
[2] Al-Farabi Kazakh National University, Almaty, Kazakhstan
✉ e-mail: yerlant@yahoo.com

Abstract. New cultivars adapted to major durum wheat growing environments are essential for the cultivation of this crop. The development of new cultivars has required the availability of diverse genetic material and their extensive field trials. In this work, a collection of tetraploid wheat consisting of 85 accessions was tested in the field conditions of Almaty region during 2018 and 2019. The accessions were ranged according to nine agronomic traits studied, and accessions with the highest yield performance for Almaty region of Kazakhstan were revealed. The ANOVA suggested that the performance of agronomic traits were influenced both by Environment and Genotype. Also, the collection was analyzed using seven SSR (simple sequence repeats) markers. From 3 to 6 alleles per locus were revealed, with an average of 4.6, while the effective number of alleles was 2.8. Nei's genetic diversity was in the range of 0.45–0.69. The results showed high values of polymorphism index content (PIC) in the range of 0.46–0.70, with an average of 0.62, suggesting that 6 out of 7 SSRs were highly informative (PIC > 0.5). Phylogenetic analysis of the collection has allowed the separation of accessions into six clusters. The local accessions were presented in all six clusters with the majority of them grouped in the first three clusters designated as A, B, and C, respectively. The relations between SSR markers and agronomic traits in the collection were studied. The results can be efficiently used for the enhancement of local breeding projects for the improvement of yield productivity in durum wheat.
Key words: *Triticum turgidum*; genetic diversity; SSR markers; agronomic traits.

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Фенотипическое и генетическое разнообразие коллекции тетраплоидной пшеницы, выращенной в Казахстане

А. Затыбеков[1], Ш. Әнуарбек[1, 2], С. Абугалиева[1], Е. Туруспеков[1, 2] ✉

[1] Институт биологии и биотехнологии растений, Алматы, Казахстан
[2] Казахский национальный университет им. аль-Фараби, Алматы, Казахстан
✉ e-mail: yerlant@yahoo.com

Аннотация. В основе эффективных технологий выращивания полевых культур лежат новые сорта, адаптированные к условиям зоны производства. Создание таких сортов предполагает наличие коллекции с широким генетическим разнообразием и тщательные полевые экологические испытания. В данной работе коллекция тетраплоидной пшеницы, состоящая из 85 сортов и линий различного происхождения, была изучена в полевых условиях Алматинской области Казахстана в 2018 и 2019 гг. Образцы коллекции были ранжированы по девяти изученным сельскохозяйственным признакам, в результате чего выявлены линии с высокой продуктивностью в условиях Алматинской области. С помощью дисперсионного анализа удалось установить, что как окружающая среда, так и генотип оказывают статистически высокое влияние на хозяйственно ценные признаки. Коллекция была исследована также с использованием семи микросателлитных SSR (simple sequence repeats) маркеров. Обнаружено от 3 до 6 аллелей на локус со средним значением 4.6, тогда как среднее значение эффективного числа аллелей равнялось 2.8. Индекс генетического разнообразия коллекции по Нею был высоким, в пределах 0.45–0.69. Значения PIC (polymorphism index content) варьировали от 0.46 до 0.70, при этом шесть из семи SSR показали высокую информативность (PIC > 0.5). Филогенетический анализ коллекции позволил разделить образцы на шесть кластеров. Местные образцы были представлены во всех шести кластерах; большинство из них было сгруппировано в первых трех кластерах, обозначенных как A, B и C. Изучена связь между определенными SSR-маркерами и агрономическими признаками в рассматриваемой коллекции. Полученные результаты могут быть эффективно использованы для усиления отечественных селекционных проектов для улучшения продуктивности твердой пшеницы.
Ключевые слова: *Triticum turgidum*; генетическое разнообразие; SSR-маркеры; хозяйственно ценные признаки.

Introduction

Durum wheat (*Triticum turgidum* L. ssp. *turgidum* convar. *durum* (Desf.) MacKey) is a tetraploid species of wheat and is the main crop to producers of pasta and cereals. The growing area under durum wheat is about 17 million hectares in the world and production is 37 million tons (Kabbaj et al., 2017; Zaïm et al., 2017). In 2019, durum wheat production in Kazakhstan amounted to 560 thousand tons (https://agbz.kz). Other tetraploid wheat species *Triticum turgidum* L. ssp. *turanicum* (Jakubz.) Á. Löve & D. Löve, *Triticum turgidum* L. ssp. *polonicum* (L.) Thell., *Triticum turgidum* L. ssp. *carthlicum* (Nevski) Á. Löve & D. Löve, *Triticum turgidum* ssp. *dicoccum* (Shrank ex Schübler) Thell. are used as food and feed crops in different world regions. Wild species *Triticum turgidum* ssp. *dicoccoides* (Korn. ex Asch. & Graebn.) Thell. is also often included in crossing schemes as a source for resistance to abiotic and biotic stresses (De Vita, Taranto, 2019; Mujeeb-Kazi et al., 2019).

The enhancement of a breeding program largely depends on an understanding of adaptation-related patterns that affect the productivity of cereal crops, including durum wheat. One of the ways to study these patterns is the assessment of diverse germplasm collections, including relative wild and cultivated species and landraces, in a particular environmental condition, and evaluate genotype × environment interaction features (Anuarbek et al., 2020). Hence, the comprehensive study of the diverse germplasm is a very important prerequisite for the successful conservation and rational use of plant genetic resources, including both wild and cultivated tetraploid wheat species (Maccaferri et al., 2003; Anuarbek et al., 2020). The appropriate assessment of the genetic diversity in these collections depends on the application of informative and efficient types of DNA markers. In many centers of the world, research is underway to find and use different types of DNA markers with the aim of using them to study genetic diversity, inventory, genotyping, mapping, and identifying genes associated with useful traits of cultivated plant varieties and lines (Idrees, Irshad, 2014). Various types of DNA markers have been developed and are successfully used to study the genetic diversity of accessions of the genus *Triticum* L. (Röder et al., 1998; Song et al., 2005; Singh et al., 2018). PCR-based markers, such as RAPD, AFLP, and SSR, are widely used tools for studying genetic diversity and discrimination both durum and common wheat (Khlestkina et al., 2002; Kudriavtsev et al., 2004; Yildirim et al., 2011; Abugalieva et al., 2012; Melloul et al., 2014; Adonina et al., 2017).

The wheat genome contains a class of specific nucleotide sequences called microsatellites, also known as SSRs or simple sequences repeats (Ganal, Röder, 2007). SSR markers have many advantages, being highly polymorphic, codominant, informative, reliable, and the availability of information on chromosomal localization (Röder et al., 1998; Vieira et al., 2016). Microsatellites are hypervariable, they often have dozens of alleles at one locus, differing from each other in the number of repeats. They are widely used to study genetic diversity, as well as for the analysis of paternity and mapping of quantitative trait loci (QTLs), kinship, belonging to a specific population, for studying hybridization, evolutionary processes, and for searching for paralogs (Abouzied et al., 2013; Leonova et al., 2013; Jaiswal et al., 2017).

Durum wheat polymorphism studies are currently underway worldwide. The survey of reports demonstrated the successful use of SSR markers for assessment of the genetic diversity in different collections of Europe (Ganeva et al., 2010; Marzario et al., 2018), Africa (Henkrar et al., 2016; Slim et al., 2019), China (Wang et al., 2007; Chen et al., 2012), Russia (Kudryavtsev et al., 2004), Turkey (Yildirim et al., 2011), Syria (Achtar et al., 2010), etc. Microsatellites are also highly effective in tagging specific genes that play an important role in variation for yield components and biotic stress resistance. A number of studies reported relations between SSR loci and wheat traits, such as yield, etc. For instance, Zhang et al. (2013) showed that the *Xgwm11*-1B locus is significant ($p < 0.001$) for plant height. In the study reported by Li et al. (2015) it was shown that the marker *Xgwm148*-2B is associated with the manifestations of the traits "thousand grain weight", "spike yield index" and "weight of kernels per spike". *Xgwm251* was associated with lipoxygenase (LOX) activity, which is an important factor determining the color of flour and end-use products of wheat (Geng et al., 2010). Vinod et al. (2014) have identified the significant association between *Xgwm234* and the resistance of *T. turgidum* to leaf rust. Golabadi et al. (2011) showed that the *Xcfa2114*-6A marker was responsible for 20 % of the phenotypic variation in the yield index and thousand grain weights (TGW) under different environmental conditions. SSR marker *Xgwm219* was also shown to be associated with TGW (Roncallo et al., 2017). These examples suggest that the assessment of the genetic diversity of the varietal gene pool of durum wheat may provide not only proper genetic documentation of the accessions but also hinting the identification of a valuable source of genes associated with agronomic traits.

The purpose of this work was the study the genetic diversity using seven SSR markers and phenotypic variation in yield components in the collection of tetraploid species harvested in the conditions of South-East Kazakhstan.

Materials and methods

Plant material and experimental site conditions. The plant material consisted of 85 accessions of tetraploid wheat (2 *Triticum turgidum* ssp. *dicoccoides* (Korn. ex Asch. & Graebn.) Thell., 2 *Triticum turgidum* ssp. *dicoccum* (Shrank ex Schübler) Thell., 65 *Triticum turgidum* L. ssp. *turgidum* convar. *durum* (Desf.) MacKey, 10 *Triticum turgidum* L. ssp. *turanicum* (Jakubz.) Á. Löve & D. Löve, 4 *Triticum turgidum* L. ssp. *polonicum* (L.) Thell., and 2 *Triticum turgidum* L. ssp. *carthlicum* (Nevski) Á. Löve & D. Löve from different geographical origins (Supplementary Table 1)[1]. Seeds were provided by the Research Center for Grain and Industrial Crops (Foggia, Italy), University of Bologna (Bologna, Italy), Aktobe and Karabalyk Agricultural Experimental Stations (Kazakhstan). The collection included 21 cultivars and 15 promising lines of durum wheat from Kazakhstan (see Suppl. Table 1).

The studied collection of tetraploid wheat was evaluated in two randomized replicates in the field conditions of Almaty region (Table 1).

Each accession was planted in two rows with a row spacing of 15 cm, 25 seeds per row. In total, nine agronomic traits con-

[1] Supplementary Tables 1 & 2 are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2020-24/appx9.pdf

Table 1. Meteorological conditions and characteristics of the experimental site

Year	Precipitation, mm	Coordinate			Temperature, °C			Sowing date	Harvesting date
		latitude	longitude	altitude, m	mean	min	max		
2018	311.6	43°21′	76°53′	740	20.2	–1.7	37.7	15.04.18	01.08.18
2019	396.0	43°21′	76°53′	740	18.7	–2.9	37.6	28.03.19	04.08.19

Note: T mean, T max and T min – average, maximum and minimum temperature during the vegetative period, respectively.

nected with the vegetation period, plant morphology, and yield components were studied. The list of traits included the heading time (HT, days), flowering time (FT, days), seed maturation time (SMT, days), plant height (PH, cm), spike length (SL, cm), number of fertile spikes (NFS, pcs), number of kernels per spike (NKS, pcs), thousand kernel weight (TKW, g), and yield per plant (YPP, g) (Anuarbek et al., 2020).

DNA extraction and SSR genotyping. Genomic DNA was isolated from individual 4-day-old wheat seedlings, according to Dellaporta et al. (1983). The quality and quantity of isolated DNA were evaluated using a NanoDrop 2000 (Thermo Fisher Scientific, USA) and agarose electrophoresis in 1 % gel. The list of markers used for SSR analysis was the following: *Xgwm11*, *Xgwm148*, *Xgwm251*, *Xgwm234*, *Xcfa2114*, *Xgwm169*, and *Xgwm219* (Supplementary Table 2). Polymerase chain reaction (PCR) was conducted in a Veriti™ Thermal Cycler (Thermo Fisher Scientific, USA). The PCR reaction mixture (10 µl) contained from 2.5 mM of 10× Taq buffer; 0.2 mM of each dNTP; 1.5 mM $MgCl_2$; 250 µM of each primer; 1 unit *Taq* polymerase (Promega, USA) and 50 ng of genomic DNA.

The amplification program included the following cycles: 94 °C – 3 min; 40 cycles: 94 °C – 1 min; annealing temperature (55 or 60 °C depending on the primer) – 1 min; 72 °C – 2 min; and 72 °C – 10 min. PCR products were separated on 6 % polyacrylamide gels (Amresco, Solom, OH) run in 0.5× TBE buffer pH 8.0 at 250 V for 1.5 h. Gels were stained with ethidium bromide, and the images were recorded with a Bio-Rad Image System (Bio-Rad, Hercules, CA). Allele sizes were estimated in comparison with 100 bp DNA ladder (Thermo Fisher Scientific, USA).

Statistical analyses of field data were estimated using SPSS 22.0 and STATISTIKA 13.2 software (http://software.dell.com/products/statistica).

Genetic diversity was assessed based on Nei's genetic diversity index and Shannon Information Index, using the GenAlex, ver.6.5 program (Peakall, Smouse, 2012). The values of the PIC index (polymorphism information content) suggested the effectiveness of the markers used, given that markers with a value of PIC > 0.5 considered as highly informative; 0.5 > PIC > 0.25 as informative; and PIC ≤ 0.25 as marginally informative (Botstein et al., 1980). Variation among populations was studied using Principal Coordinate Analysis (PCoA) in the software GenAlex, ver.6.5 (Peakall, Smouse, 2012). The resulting similarity matrix was further analyzed using the neighbor-joining clustering algorithm for the construction of the dendrogram. The phylogenetic tree was constructed using PAST v.3.25 software (Hammer et al., 2001). Analyses of marker-trait associations were conducted using a simple *t*-test (Kim, 2015).

Results

Phenotypic variation in the studied collection

Field trials for two years revealed a sharp difference in the vegetation period between species of tetraploid wheat (Table 2).

All accessions reached the ripening stage, with an except for the wild accession PI346783 (Hungary, *T. dicoccoides*). The shortest HT was observed in genotypes of *T. dicoccoides* (56.5 ± 3.5 days), the longest – in *T. polonicum* (60.7 ± 3.9 days) (see Table 2).

Plant height is one of the important morphological traits of the crops. According to the species, the highest ones were the samples from *T. carthlicum* (117.9 ± 5.4 cm), while the accessions from *T. dicoccum* were the lowest (97.4 ± 7.4 cm). On the other hand for *T. durum* genotypes the PH ranged from 58.0 ± 3.7 cm (Casanova 58.0 ± 3.7, Mexicali75 58.5 ± 4.9, Ciclope 60.5 ± 3.9) to 137.6 ± 3.0 cm for cultivar Kargala 66 (see Suppl. Table 1). As for the SL, the lowest value (5.0 ± 0.2 cm) had the cultivar PI 184526 (*T. turanicum* from Portugal), while the highest value (17.5 ± 1.7 cm) was in accession PI 210845 (*T. polonicum* from Iran).

The value of a cultivar is determined by its productivity, which consists of several components, including TKW which is significantly affected by weather conditions, violation of moisture supply, and mineral nutrition of plants during the formation and maturation of grain. The highest averaged TKW values were revealed for three *T. turanicum* accessions (CLTR11390, USA – 64.8 ± 4.1 g; PI 352514, Azerbaijan – 58.2 ± 1.0 g; and PI 254206, Iran – 55.2 ± 4.0 g) and *T. polonicum* from Iraq (PI 208911 – 61.8 ± 4.5 g). The lowest TKW value was in accessions of *T. carthlicum* (29.9 ± 1.1 g). The NFS ranged from 3.9 ± 0.6 pcs/plant in the accession PI 343446 (*T. dicoccoides*) to 2.0 ± 0.5 pcs/plant in genotypes PI 210845 and PI 266846 of *T. polonicum*.

As for NKS and YPP the highest value were on accessions of *T. durum* and the lowest to *T. dicoccoides* (see Table 2). The min value of NKS (24.8 ± 3.8 pcs) under both conditions was obtained in PI 343446 (*T. dicoccoides*, Israel), the max – in Kazakh cultivar Gordeiforme 254 (67.7 ± 7.1 pcs) and Canadian cultivar Strongfield (62.2 ± 1.2). Overall 31 *T. durum* accessions prevailed the local check cultivar Gordeiforme 254 (4.4 ± 1.6 g/plant) by YPP. Top twenty accessions by yield contained cultivars from Canada (Strongfield – 7.6 ± 1.9 g/plant), Spain (Granizo – 7.0 ± 1.9 g/plant), Italy (Capeiti-8 and Ancomarzio), Syria (Sharm5), Russia (Har'kovskaya 46, Altaika, Altaiskii yantar'), Ukraine (Har'kovskaya 90 and Har'kovskaya 9), USA (LO92), as well as 5 cultivars and 4 breeding lines (e. g. G 2607 – 7.2 ± 1.4 g/plant), from Kazakhstan (see Suppl. Table 1).

Table 2. Phenotypic variation in the collection of tetraploid wheat according to two-year field trials data

Traits	Species					
	carthlicum	*dicoccoides*	*dicoccum*	*durum*	*polonicum*	*turanicum*
HT, days	58.4 ± 3.6	56.5 ± 3.5	59.4 ± 2.1	57.8 ± 1.7	60.7 ± 3.9	58.5 ± 1.3
FT, days	62.1 ± 4.4	59.0 ± 3.0	65.3 ± 1.8	62.9 ± 2.2	66.5 ± 2.6	64.7 ± 1.7
SMT, days	33.3 ± 1.8	35.8 ± 2.3	34.4 ± 2.6	37.9 ± 1.7	32.4 ± 1.4	36.3 ± 2.3
PH, cm	117.9 ± 5.4	107.5 ± 1.2	97.4 ± 7.4	100.4 ± 5.3	110.1 ± 3.4	98.1 ± 8.6
SL, cm	9.6 ± 0.1	8.4 ± 0.3	9.8 ± 1.7	7.6 ± 0.5	13.1 ± 1.0	8.6 ± 0.5
NFS, pcs	3.5 ± 0.6	3.9 ± 0.6	2.3 ± 0.2	2.8 ± 0.4	2.0 ± 0.5	2.1 ± 0.3
NKS, pcs	40.3 ± 4.1	24.8 ± 3.8	29.9 ± 0.9	45.9 ± 3.4	35.8 ± 3.0	37.3 ± 4.3
TKW, g	29.9 ± 1.1	35.0 ± 1.1	39.8 ± 1.3	44.6 ± 3.0	47.6 ± 3.1	48.0 ± 4.0
YPP, g	3.3 ± 0.9	2.5 ± 0.5	2.6 ± 0.2	4.5 ± 0.8	2.9 ± 0.6	3.1 ± 0.6

Note: HT – heading time, FT – flowering time, SMT – seed maturation time, PH – plant height, SL – spike length, NFS – number of fertile spikes, NKS – number of kernel per spike, TKW – thousand kernel weight, YPP – yield per plant.

Table 3. Two-way ANOVA based on two years field trials

Traits	Genotype (species)		Environment (year)		Genotype × Environment (species × year)	
	SS	F	SS	F	SS	F
HT	60.51	0.73	66.8	4.01*	69.67	0.84
FT	71.1	0.46	24.1	0.77	161.2	1.03
SMT	183.01	2.79*	69.75	5.32*	47.72	0.73
PH	1845.9	0.85	282.3	0.65	860.1	0.40
NFS	13.61	5.97***	0.58	1.26	3.97	1.74
SL	67.84	6.30***	16.23	7.53**	15.55	1.44
NKS	3547.44	11.54***	298.07	4.85*	145.69	0.47
TKW	1650.98	9.85***	35.53	1.06	326.09	1.95
YPP	49.22	5.06***	0.86	0.44	11.74	1.21

Note: The F values are provided with significance level indicated by the asterisks. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

The Pearson index analysis revealed a significant positive correlation ($p < 0.01$) between yield components and phenotypic traits. The ANOVA test based on two-years field trials suggested that Genotype significantly influenced the SMT, NFS, SL, and all yield components (NFS, NKS, TKW, YPP) with $p < 0.001$ (Table 3).

Microsatellite analysis of the tetraploid wheat collection

The lines and cultivars of the studied tetraploid wheat collection were analyzed using 7 polymorphic microsatellite markers (see Suppl. Table 2) localized on 6 wheat chromosomes – 1B, 2B, 4B, 5B, 6A, 6B. The results based on using 7 SSR markers have allowed identifying a total of 32 alleles, with average 4.57 alleles per marker (Table 4).

The effective number of alleles ranged from 1.82 to 3.27, with a mean value of 2.77. Nei's genetic diversity index averaged 0.62 (see Table 4). The average value of polymorphism information content (PIC) was 0.62, ranging from 0.46 for *Xgwm219* to 0.7 for *Xgwm148*, *Xgwm251*, and *Xgwm11*, respectively.

Table 4. Assessment of the level of genetic diversity of SSR markers in tetraploid wheat collection

SSR marker	na	ne	I	h	PIC
Xgwm11	6	3.27	1.38	0.69	0.70
Xgwm148	4	3.23	1.28	0.69	0.70
Xgwm251	6	3.19	1.29	0.69	0.69
Xgwm234	3	2.46	0.99	0.59	0.60
Xcfa2114	5	2.63	1.18	0.62	0.58
Xgwm169	4	2.75	1.13	0.63	0.64
Xgwm219	4	1.82	0.81	0.45	0.46
Mean	4.57	2.77	1.15	0.62	0.62
SE	1.13	0.52	0.19	0.08	0.09

Note: na – the number of alleles per locus; ne – the effective number of alleles; I – Shannon information index; h – Nei's diversity index; PIC – polymorphic information content.

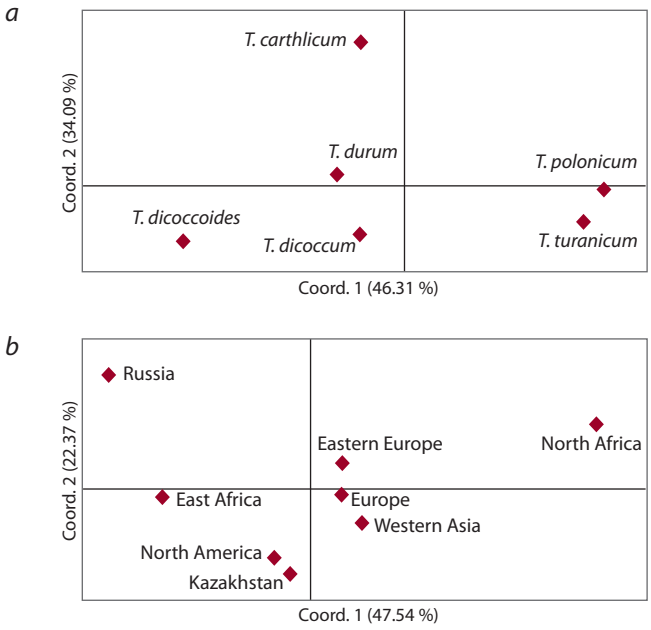

Fig. 1. Principal coordinate analysis for 85 tetraploid wheat accessions separated by species (*a*) and origin (*b*) groups based on SSR analysis.

The PCoA was conducted based on SSR genotyping of 85 tetraploid wheat accessions using 7 SSR markers. Accessions of the studied collection were divided into groups depending on their attribution to species and place of origin, respectively (Fig. 1).

The first principal component in the PCoA (46.31 %) clearly separated *T. polonicum* and *T. turanicum* from other species (see Fig. 1, *a*). The most genetically distant from other species was *T. carthlicum*. PCoA using origin data revealed that local genotypes were genetically closer to the North American accessions (see Fig. 1, *b*). The accessions from Russia and North Africa were genetically distant from other groups of origin.

Based on the genetic diversity results using 7 polymorphic SSR markers, a phylogenetic tree of 85 accessions of tetraploid wheat was constructed (Fig. 2).

The analysis revealed a division into two large clusters. The first cluster consisted mostly of cultivars of tetraploid wheat from Kazakhstan and North America. The second cluster was divided into three sub-clusters. Although the European accessions were dominated in all three subclusters of cluster 2, all three sub-clusters included cultivars and lines of Kazakhstan (see Fig. 2).

The *t*-test was performed to confirm the significance of the SSR markers for the studied traits. The results identified the most informative SSR markers related to major agronomic traits (Table 5). *Xgwm251* showed a significant relationship to HT and FT. Four markers were related to variance in PH (*Xcfa2114*, *Xgwm251*, *Xgwm234*, and *Xgwm169*).

Discussion

Initially, the studied collection was separated according to their species classification and origin (see Suppl. Table 1). The average yield analysis in the collection of tetraploid accessions over two years (2018 and 2019) suggested that it is highly correlated with all studied phenotypic traits ($p < 0.01$),

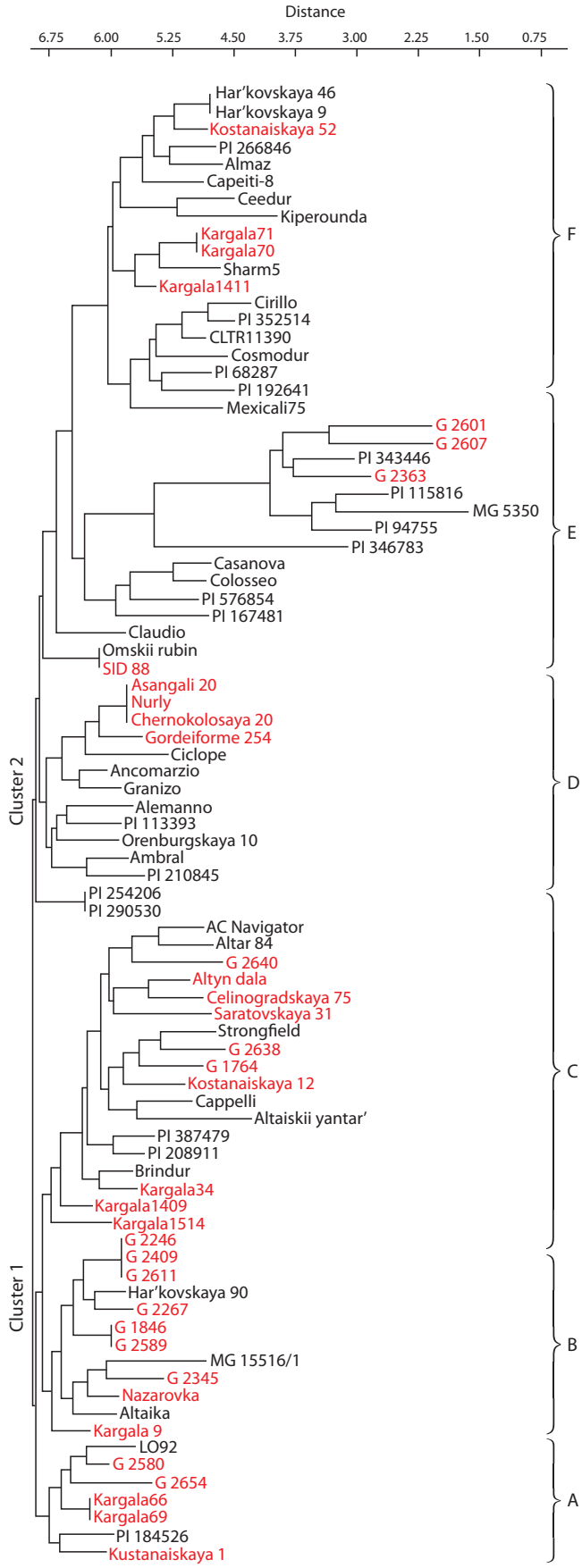

Fig. 2. Neighbor-joining phylogenetic tree of 85 tetraploid wheat accessions based on SSR analysis. Sub-clusters are designated as A, B, C, D, E, and F.

Table 5. The *t*-test results on the identification of the relations between SSR markers and phenotypic traits

Traits	*Xgwm11*	*Xgwm148*	*Xgwm251*	*Xgwm234*	*Xcfa2114*	*Xgwm169*	*Xgwm219*
HT	0.62	0.68	2.17*	0.49	–0.36	–0.54	1.71
FT	0.79	0.80	2.05*	–0.14	0.19	–1.43	1.54
SMT	0.07	–0.88	–0.66	–1.10	–0.46	0.48	–0.07
PH	1.88	0.91	2.37*	–2.32*	–2.16*	2.29*	1.17
NFS	–0.53	0.26	0.93	0.19	–0.56	3.51***	1.12
SL	1.58	–0.96	1.53	0.60	–0.06	–2.27*	–0.20
NKS	0.95	–0.16	–0.92	0.44	1.01	1.49	2.30*
TKW	1.75	0.50	–1.68	2.33*	0.17	–1.29	–0.36
YPP	0.90	0.54	0.03	1.59	1.61	2.80***	2.36*

Note: The *t*-values are provided with significance level indicated by the asterisks. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

confirming the importance of selected characters in the trials. The two-way ANOVA showed that Environment greatly influenced HT and SMT. In addition, it was found that SMT is also influenced by Genotype, showing the prospects of possibility to adjust maturation time in the breeding process, as early seed maturation is vital to avoid abiotic stresses during the important stages of plant growth. Particularly, it was shown that in *T. polonicum* the seeds are ripening nearly five days earlier than in *T. durum* (see Table 2). The field trials have allowed the identification of accessions with outstanding field performances. For instance, the cultivar Strongfield (Canada) showed 7.6 ± 1.9 g/plant, which was the highest yield value among 31 *T. durum* accessions that prevailed local standard Gordeiforme 254 (4.4 ± 1.6 g/plant). In general, two-way ANOVA indicated the great influence of the environmental factors, as they were affected both adaptation-related traits, such as HT and SMT, and yield components, such as SL and NKS (see Table 3).

The entire collection was studied using seven SSR markers that were located on six different chromosomes (see Suppl. Table 2). According to the previous works, a list of markers in this study was most useful to evaluation of genetic diversity and associations with agronomic traits of durum wheat (Royo et al., 2005). The average PIC value was higher than 0.6, suggesting that the level of polymorphism was very high. The high level of variation in the collection has effectively allowed the separation of accessions according to their species classification (see Fig. 1, *a*). Notably, the PC1 (46.3 %) separated *T. polonicum* and *T. turanicum* from the remaining species, and the PC1 (34.1 %) distinguished *T. carthlicum* and *T. durum* from *T. dicoccum* and *T. dicoccoides*. Interestingly, the accessions originated in Kazakhstan were genetically close to North American samples (see Fig. 1, *b*), and it is to some extent confirm the phylogeny of hexaploid bread wheat studies using SNP (single nucleotide polymorphism) markers (Turuspekov et al., 2015). The PC plot is suggesting that six accessions of durum wheat from the Russian Federation are distinctly different from accessions with other origins (see Fig. 1, *b*). The Neighbor-joining phylogenetic tree suggested that all accessions can be divided into two clusters, where cluster 1 was mostly populated by accessions from Kazakhstan (see Fig. 2).

The significance of each SSR marker for studied traits was assessed using a two-tailed *t*-test (Lüders et al., 2016; Rahimi et al., 2019). The results of the test suggested that five out of seven SSRs were significant at least for one studied trait (see Table 5). The PH was the trait where four SSR markers, two with negative and two with positive values, were significantly correlated. In addition, the test showed that *Xgwm234* is significantly correlated with TKW and *Xgwm219* and *Xgwm169* with YPP (see Table 5). Thus, the application of SSR markers in the analysis of tetraploid wheat collection consisting of 85 accessions was used for (1) genetic documentation of samples, (2) for phylogenetic clusterization based on the species classification and geographic origin, and (3) associations between DNA markers and studied phylogenetic traits. Hence, the results can be efficiently used for the enhancement of local breeding projects for the improvement of yield productivity in durum wheat.

Conclusion

The phenotypic analysis of the tetraploid wheat collection consisting of 85 accessions showed a high correlation of YPP with all 8 phenotypic traits in conditions of South-East Kazakhstan. The ANOVA suggested that the environmental conditions significantly affected the variation in HT and SMT, while Genotype has contributed significantly to main yield components, including TKW. Overall, 31 accessions of *T. durum* showed higher average yield values in comparison with local check cultivar Gordeiforme 254 (4.4 ± 1.6 g/plant), and Canadian cultivar Strongfield was with the highest yield value (7.6 ± 1.9 g/plant). The application of seven SSR markers suggested that local accessions were distinctly different from durum accession from other parts of the world. Particularly, the Principal Coordinate plot showed that local durum samples were most close to North American samples. The Neighbor-joining phylogenetic tree separated 85 samples to two main clusters, where the cluster 1 was mainly represented by Kazakh accessions and cluster 2 mostly by European accessions. The application of the *t*-test indicated that five out of seven SSRs were significant at least with one agronomic trait. Obtained results can be efficiently used for the enhancement of local breeding projects for the improvement of yield productivity in durum wheat.

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ORCID ID

A. Zatybekov orcig.org/0000-0003-4310-5753
S. Anuarbek orcig.org/0000-0002-8673-9820
S. Abugalieva orcig.org/0000-0002-9748-507X
Y. Turuspekov orcig.org/0000-0001-8590-1745

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Genetic diversity of VIR *Raphanus sativus* L. collections on aluminum tolerance

A.B. Kurina ✉, I.A. Kosareva, A.M. Artemyeva

Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
✉ e-mail: nastya_n11@mail.ru

Abstract. Radish and small radish (*Raphanus sativus* L.) are popular and widely cultivated root vegetables in the world, which occupy an important place in human nutrition. Edaphic stressors have a significant impact on their productivity and quality. The main factor determining the phytotoxicity of acidic soils is the increased concentration of mobile aluminum ions in the soil solution. The accumulation of aluminum in root tissues disrupts the processes of cell division, initiation and growth of the lateral roots, the supply of plants with minerals and water. The study of intraspecific variation in aluminum resistance of *R. sativus* is an important stage for the breeding of these crops. The purpose of this work was to study the genetic diversity of *R. sativus* crops including 109 accessions of small radish and radish of various ecological and geographical origin, belonging to 23 types, 14 varieties of European, Chinese and Japanese subspecies on aluminum tolerance. In the absence of a rapid assessment methodology specialized for the species studied, a method is used to assess the aluminum resistance of cereals using an eriochrome cyanine R dye, which is based on the recovery or absence of restoration of mitotic activity of the seedlings roots subjected to shock exposure to aluminum. The effect of various concentrations on the vital activity of plants was revealed: a 66-mM concentration of $AlCl_3 \cdot 6H_2O$ had a weak toxic effect on *R. sativus* accessions slowing down root growth; 83 mM contributed to a large differentiation of the small radish accessions and to a lesser extent for radish; 99 mM inhibited further root growth in 13.0 % of small radish accessions and in 7.3 % of radish and had a highly damaging effect. $AlCl_3 \cdot 6H_2O$ at a concentration of 99 mM allowed us to identify the most tolerant small radish and radish accessions that originate from countries with a wide distribution of acidic soils. In a result, it was possible to determine the intraspecific variability of small radish and radish plants in the early stages of vegetation and to identify genotypes that are contrasting in their resistance to aluminum. We recommend the $AlCl_3 \cdot 6H_2O$ concentration of 83 mM for screening the aluminum resistance of small radish and 99 mM for radish. The modified method that we developed is proposed as a rapid diagnosis of aluminum tolerance for the screening of a wide range of *R. sativus* genotypes and a subsequent study of contrasting forms during a longer cultivation of plants in hydroponic culture (including elemental analysis of roots and shoots, contrasting in resistance of accessions) as well as reactions of plants in soil conditions.

Key words: radish and small radish; collection; genetic diversity; acidic soils; eriochrome cyanine R; early diagnosis; aluminum resistance.

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Генетическое разнообразие *Raphanus sativus* L. коллекции ВИР по алюмоустойчивости

А.Б. Курина ✉, И.А. Косарева, А.М. Артемьева

Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
✉ e-mail: nastya_n11@mail.ru

Аннотация. Редис и редька (*Raphanus sativus* L.) – популярные и широко возделываемые в мире корнеплодные овощные культуры, которые занимают важное место в питании человека. На их продуктивность и качество существенное влияние оказывают эдафические стрессоры. Основным фактором, определяющим фитотоксичность кислых почв, служит повышенная концентрация подвижных ионов алюминия в почвенном растворе. Аккумуляция алюминия в тканях корня нарушает процессы деления клеток, инициации и роста боковых корней, снабжения растения минеральными веществами и водой. Изучение внутривидовой изменчивости по алюмоустойчивости *R. sativus* является важным этапом в селекции этих культур. Цель настоящего исследования заключалась в изучении генетического разнообразия культур *R. sativus* на примере 109 образцов редиса и редьки различного эколого-географического происхождения, принадлежащих 23 сортотипам, 14 разновидностям европейского, китайского и японского подвидов, по признаку устойчивости к токсиче-

скому действию ионов алюминия. При отсутствии специализированной для вида методики экспресс-оценки взят метод оценки алюмоустойчивости с использованием эриохромцианинового красителя, разработанный для зерновых культур, в основе которого лежит учет степени восстановления митотической активности корней проростков, подвергнутых шоковому воздействию повышенных концентраций алюминия. Выявлено влияние различных концентраций на жизнедеятельность растений: концентрация хлорида алюминия 66 мМ оказывала слабое токсическое действие на образцы *R. sativus*, замедляя отрастание корней; концентрация 83 мМ оказалась в высокой степени дифференцирующей для редиса и в меньшей – для редьки; концентрация 99 мМ полностью ингибировала дальнейший рост корней у 13.0 % образцов редиса и 7.3 % редьки и обладала высоко повреждающим эффектом. Концентрация $AlCl_3 \cdot 6H_2O$ 99 мМ позволила выделить наиболее высокотолерантные образцы редиса и редьки, которые происходят из стран с широким распространением кислых почв. В результате исследований широкого разнообразия мировой коллекции определена внутривидовая изменчивость редиса и редьки на ранних этапах вегетации и идентифицированы контрастные по устойчивости к алюминию генотипы. Мы рекомендуем концентрацию 83 мМ $AlCl_3 \cdot 6H_2O$ для скрининга алюмоустойчивости образцов редиса, а концентрацию 99 мМ – для образцов редьки. Разработанный нами модифицированный метод предлагается в качестве экспресс-диагностики алюмотолерантности для быстрого скрининга широкого спектра генотипов *R. sativus* и последующего изучения контрастных форм при более длительном выращивании растений в гидропонной культуре (включая элементный анализ корней и побегов, контрастных по устойчивости образцов), а также реакций растений в почвенных условиях.
Ключевые слова: коллекция редиса и редьки; генетическое разнообразие; кислые почвы; эриохромцианин; ранняя диагностика; алюмоустойчивость.

Introduction

Aluminum is one of the most abundant metals in the earth' crust (Fitzpatrick, 1986; Kochian et al., 2015) and is considered non-toxic to plants when the soil solution is neutral or slightly alkaline. Natural processes or human activities can lead to an increase acidity in soil, in result of which the solubility of aluminum increases, and the content of its mobile forms (Al^{3+}) increases (Lin-Tong et al., 2013), that makes aluminum the main toxic factor in acidic soils (Klimashevskiy, 1991; Kochian et al., 2004). Acidic soils in the world make up 30–40 % of arable ground and up to 70 % of ground that can potentially be used as arable (Suhoverkova, 2015). In Russia in 2019, out of 50 million hectares of excessively acidic soils, strongly and moderately acidic ones occupy from 25 to 35 million hectares, which is about 30 % of all arable ground (Vorob'ev, 2019).

The toxicity of Al^{3+} ions reduces productivity by inhibiting root growth and affecting water and nutrient absorption. A number of studies have described the symptoms of aluminum poisoning associated with impaired permeability of the cell wall, plasma membrane, mitochondrial, cytoskeleton, and nuclear functions (McNeilly, 1982; Roy et al., 1988; Aniol, 1997; Kabata-Pendias, 2010). So, aluminum affects on a series of cellular processes, including the rate of cell division, and disrupts the properties of protoplasm and cell walls.

Plants are subdivided into resistant and sensitive by alumotoxicity, varietal differences may be stronger than species (Hanson, Kamprath, 1979; Klimashevskiy, 1991). Plants have developed several mechanisms of resistance to aluminum during the evolutionary process (Kochian et al., 2005, 2015; Ma, 2007; Ma et al., 2014). In recent years, the molecular mechanism of aluminum tolerance in agricultural crops, primarily in cereals, has been actively studied (Liu et al., 2014; Ma et al., 2014; Kochian et al., 2015). Significant progress has been achieved in understanding the physiological and molecular mechanisms of aluminum tolerance in Arabidopsis (Hoekenga et al., 2006), rapeseed (Ligaba et al., 2006), maize (Ligaba et al., 2012), soybean (Peng et al., 2018), rice (Huang et al., 2012; Che et al., 2018), sorghum (Huang et al., 2018; Melo et al., 2019), rye (Collins et al., 2008; Yokosho et al., 2010) and wheat (Gruber et al., 2010; Wang et al., 2015).

At present, aluminum resistance is considered as a complex phytoecological problem, from the solution of which an obtaining of guaranteed productivity crops on acidic soils depends. The identification of genes and mechanisms of aluminum tolerance makes possible Al-tolerant species and cultivars of agricultural crops breeding using molecular and transgenic approaches (Delhaize et al., 2004; Magalhaes et al., 2007; Pereira et al., 2010).

The basic critical parameter for the successful creation of stress tolerant cultivars is the genetic diversity of the initial material for this indicator as a material for selection (Lisitsyn, Amunova, 2014). The successful creation of aluminum-resistant cultivars of agricultural plants is based on a significant variability in the trait of aluminum tolerance and relatively simple methods of screening and breeding (Batalova, Lisitsyn, 2002; Kosareva, 2012). The search for genotypes with a high tolerance to Al is of great importance for agriculture on acidic soils.

Radish and small radish belong to the species *Raphanus sativus* L., for which two primary geographical centers of origin are known – Mediterranean and Asian (Vavilov, 1965), herewith the Asian center was divided into secondary centers in the classification of M.A. Shebalina and L.V. Sazonova (1985): South West Asian, East Asian, South Asian tropical. Small radish is a mutant form of radish; artificial selection was carried out on the feature of dwarfishness of plants in the vegetative period of ontogenesis, while the plants of the reproductive period practically do not differ in habitus from the radish plants. The

processes of mutagenesis in *R. sativus* are determined by the climatic conditions of the places of origin of cultural forms. Cultivation of radish began 4–3 thousand BC, small radish was introduced into culture much later – the first information about it appeared in Italy at the beginning of the 16th century.

Small radish cultivars are assigned to 6 botanical varieties and 16 types, radish – 14 varieties and 20 types, which differ in a complex of morphological, phenological, physiological, biochemical and economically valuable traits. Small radish and radish are popular and widely cultivated root vegetable crops around the world that play an important role in human nutrition. They are valued for their high productivity, manufacturability, good taste and valuable biochemical composition.

For the growth and development of small radish and radish, the neutral reaction of the soil solution (pH 6.0–8.0) is the favorable. Plants are especially sensitive to low acidity in the initial periods of growth. Most of the spaces under small radish and radish in the world are located on the territory occupied by acidic soils; alumotoxicity makes a negative contribution to the decrease of the productivity and quality of these crops. Therefore, modern cultivars have to be tolerant to Al, alongside with signs of high productivity, resistance to pathogens, manufacturability, etc. The first stage in such studies should be the search in the gene pool of *R. sativus* for forms resistant to aluminum in an acidic environment.

Several diagnostic methods have been used to assess the degree of plant resistance to aluminum (Kosareva et al., 1995). Often used laboratory screening techniques are based on various modifications of methods for germinating seeds in an aquatic culture in the presence of toxic aluminum concentrations (Foy, 1996; Lisitsyn, 1999; Gupta, Gaurav, 2014). The advantage of such techniques is the simplicity of execution, low time spent, high throughput, and the ability to diagnose genotypes at the early stages of ontogenesis. A series of studies revealed a quite high correlation ($r = 0.71…0.85$) between the results of laboratory assessments of resistance at the early stages of development with the data of field and vegetation tests of adult plants (Aniol, 1981; Klimashevskiy, 1991; Baier et al., 1995; Burba et al., 1995).

Plant resistance can be assessed in laboratory tests by the degree of damage of the seedlings roots by aluminum using hematoxylin (Canado et al., 1999) and eriochrome cyanine R (Aniol, 1981). This method was successfully applied to assess the intraspecific variability of aluminum tolerance in rice (Awasthi et al., 2017), peas, maize, wheat, and sorghum (Anas, Yoshida, 2004; Kosareva, 2012; Vishnyakova et al., 2015) with hematoxylin, and with wheat, rye, triticale (Aniol, 1981; Aniol, Gustafson, 1984), aegilops, oats, maize (Kosareva, Semenova, 2004; Kosareva, 2012) and peas (Vishnyakova et al., 2015) with eriochrome cyanine R.

Researches of *R. sativus* root crops resistance to damage of aluminum have practically not been conducted. The toxicological effect of aluminum-based coagulants on various crops, including individual radish genotypes, was studied in the work of K. Zhang and Q. Zhou (2005). Oil radish (*Raphanus sativus* var. *oleifera* Metzg.) has the greatest potential for phytoextraction of fluorides from contaminated soils (Sokolova et al., 2019). J. Raj and L.R. Jeyanthi (2014) studied the effect of aluminum chloride on the germination of *R. sativus* seeds, and it was found that the maximum allowable limit for Al to maintain viability is 10 mM. The study of intraspecific variation of *R. sativus* aluminum resistance is an important stage for the breeding of these crops.

The purpose of this work was to study the genetic diversity of the VIR world wide *R. sativus* collection on the aluminum tolerance trait. The tasks were to determine the toxic concentration of aluminum chloride ($AlCl_3 \cdot 6H_2O$), which differentiates small radish and radish accessions according to the degree of aluminum resistance, to identify the most resistant genotypes, and to determine their botanical, agrobiological, and geographic confinedness.

Materials and methods

The object of research is the VIR core collections of small radish and radish, consisting of accessions of various ecological and geographical origin and most fully characterizing the diversity of the species.

The studied collection of small radish is represented by 54 accessions from 25 countries belonging to 13 cultivar types, 6 varieties of European and Chinese subspecies. The collection of radish is represented by 55 accessions from 17 countries, belonging to 10 cultivar types, 8 varieties of European, Chinese and Japanese subspecies (see the Table).

In the absence of a rapid assessment methodology specialized for the studied species, the method of the aluminum resistance evaluation of cereals using an eriochrome cyanine R dye is used (Aniol, 1981), which is based on the recovery or absence of restoration of the seedlings roots mitotic activity subjected to shock exposure to aluminum.

The experiments were carried out in a climatic chamber with an illumination 7000 Lx, a temperature 19–21 °C and a photoperiod 16 h. Seeds (50 pieces of each accession) were placed in special cells for seeds and a mesh bottom, which were placed in 6-liter containers, placing them on the surface of the nutrient solution. The nutrient solution contained (mM): 0.4 $CaCl_2$, 0.4 KNO_3, 0.25 $MgCl_2$, 0.01 $(NH_4)_2SO_4$, 0.04 NH_4NO_3; pH 4.2 (Aniol, Gustafson, 1984). After germinating the seeds for 3 days, the not viable ones were rejected. Then, the cuvettes with seedlings were placed in a freshly prepared nutrient solution supplemented with aluminum chloride ($AlCl_3 \cdot 6H_2O$) and incubated for 24 h.

Thus there are no descriptions of the *R. sativus* crops aluminum resistance in the publications, based on the

Characterization of *R. sativus* accessions along the length of root growth at various concentrations of aluminum chloride

No.	Catalog number	Accession name	Origin	Concentration, mM		
				66	83	99
			Small radish			
1	551	Red	Turkey	1.40 ± 0.12*	0.50 ± 0.05	0.35 ± 0.04
2	1233	Dungan	China	0.71 ± 0.10	0.65 ± 0.10	0.23 ± 0.02
3	1543	Pink with a white tip	Russia	1.40 ± 0.12	1.20 ± 0.13	0.29 ± 0.05
4	1546	Moskovskiy parovoy		1.70 ± 0.15	1.35 ± 0.12	0.00
5	1666	Virovsky bely		2.20 ± 0.08	1.50 ± 0.07	1.45 ± 0.03
6	1667	Krasnyy velikan		1.15 ± 0.11	0.89 ± 0.13	0.38 ± 0.01
7	1703	Pernot retek	Hungary	0.42 ± 0.06	0.20 ± 0.03	0.02 ± 0.02
8	1762	Ohlsens Enke	Denmark	1.41 ± 0.10	1.30 ± 0.12	0.01 ± 0.02
9	1776	Local	China	1.41 ± 0.07	0.72 ± 0.11	0.00
10	1879	Long scarlet	India	0.81 ± 0.09	0.50 ± 0.08	0.00
11	1923	Local	China	2.05 ± 0.15	1.30 ± 0.09	0.39 ± 0.07
12	1925	Scarlet globe	Canada	0.15 ± 0.02	0.20 ± 0.03	0.00
13	1939	French Breakfast	Pakistan	0.90 ± 0.06	0.75 ± 0.09	0.00
14	1941	Cavalier bright scarlet	Canada	0.58 ± 0.09	0.20 ± 0.10	0.01 ± 0.01
15	1946	Darozi Surkh local	Tajikistan	1.80 ± 0.09	0.35 ± 0.06	0.45 ± 0.08
16	2083	Saxa	Chile	0.42 ± 0.09	1.04 ± 0.09	0.95 ± 0.03
17	2087	White Icicle		2.20 ± 0.10	0.70 ± 0.08	0.75 ± 0.07
18	2100	Bartender Red		1.25 ± 0.08	1.08 ± 0.05	0.85 ± 0.06
19	2105	Long Scarlet		1.35 ± 0.08	1.05 ± 0.06	0.76 ± 0.06
20	2106	White globe Hailstone		0.96 ± 0.07	0.35 ± 0.08	0.28 ± 0.08
21	2111	Champion		1.25 ± 0.07	0.90 ± 0.07	0.91 ± 0.06
22	2120	Local	Turkey	0.75 ± 0.18	1.45 ± 0.10	0.02 ± 0.02
23	2130	Local	Azerbaijan	1.70 ± 0.13	0.51 ± 0.05	0.50 ± 0.09
24	2133	Cherry Belle	Tanzania	1.85 ± 0.23	1.23 ± 0.08	0.40 ± 0.05
25	2136	Balady	Hungary	0.65 ± 0.07	0.79 ± 0.06	0.45 ± 0.04
26	2141	Local	Iran	1.87 ± 0.14	0.50 ± 0.01	0.32 ± 0.08
27	2156	National	Algeria	1.35 ± 0.12	0.79 ± 0.11	0.00
28	2168	Gaudry	Netherlands	1.25 ± 0.12	0.97 ± 0.06	0.75 ± 0.05
29	2175	Pernot OJO/52	Sweden	0.80 ± 0.08	0.90 ± 0.05	0.60 ± 0.04
30	2187	Local	Azerbaijan	1.20 ± 0.09	0.79 ± 0.05	0.69 ± 0.06
31	2188	Candela di ghiaccio	Italy	1.01 ± 0.10	0.86 ± 0.09	0.40 ± 0.04
32	2192	Vetomag	Hungary	1.20 ± 0.10	0.86 ± 0.06	0.74 ± 0.04
33	2196	Local	Lebanon	1.00 ± 0.10	0.65 ± 0.10	0.10 ± 0.01
34	2197	De Pontvil	France	0.65 ± 0.07	0.51 ± 0.04	0.21 ± 0.03
35	2200	Local	Syria	1.95 ± 0.10	1.29 ± 0.09	0.92 ± 0.06
36	2210	Saratovskiy	Russia	1.20 ± 0.07	0.65 ± 0.05	0.01 ± 0.10
37	2217	Local	Afghanistan	1.60 ± 0.14	0.76 ± 0.12	0.29 ± 0.06
38	2222	Janosnapi	Hungary	2.65 ± 0.08	1.20 ± 0.07	1.15 ± 0.05
39	2231	Vates'long scarlet	Ethiopia	0.55 ± 0.05	0.55 ± 0.05	0.08 ± 0.02
40	2234	Sermino	France	1.25 ± 0.10	0.60 ± 0.05	0.46 ± 0.04
41	2239	Eroica	Netherlands	1.46 ± 0.08	0.95 ± 0.06	0.75 ± 0.05
42	2245	Local	Argentina	1.40 ± 0.25	0.51 ± 0.06	0.00
43	2260	Local	Russia	1.56 ± 0.03	1.25 ± 0.01	1.00 ± 0.08
44	2302	Long rouge arabe	Libya	1.10 ± 0.05	0.75 ± 0.06	0.60 ± 0.05
45	2343	Saxa	Iceland	1.51 ± 0.17	1.30 ± 0.12	0.55 ± 0.05
46	2347	Syla	Denmark	1.20 ± 0.08	0.45 ± 0.09	0.12 ± 0.02
47	2360	Helios	Czech Republic	0.93 ± 0.05	1.00 ± 0.01	0.60 ± 0.06
48	2366	Pernot	France	0.36 ± 0.03	0.49 ± 0.06	0.61 ± 0.04
49	2371	Safir	Denmark	1.45 ± 0.10	0.85 ± 0.05	0.56 ± 0.03
50	2379	Local	Lebanon	1.50 ± 0.09	0.60 ± 0.09	0.58 ± 0.02
51	2383	Jegcsap	Hungary	1.40 ± 0.13	0.65 ± 0.05	0.50 ± 0.07
52	2393	Crimson Giant	Canada	0.90 ± 0.09	0.71 ± 0.06	0.60 ± 0.04
53	2402	Rabanito	Argentina	1.84 ± 0.22	1.03 ± 0.08	0.67 ± 0.01
54	2408	Notar	Netherlands	1.25 ± 0.08	0.85 ± 0.07	0.47 ± 0.03
Means				1.25 ± 0.53	0.81 ± 0.34	0.44 ± 0.35
LSD				0.28	0.18	0.19

Table (end)

No.	Catalog number	Accession name	Origin	Concentration, mM		
				66	83	99
			Radish			
55	1675	Belaya adzharskaya	Belarus	1.70 ± 0.17	1.22 ± 0.02	0.38 ± 0.03
56	1778	Winter round black	Germany	1.85 ± 0.21	1.35 ± 0.15	0.98 ± 0.05
57	1805	Local	Uzbekistan	2.25 ± 0.25	0.71 ± 0.11	0.46 ± 0.06
58	1816	Belozelenaya	Kazakhstan	2.25 ± 0.13	1.15 ± 0.16	1.25 ± 0.11
59	1857	Chang Shui Lobo	China	2.20 ± 0.20	1.40 ± 0.14	0.70 ± 0.12
60	1865	Weixiang		2.88 ± 0.25	1.20 ± 0.08	0.00
61	1891	Anbenmu	South Korea	1.20 ± 0.09	1.11 ± 0.04	0.55 ± 0.05
62	1892	Darwish ali	Egypt	1.37 ± 0.07	0.85 ± 0.07	0.49 ± 0.07
63	1895	Hung-tung-lun	China	0.38 ± 0.04	0.85 ± 0.05	0.43 ± 0.11
64	1902	Belaya zelenogolovaya		1.65 ± 0.20	1.50 ± 0.18	0.00
65	1903	Red		1.09 ± 0.12	0.65 ± 0.06	0.35 ± 0.03
66	1906	Red ball of changchou		1.35 ± 0.16	0.97 ± 0.06	0.78 ± 0.06
67	1913	Lobo	South Korea	2.13 ± 0.18	0.75 ± 0.06	0.80 ± 0.10
68	1914	Winter round white	Russia	1.21 ± 0.13	0.80 ± 0.07	0.35 ± 0.06
69	1935	Nezima pointed rooted	Japan	2.10 ± 0.17	1.20 ± 0.09	0.90 ± 0.10
70	1942	Wase sakurajima		1.65 ± 0.14	1.70 ± 0.23	1.00 ± 0.13
71	1958	Hakata haruwaka		2.70 ± 0.06	2.25 ± 0.11	1.45 ± 0.03
72	1967	Local	Afghanistan	1.68 ± 0.18	1.21 ± 0.06	0.78 ± 0.13
73	1978	Local	Kyrgyzstan	2.38 ± 0.15	0.89 ± 0.03	0.71 ± 0.19
74	1983	Nezhnaya	Russia	2.08 ± 0.02	1.26 ± 0.23	0.92 ± 0.06
75	2000	Local	Uzbekistan	2.50 ± 0.41	1.29 ± 0.08	0.66 ± 0.08
76	2012	Runder swarzer	Germany	1.90 ± 0.15	1.60 ± 0.33	1.09 ± 0.11
77	2014	Local	Iraq	1.65 ± 0.15	0.98 ± 0.05	0.65 ± 0.07
78	2021	Local	Kazakhstan	0.89 ± 0.09	0.98 ± 0.08	0.61 ± 0.04
79	2025	Skvirovskaya white	Ukraine	0.56 ± 0.09	1.45 ± 0.19	0.87 ± 0.12
80	2033	Turnip	Japan	0.90 ± 0.04	0.86 ± 0.10	0.90 ± 0.14
81	2034	Miyashige Onaga		1.75 ± 0.17	1.00 ± 0.05	0.86 ± 0.07
82	2074	Local	Egypt	3.05 ± 0.21	1.56 ± 0.09	1.13 ± 0.09
83	2084	Round black spanish	USA	1.85 ± 0.18	1.95 ± 0.15	1.10 ± 0.06
84	2101	Chinese white winter	Chile	2.07 ± 0.15	1.55 ± 0.12	1.00 ± 0.09
85	2111	Minotoki 2	Japan	2.46 ± 0.10	1.75 ± 0.12	1.16 ± 0.06
86	2112	Sakata Tenshun		1.95 ± 0.13	1.65 ± 0.17	0.80 ± 0.09
87	2115	Black	Russia	1.25 ± 0.09	1.13 ± 0.11	0.50 ± 0.11
88	2122	Bai cu	Vietnam	1.85 ± 0.24	0.95 ± 0.05	1.10 ± 0.04
89	2124	Local	Turkey	1.67 ± 0.15	1.66 ± 0.17	0.96 ± 0.15
90	2128	Haruysi 360	Japan	2.20 ± 0.16	1.79 ± 0.16	1.20 ± 0.07
91	2133	Eifuku		1.25 ± 0.14	0.75 ± 0.03	0.00
92	2134	Eifuku 2		2.15 ± 0.15	0.46 ± 0.05	0.56 ± 0.08
93	2148	Local	Kazakhstan	1.80 ± 0.13	1.40 ± 0.10	1.05 ± 0.06
94	2151	Altari mu	South Korea	1.16 ± 0.08	1.30 ± 0.10	0.87 ± 0.07
95	2155	Local	Japan	1.75 ± 0.07	1.30 ± 0.33	1.09 ± 0.12
96	2156	Lebidka	Ukraine	1.58 ± 0.02	0.48 ± 0.01	0.00
97	2157	Natsu Sakkari	Japan	1.91 ± 0.13	2.00 ± 0.29	0.91 ± 0.09
98	2158	Shinshuuji		2.41 ± 0.14	1.70 ± 0.09	0.96 ± 0.07
99	2159	Yamato rice		2.40 ± 0.23	1.75 ± 0.13	1.19 ± 0.06
100	2160	Akasuji		2.65 ± 0.17	1.80 ± 0.11	1.45 ± 0.12
101	2161	Hariou		2.75 ± 0.16	1.85 ± 0.24	1.37 ± 0.04
102	2163	Mayskaya belaya	Russia	1.75 ± 0.20	0.75 ± 0.09	0.45 ± 0.14
103	2170	Nongwoo iljin	South Korea	3.00 ± 0.19	1.80 ± 0.09	1.27 ± 0.01
104	2173	Jangsu		2.60 ± 0.14	1.90 ± 0.24	1.30 ± 0.02
105	2175	Sodam		1.50 ± 0.20	1.05 ± 0.08	1.10 ± 0.07
106	2177	Back ok		2.30 ± 0.24	1.65 ± 0.13	0.95 ± 0.05
107	2178	Shinmyeong		2.60 ± 0.20	1.49 ± 0.10	0.55 ± 0.07
108	2183	Gascinets	Belarus	2.75 ± 0.24	2.15 ± 0.11	1.16 ± 0.10
109	2184	Cheng sugeng zung	South Korea	2.25 ± 0.21	1.11 ± 0.07	1.05 ± 0.06
Means				1.91 ± 0.62	1.30 ± 0.46	0.82 ± 0.38
LSD				0.33	0.23	0.20

* MEAN ± SD.

preliminary experiments, we used $AlCl_3 \cdot 6H_2O$ concentrations of 66, 83, and 99 mM, which had a toxic effect on plants and inhibited root growth in degrees under the used conditions. After that, the cuvettes were placed in a fresh nutrient solution without aluminum and incubated for 48 h. During the indicated time, reparation processes took place in the roots (restoration of the mitotic activity of cells) and the roots grew. The seedlings were washed with clean water and the roots were stained by immersing the cuvettes in a 0.1 % solution of eriochrome cyanine R for 10 min. The excess dye was washed off with clean water, and the roots were dried with filter paper. The zone of root tissue damage with aluminum was colored violet after staining with eriochrome cyanine R. Plant resistance to aluminum was determined by the length of root tip regrowth. For each accession two independent experiments were carried out in two-fold repetition.

Statistical data processing was performed by the method of analysis of variance using the STATISTICA v.12.0 program (StatSoft Inc., USA), by the method of cluster analysis (Ward's method) using the PAST program (Hammer et al., 2001).

Results

At the first stage, we investigated the effect of different aluminum concentrations on small radish and radish. In general, the results of our research have shown that an excess of aluminum and hydrogen (low pH) in the nutrient solution negatively affects the growth and development of the embryonic roots of small radish and radish seedlings. We observed significant differences between *R. sativus* accessions in root regrowth at all tested concentrations of $AlCl_3 \cdot 6H_2O$ (see the Table).

The aluminum chloride concentration of 66 mM had a weak toxic effect on *R. sativus* accessions. In most of the small radish and radish accessions, the mitotic activity of seedling root cells was restored after the shock exposure to aluminum. In 70.4 % of the small radish accessions and 92.7 % of the radish, the root growth was rather high (more than 1.0 cm), that indicates a normal further development. 22.2 % of the small radish accessions and 5.5 % of the radish showed an average root growth (0.5–1.0 cm); in four small radish accessions and one radish, the root growth was less than 0.5 cm.

At a concentration of $AlCl_3 \cdot 6H_2O$ of 83 mM, a large differentiation of the accessions was observed. In 29.6 % of the small radish accessions and 70.9 % of the radish, the root growth was more than 1.0 cm, the average regrowth (0.5–1.0 cm) was observed in 51.9 % of the small radish and 25.5 % of the radish. Root growth of less than 0.5 cm was observed in 18.5 % small radish and 3.6 % radish accessions.

At an aluminum chloride concentration of 99 mM, there was no further root growth in 13.0 % of the small radish and in 7.3 % of the radish accessions. A slight root growth (up to 0.5 cm) was observed in 46.3 % of small radish and 14.5 % of radish. Root regrowth by 0.5–1.0 cm was observed in 33.3 % of small radish and 41.8 % of radish. Normal root growth after exposure of this concentration of the toxicant was observed in only 7.4 % of the small radish and 36.4 % of the radish accessions.

So, the differences were most clearly manifested between small radish accessions at Al concentration of 83 mM, and between radish accessions at a 99 mM concentration at different stressor intensity. These concentrations were used for further evaluation of polymorphism because their negative impact showed the maximum differentiating ability.

The accessions with the minimum length of root regrowth had an intense violet coloration of the root areas that grew upon the addition of mobile aluminum, and the accessions with the maximum length of the root regrowth had a weak but detectable staining (Fig. 1).

The accessions of small radish and radish were divided into several statistically significant groups according to the length of root regrowth, depending on the concentration of aluminum (Fig. 2). The accessions were characterized by a wide range of root growth at a concentration of 66 mM – 0.15–2.65 cm (small radish) and 0.38–3.05 cm (radish), this variability divided the samples into seven and eight groups, respectively.

Small radish accessions were divided at a concentration of 83 mM into four groups with a range of variability from 0.20 to 1.50 cm. The first group consisted of five accessions with root growth less than 0.40 cm; these accessions are of var. *rubescens* Sinsk. from Canada and Hungary. The second group included the largest number of accessions (24 accessions) from the countries of Minor Asia and Central Asia and Africa. The third group was represented by accessions of various types from Europe and South America. The fourth group included nine accessions with root regrowth more than 1.20 cm; these accessions are from Russia, China, Turkey, Hungary, Iceland, and Tanzania. Radish accessions were divided at a given concentration into five groups with a range of 0.46–2.25 cm. Accessions were absent with root regrowth after exposure to this concentration less than 0.40 cm. The first group included 8 accessions with root growth from 0.41 to 0.80 cm from Japan, Russia, China and Uzbekistan. The second group was represented by accessions from Central Asia, Vietnam, South Korea, Egypt and Japan. The third and fourth groups were the largest and included 31 accessions with root growth more than 1.20 cm from Japan, South Korea, countries of Europe and Central Asia, as well as from the USA, Chile and Russia. The fifth group was represented by 3 accessions from Japan and Belarus with root regrowth of more than 2.0 cm.

The small radish and radish accessions were divided at a concentration of 99 mM into four groups in the range from 0.00 to 1.45 cm. The first group consisted of 26 small radish accessions, of which 7 accessions did not have root regrowth; these accessions had different geographic origin, but most accessions were from Canada, Russia, China,

Fig. 1. Appearance of resistant (left) and sensitive (right) small radish seedling.

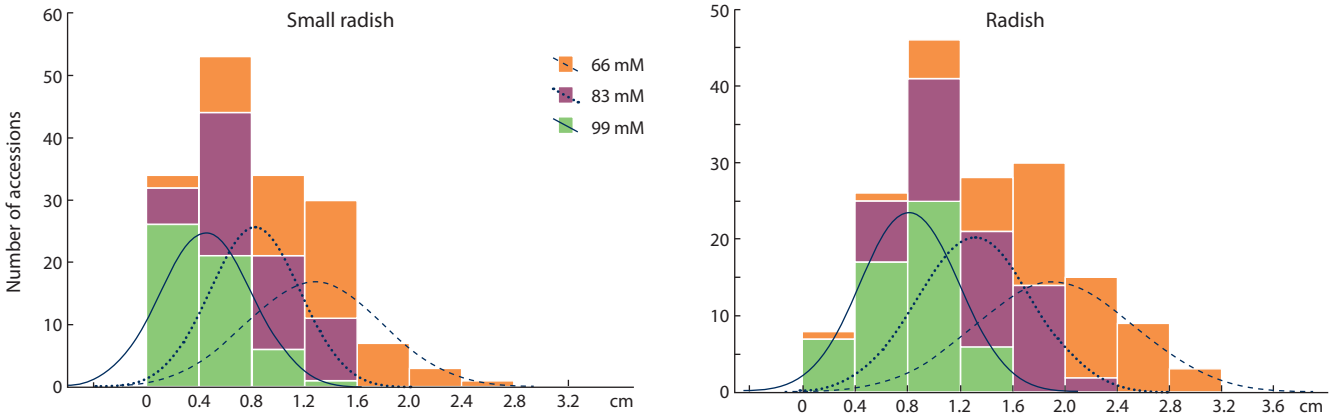

Fig. 2. Histogram of the distribution of small radish and radish accessions along the length of root growth at various concentrations of aluminum chloride.

and Central Asia. The first group of radish included only 7 accessions, of which four did not grow roots; this group included accessions from China, Ukraine, Belarus, and Russia. The second group of small radish was formed by accessions from Europe and South America, as well as some accessions from Azerbaijan, Tajikistan and Libya. This group of radish includes accessions from Russia, the countries of Central Asia, China and South Korea. The third group of small radish included 6 accessions from Chile, Russia and Syria, radish – 25 accessions mainly from Japan, South Korea, as well as from Chile, Turkey, Russia, Germany and the USA. The fourth group in small radish was formed by only one accession from Russia (k-1666), in radish – 6 accessions from Japan, South Korea and Kazakhstan.

Figure 3 shows a dendrogram based on the results of cluster analysis of root growth in *R. sativus* accessions after exposure to toxic concentrations of $AlCl_3 \cdot 6H_2O$. According to the screening results using the Ward's method, the small radish and radish accessions were divided into two big groups, each of the groups was divided into clusters according to the degree of aluminum resistance, the total number of which was five. The first group is represented by two clusters, the second – by three.

The first small cluster included accessions of Japanese radish from Japan and South Korea and Belarus and an accession of Chinese radish from Egypt; they showed a large root growth at a concentration of 66 mM $AlCl_3 \cdot 6H_2O$ and relatively high at concentrations of 83 and 99 mM. The second cluster combined accessions of small radish and radish with root growth more than 1.0 cm after exposure to all three toxic concentrations of Al. The cluster is divided into two subclusters. The first subcluster contains an accession of small radish from Russia (k-1666, Virovsky bely), accessions of Japanese radish from Japan and South Korea, two accessions of European winter radish from Germany and the USA (var. *niger* (L.) Sinsk.) and an accession of Chinese radish from Chile (var. *lobo*). The second subcluster includes accessions of small radish from Hungary (var. *chloris* Alef.), Syria (var. *rubescens* Sinsk.),

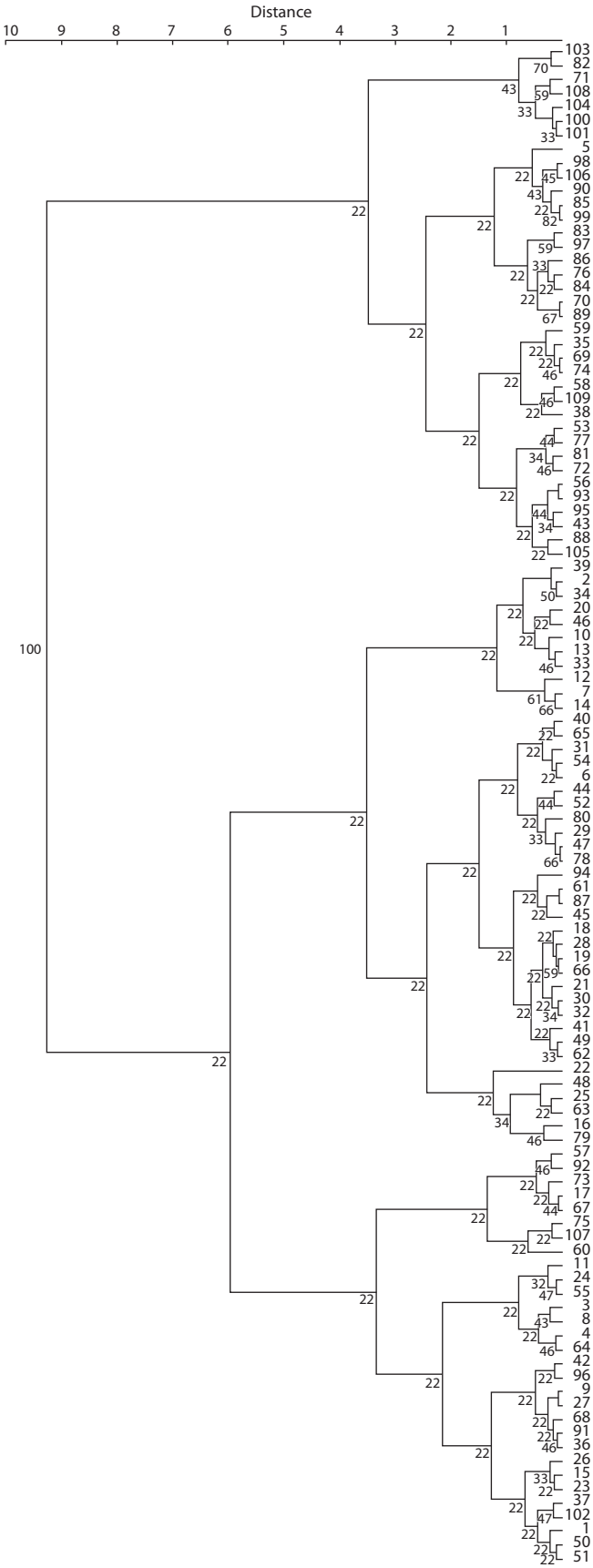

Fig. 3. Dendrogram of *R. sativus* accessions by root growth after exposure to different concentrations of $AlCl_3 \cdot 6H_2O$. Ward's method.

The numbers on the dendrogram indicate the size of the bootstrap. The numbers to the right of the dendrogram are accessions numbers in accordance with the Table.

Argentina (var. *striatus* Sinsk.) and Russia (var. *roseus* Sazon.), accessions of Chinese radish from Kazakhstan, China and South Korea (var. *virens* Sazon.), Japan, Russia, Iraq and Afghanistan (var. *rubidus* Sazon.) and accessions of Japanese radish from Japan, South Korea and Vietnam.

The third small cluster unites small radish accessions, which showed little or no root regrowth at all concentrations used. The cluster included accessions from France, Pakistan (var. *striatus* Sinsk.), Canada, Hungary, Ethiopia, Lebanon (var. *rubescens* Sinsk., var. *radicula*), China and India (var. *roseus* Sazon.).

The fourth cluster is represented by accessions of small radish and radish, in which root regrowth after exposure to toxic concentrations of 83 and 99 mM was average (up to 1.0 cm). The cluster is divided into three subclusters. The first subcluster included small radish accessions of var. *striatus* and var. *rubescens*, one accession each of var. *radicula* and var. *roseus*, Chinese radish from Kazakhstan (var. *lobo*) and China (var. *roseus* Sazon.) and Japanese radish from Japan. The second subcluster unites accessions of small radish from Chile, the Netherlands, Hungary (var. *rubescens* Sinsk.), accessions of European radish from Russia, Egypt (var. *niger* (L.) Sinsk.) and Chinese radish from South Korea (var. *lobo*), China (var. *roseus* Sazon.). The third subcluster includes accessions of small radish var. *rubescens* from Chile, Turkey and Hungary, accessions of European winter radish from Ukraine (var. *hybernus*), and an accession of pink Chinese radish from China.

The fifth cluster includes accessions of small radish and radish, with partial or complete inhibition of root growth at a concentration of 99 mM and an average root regrowth at other concentrations. The cluster is divided into three subclusters. The first subcluster unites accessions of Chinese radish of Central Asian origin, Japanese radish from Japan and South Korea, and an accession of small radish from Chile. The second subcluster mainly includes accessions of small radish from Russia, China and Tanzania and two accessions of radish from Belarus and China. The third subcluster is mainly represented by accessions of small radish of Central Asian origin and several accessions of radish from Russia and Ukraine.

Discussion

Genetic processes were of great importance in the phylogenesis of radish and small radish: recombination, mutations at the chromosomal level, expression of inactive genes and changes in the frequencies of alleles that control traits and determine the phenotype of the plant; they occurred under natural and artificial selection in various ecological and geographical conditions (Bunin, Esikawa, 1993). The large intraspecific diversity of forms of *R. sativus* at the diploid level of development is explained by spontaneous gene and inherited somatic mutations (Campbell, Snow, 2009). In our previous studies, we found that the limits of variability of quantitative traits (morphological, producti-

vity traits, early maturity, and accumulation of nutrients) in small radish and radish are very large (Kurina et al., 2017, 2018; Kurina, Artemyeva, 2017, 2019). For example, the amplitude of variation of the most important features: the duration of the period of vegetation is 18–95 days; root weight is 2–75 (small radish) and 150–1100 g (radish); the diameter of the leaf rosette is 8–45 cm; root shape: round-flat, round, round-oval, oval, cylindrical, fusiform, conical; content of ascorbic acid 18–55 mg/100 g, etc.

According to the literature data, it is known that, in general, small radish and radish are resistant to the action of heavy metals and have a high accumulating ability of heavy metals in the root (Wang et al., 2012; Ngo et al., 2016; Elizarieva et al., 2017). Japanese radish accumulates less toxic elements in roots; it is more resistant to pollution by such heavy metals as lead, cadmium, nickel, zinc, vanadium, chromium, arsenic. The response of Japanese radish to soil pollution is varietal specific (Gorelova et al., 2005; Xu et al., 2017). Crops of *R. sativus* are accumulators of heavy metals; they have been proposed for phytoremediation (Kumar et al., 1995; Ebbs, Kochian, 1997; Ebbs et al., 1997; Wang et al., 2012). Also, radish is a vegetable crop moderately sensitive to salt stress (Sun et al., 2016).

The study of *R. sativus* crops revealed high intraspecific variability in aluminum resistance. In general, radish was more resistant to alumo stress than small radish regardless of concentration, which is probably related to the processes of morphogenesis.

As a result of grouping accessions according to the length of root regrowth after exposure to various toxic concentrations of aluminum chloride (see Fig. 2), it was found that the accessions of both crops form four groups with a root regrowth range from 0 to 1.6 cm at a concentration of 83 and 99 mM. Accessions of *R. sativus* reacted weakly to low concentrations of $AlCl_3 \cdot 6H_2O$, the mitotic activity of seedling root cells was restored after the shock effect of aluminum. With an increase of concentration, intraspecific differences in the crops begin to appear. The intensity of staining with eriochrome cyanine R characterizes the concentration of mobile forms of aluminum, which in turn correlates with aluminum tolerance (Vishnyakova et al., 2015). If, after treatment with aluminum, the concentration of its active forms is low, then the mitotic activity of cells is restored at the root, the root grows back, and after the staining zone, an unstained growth appears (Kosareva, 2012). So, the intensity of the staining can serve as an additional indicator of the degree of aluminum tolerance associated with the concentration of the toxicant in the root tissues.

Based on the obtained results, we propose a resistance scale for *R. sativus* crops based on aluminum tolerance: root growth up to 0.40 cm – sensitive, from 0.41 to 0.80 cm – weakly resistant, from 0.81 to 1.20 cm – medium resistant, more than 1.21 cm – highly resistant.

The $AlCl_3 \cdot 6H_2O$ concentration of 99 mM made possible to identify the most tolerant small radish samples (in descending order): Virovsky bely (k-1666, Russia), Janosnapi (k-2222, Hungary), Local (k-2260, Russia), and radish: Hakata haruwaka (k-1958, Japan), Akasuji (k-2160, Japan), Hariou (k-2161, Japan), Jangsu (k-2173, South Korea).

According to the results of cluster analysis, it was revealed that the first and second clusters combine highly resistant and medium-resistant radish accessions and highly resistant small radish accessions, the third cluster contains sensitive and low-resistant small radish accessions, and the fourth and fifth clusters mainly contain medium-resistant small radish accessions and low-resistant and unresistant radish accessions. It was revealed that accessions of *R. sativus* of Central Asian origin (Azerbaijan, Uzbekistan, Afghanistan, etc.), as well as from African countries (Algeria, Ethiopia) were found to be weak resistant and sensitive to alumostress. The soils of these countries are characterized by a neutral or slightly alkaline reaction of the soil solution, what, probably, determines the low resistance of the accessions to low acidity and alumostress. Medium-resistant accessions were mainly of European origin (Netherlands, Germany, Italy, etc.), as well as from the USA and Chile. In these countries, there is an active breeding of these crops in various directions. Accessions of small radish and radish from Russia, Hungary, Turkey, China, Japan, South Korea, and Kazakhstan had varying degrees of resistance; accessions of the same geographic origin could be both aluminum tolerant and sensitive to aluminum. Perhaps this is due to the presence of both acidic and neutral/alkaline soils in these countries, as well as to the direction of breeding work with these crops. The most aluminum-tolerant were accessions of Japanese radish from Japan of Kameido type and Shiroagiri type from South Korea, local accessions of green Chinese radish from Kazakhstan and accessions of Chinese small radish of the Russian breeding, which were obtained by selection and hybridization from the population of Asian radishes.

So, the *Raphanus sativus* species is polymorphic not only in phenotypic and biochemical characteristics, but also in the degree of resistance to various abiotic stresses.

Conclusion

As a result of this study, we found that excess concentrations of mobile aluminum and hydrogen (elements of acidic soils) in the root zone lead to a negative effect on the growth and development of embryonic roots of small radish and radish accessions. In toxic concentrations of aluminum chloride in the nutrient medium, the accessions of the studied species were characterized by high variability in terms of aluminum tolerance at different stressor intensity. As a result of screening, we revealed the intraspecific variability of small radish and radish at the early stages of the growing season and identified genotypes contrasting in resistance to aluminum. We recommend a concentration of 83 mM $AlCl_3 \cdot 6H_2O$ for assessing the aluminum tolerance of small radish, and a concentration of 99 mM for assessing radish. The method developed by us is proposed as an express diagnostics of aluminum tolerance for rapid screening of

a wide range of *R. sativus* genotypes and subsequent study of contrasting forms during longer plant cultivation in hydroponic culture (including elemental analysis of roots and shoots contrasting in the resistance of accessions), as well as plant reactions in soil conditions.

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ORCID ID

A.B. Kurina orcid.org/0000-0002-3197-4751
I.A. Kosareva orcid.org/0000-0001-9654-7235
A.M. Artemyeva orcid.org/0000-0002-6551-5203

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Alkaloids of narrow-leaved lupine as a factor determining alternative ways of the crop's utilization and breeding

M.A. Vishnyakova ✉, A.V. Kushnareva, T.V. Shelenga, G.P. Egorova

Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
✉ e-mail: m.vishnyakova.vir@gmail.com

Abstract. Narrow-leaved lupine (*Lupinus angustifolius* L.), a valuable leguminous crop adapted to a wide range of climatic conditions, has a very short history of domestication. For many centuries it was used mainly as a green manure, since the success and prospects of the multi-purpose use of the species depend on its breeding improvement, in particular, on a particular concentration of alkaloids in seeds and green mass. The first varieties of scientific breeding were created only in the 1930s after the appearance of low-alkaloid mutants. Despite wide prospects for use in various areas of the national economy, unstable productivity and susceptibility to diseases hinder the production of this crop. Obviously, breeders deal only with a small part of the gene pool of the species and limited genetic resources, using mainly low-alkaloid (sweet) genotypes to create new varieties. The genetic potential of the species can be used more efficiently. At the same time, it is rational to create highly alkaloid (bitter) varieties for green manure, while food and feed varieties should not lose their adaptive potential, in particular, resistance to pathogens, due to the elimination of alkaloids. In this regard, it seems to be a productive idea to create 'bitter/sweet' varieties combining a high content of alkaloids in the vegetative organs and low in seeds, which can be achieved by regulating the synthesis/transport of alkaloids in the plant. The paper discusses the current state of use of the species as a green manure, fodder, food plant. Information is given on the quantity and qualitative composition of narrow-leaved lupine alkaloids, their applied value, in particular, fungicidal, antibacterial, insecticidal, the use of lupine alkaloids as active principles of drugs. Along with promising breeding considerations, the possibility of using technologies for processing raw high-alkaloid materials with the accompanying extraction of valuable ingredients for pharmaceuticals is discussed. Information is briefly presented about the genomic resources of the species and the prospects for their use in marker-assistant selection and genome editing.
Key words: narrow-leaved lupine; alkaloids, domestication; breeding; feed; food; green manure; varieties; pharmacology; genetic and genomic resources.

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Алкалоиды люпина узколистного как фактор, определяющий альтернативные пути использования и селекции культуры

М.А. Вишнякова ✉, А.В. Кушнарева, Т.В. Шеленга, Г.П. Егорова

Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
✉ e-mail: m.vishnyakova.vir@gmail.com

Аннотация. Люпин узколистный (*Lupinus angustifolius* L.) – ценная зернобобовая культура, адаптированная к широкому спектру климатических условий и имеющая непродолжительную историю доместикации. В течение многих веков его употребляли преимущественно как сидеральное растение, поскольку успех и перспективы многоцелевого использования вида зависят от его селекционного улучшения, в частности от содержания определенного уровня алкалоидов в семенах и зеленой массе. Первые сорта научной селекции были созданы в 1930-х гг., после выявления низкоалкалоидных мутантов. Производство этой культуры сдерживается нестабильной урожайностью и подверженностью болезням. Очевидно, что селекционеры имеют дело лишь с небольшой частью генофонда вида и ограниченными генетическими ресурсами, используя для получения новых сортов преимущественно низкоалкалоидные (сладкие) генотипы. Генетический потенциал вида можно задействовать эффективнее. При этом сидеральные сорта рационально создавать высокоалкалоидными (горькими), а продовольственные и кормовые за счет элиминации алкалоидов не должны терять адаптивные свойства, в том числе устойчивость к патогенам. В этом отношении продуктивной идеей представляется выведение сладко-горьких сортов, сочетающих высокое содержание алкалоидов в вегетативной

массе и низкое – в семенах, чего можно добиться путем регулирования синтеза/транспорта алкалоидов в растении. В обзоре рассмотрены современное состояние использования вида в качестве сидерального, кормового, пищевого растения. Приведены сведения о количестве и качественном составе алкалоидов люпина узколистного, их прикладном значении, в частности фунгицидной, антибактериальной, инсектицидной функциях, применении отдельных алкалоидов люпина в качестве действующих начал лекарственных средств. Наряду с селекционным улучшением культуры обсуждаются возможные технологии переработки высокоалкалоидного сырья с сопутствующим извлечением ценных ингредиентов для фармацевтики. Кратко представлены сведения о геномных ресурсах вида и перспективах их использования в маркер-опосредованной селекции и при редактировании генома.
Ключевые слова: люпин узколистный; алкалоиды; доместикация; селекция; кормовые; продовольственные; сидеральные сорта; фармакология; генетические и геномные ресурсы.

Introduction

The narrow-leaved lupine (*Lupinus angustifolius* L.), also known as the blue lupine, is one of the three *Lupinus* spp. cultivated in Russia. Along with white (*L. albus* L.) and yellow (*L. luteus* L.) lupines, it is a valuable pulse crop, whose seeds contain 30–40 % of protein, up to 40 % of carbohydrates, 6 % of oil, numerous minerals, vitamins, and other beneficial ingredients, ranking this species among the most important crops of the present and the future.

Today, *L. angustifolius* is a leader among other cultivated lupine species in the cropping area occupied worldwide. It is widely cultivated in Northern Europe, countries of the ex-USSR, the United States, and New Zealand. The world's leading producer and exporter of this crop is Australia, where the areas under narrow-leaved lupine reach 0.6–0.7 million hectares, and large funds are invested in its research and breeding. In Russia, in 2018, its production area was 35,000 ha, which is not much considering the size of the country. However, the Russian Federation is still among the top ten producers of this crop (http://www.fao.org/faostat/en/#data/QC).

Lupinus angustifolius is the most early-ripening and most plastic crop species among those produced in Russia and the only one adapted to high northern latitudes – up to 60° N. It grows on acidic sandy soils deficient in nitrogen and phosphorus, and is a powerful nitrogen accumulator. Its growing season lasts from 70 to 120 days, depending on the cultivar and the climate. Total active temperatures of 1900 °C and precipitation amount of 200–250 mm from germination to maturity are enough for successful seed production. The crop endures a decrease in air temperature down to –9 °C (Kuptsov, Takunov, 2006).

Potential uses of narrow-leaved lupine have not yet been practiced to the fullest extent. Historically, this crop was grown for green manure and animal feed. These days, its nutritional, pharmacological and phytoremedial properties are coming into the sphere of interest, as well as its use as a feed in aquaculture. The prospects of its utilization as a source of bioethanol (Kuznetsova et al., 2015) and natural fiber (Kozlowski, Manys, 1997) are discussed.

The uses of narrow-leaved lupine depend on the presence of secondary metabolites in its seeds and biomass, especially quinolizidine alkaloids responsible for bitter taste and toxic to both humans and animals. Polymorphism of the species' gene pool in the content of such compounds makes it possible to develop cultivars for a specific purpose. High-alkaloid genotypes are promising as green manure plants and producers of alkaloids for pharmaceutics and medicine, while low-alkaloid ones may be used for food and feed purposes.

This review attempts to analyze different applications of narrow-leaved lupine genetic resources depending on the content of alkaloids in the genotypes, describe lupine alkaloids and their practical worth, assess the need for targeted breeding of specialized cultivars, survey genetic and genomic resources promising for breeding, and discuss possible technologies capable of expanding the crop's economic potential.

Domestication and breeding history

The center of origin for narrow-leaved lupine is the Mediterranean region. *L. angustifolius* occurs as a wild plant much more frequently than other lupine species in the Old World and is still widespread across the entire Mediterranean basin. It has also naturalized in South Africa and South-Western Australia (Gladstones et al., 1998). The species dispersed from the Mediterranean center to Central European countries, winning special recognition in Germany and Poland. In Russia, narrow-leaved lupine became known only in the early 20th century.

For thousands of years lupine has been used for green manure and animal feed. Before feeding the animals, lupine seeds were soaked in water, with several changes, in order to remove alkaloids.

The revolution in lupine breeding was observed in 1926–1928, when Reinhold von Sengbusch, a German botanist, discovered natural low-alkaloid mutants. It helped to reduce the alkaloid content in the seeds of *L. albus*, *L. luteus* and *L. angustifolius* from the traditional 1–3 % to 0.02 % and less (Sengbusch, 1931). Since that time, the breeding of low-alkaloid (sweet) lupine cultivars for animal feed has been gaining progress. Initially, such cultivars emerged in Germany, then in Sweden, Denmark and Poland. In the USSR, the first natural low-alkaloid mutants were developed by scientists at VIR and plant breeders of the Novozybkov and Minsk Experiment Stations (Anokhina et al., 2012). In Australia, where lupines were introduced in the 1960s to improve crop rotations and reclaim sandy soils, the first sweet cultivar adapted to the local environments was released soon afterwards, in 1967, and large-scale lu-

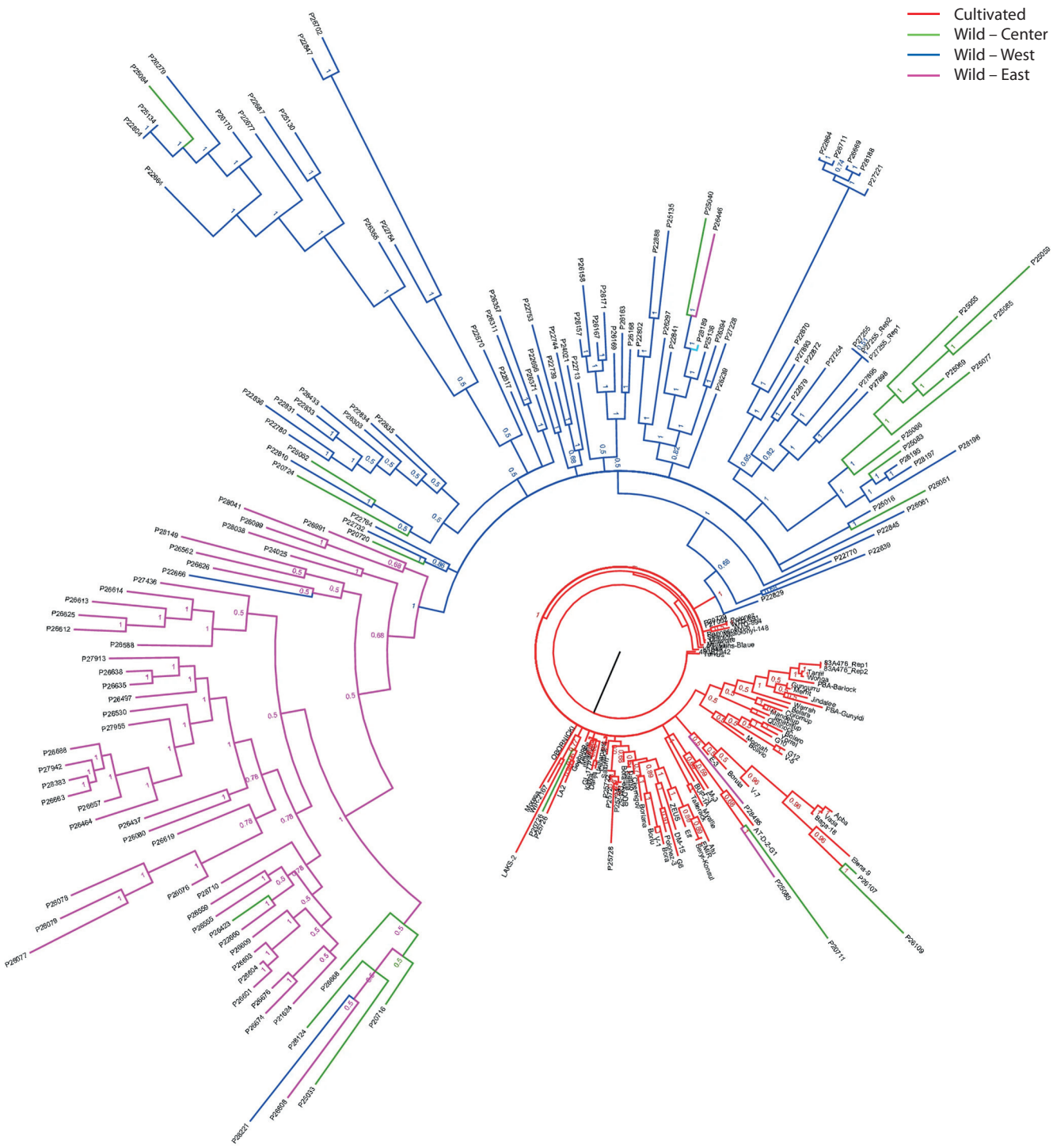

Fig. 1. The phylogenetic tree for wild and cultivated narrow-leaved lupine accessions produced on 11,690 SNPs using MrBayes v3.2.2, according to (Mousavi Derazmahalleh et al., 2018).

Wild accessions from the central part of the Mediterranean region (21 accessions) are presented in green color; from western Mediterranean (77 accessions) in navy blue; from eastern Mediterranean (49 accessions) in pink; and cultivated forms (87 accessions) in red.

pine grain production started in 1973–1974 (Gladstones, 1982).

Same as with most of the crops, the genetic diversity of domesticated narrow-leaved lupine forms is smaller than that of wild populations and landraces, and plant breeders have employed only a minor part of this diversity (Berger et al., 2012a, b). Whole genome sequencing of 146 wild and 87 cultivated accessions from different genebanks over the world ascertained that the genomic diversity in modern cultivars is thrice smaller than in wild populations (Mousavi-Derazmahalleh et al., 2018) (Fig. 1). It should be mentioned that 90 years that have passed since the develop-

ment of the first low-alkaloid cultivars is quite a short time for an agricultural crop, and the process of introducing this species into cultivation cannot be regarded as finalized.

Russian breeding centers working with narrow-leaved lupine are the All-Russian Research Institute of Lupine, Nemchinovka Federal Research Center, Belogorka Research Institute of Agriculture, Moscow Timiryazev Agricultural Academy, etc. Presently, there are 27 cultivars of narrow-leaved lupine listed in the State Register for Selection Achievements Admitted for Usage (http://reestr.gossortrf.ru/reestr.html).

Cultivars released in the early stages of the lupine breeding history were, as a rule, high-alkaloid, but those developed later, due to the selection of genotypes with reduced alkaloid content, were predominantly low-alkaloid (Anokhina et al., 2012). According to the standards accepted by a number of European countries and Australia, the content of alkaloids in seeds intended to be used for food or feed purposes (sweet) must not exceed 0.02 % of their dry weight (Frick et al., 2017). In Russia, fodder lupine seeds should have the percentage of alkaloids from 0.1 up to 0.3 % of the seed dry weight (GOST R 54632-2011, 2013), and in those for human food their content is restricted to 0.04 %, in line with the existing technical specifications developed at the All-Russian Research Institute of Lupine (TU-9716-004-0068502-2008).

Lupine as a green manure crop

With the green biomass yield of 45–60 t/ha, lupine is able to accumulate 100–300 kg/ha of ecologically safe biological nitrogen in its biomass, which is comparable with animal manure. Thus, the conditions are created for a stable or increased supply of the soil with organic matter, so that its physical and chemical properties improve, and the phytosanitary state of subsequent plantings is upgraded (Kuptsov, Takunov, 2006).

Due to its deeply penetrating roots and high dissolving capacity of root excretions, lupine assimilates phosphorus, potassium, calcium, magnesium and other elements, contributing to their intensified circulation in the topsoil and subtopsoil horizons. On average, one hectare of lupine plants leaves to the next crop, in addition to nitrogen, 30 kg of phosphorus and 50 kg of potassium (Yagovenko et al., 2003).

The demand for narrow-leaved lupine as a green manure plant continues to grow. The yield of winter rye sown into gray forest soil on a green manure fallow after lupine, without fertilizers, increases by 0.5–1.0 t/ha (Gresta et al., 2017). Besides, the alkaloids contained in the plowed green biomass produce a decontaminating effect on the soil, thus reducing the negative impact inflicted on subsequent crops by their diseases and pests, such as various root rots for cereals or scab, *Rhizoctonia* rot and golden nematode for potato (Evstratova et al., 2012). This phenomenon is undoubtedly interesting as a protective tool against fungal disease agents, and calls for further research into the mechanism of alkaloid activities (Anokhina et al., 2008; Romeo et al., 2018). Hence, high alkaloid content becomes a preferable trait in green manure cultivars, which serves to simplify breeding schemes.

Today, main requirements to lupine cultivars grown for green manure are high dry matter yield, rapid growth, and increased nitrogen-fixing activity. The latest cultivars developed by Russian breeders – 'Oligarkh', 'Metsenat' and 'Akkord' (Belogorka Research Institute of Agriculture) – contain 1.5 % of alkaloids in seeds and 0.7 % in the dry matter of green biomass, produce high yields of biomass (31–37 t/ha), demonstrate rapid initial growth and prolific foliage, and are ready for plowing into the soil in the second half of July, i. e., 50–60 days after sprout emergence (Lysenko, 2019).

Fodder qualities of narrow-leaved lupine

Lupine fodder is considered a good alternative to soybean: digestibility and feed energy coefficients of lupine proteins are level with those of soybean and exceed those of pea, while the yield of lupine in the European part of Russia is 1.5–2.0 times higher than soybean yield. Many European countries do not produce soybean, so they are forced to export it, mainly from South America, but the production areas under lupine in Europe have good prospects for expansion. According to the estimates by experts, the cost price of lupine grain production is twice lower than that of soybean grain. Besides, narrow-leaved lupine produces higher yields at lower energy costs than soybean: 840.7–846.6 MJ/100 kg (Feed Production Handbook…, 2014).

The value of lupine as a fodder crop is all the more palpable in view of the fact that not only grain but also its green biomass, with 18–23 % of crude protein and up to 14 % of sugar in dry matter, is readily consumed by all kinds of farm animals. Lupine is used for feed as freshly cut plants, in crushed grain and compound feeds, as silage and haylage, as a component of cereal and legume haylage mixtures, etc. (Kuptsov, Takunov, 2006). Its green biomass is numbered among highly nutritional succulent feeds, distinguished for its good digestibility and feed consumability. Lupine straw contains up to 7 % of protein, which is the evidence of its higher feeding value than the straw of cereal crops. It may be added to silages made of the biomass of other crops. Lupine regrowth may be used for grazing, especially as far as swine and sheep are concerned. It is much more nourishing than the stubble of cereals; it is even compared with grassy legume pastures. The practice of pasturing sheep and calves on harvested fields where grain and fodder lupine cultivars were grown is widespread in Australia (Gladstones, 1970). It should be mentioned that the regrown lupine stubble is not the only valuable grazing resource in a harvested field: leftover seeds are also a bonus – their losses at harvesting range from 150 to 400 kg/ha (Truter et al., 2015).

The lupine grain contains high enough amounts of tocopherol (3.9–16.2 mg%) and carotenoids (10–21 mg%), and 90 % of the latter is carotene. This is especially important for aquaculture, as many fish species cannot exist without carotenoids (Korol, Lakhmotkina, 2016a).

Narrow-leaved lupine as human food

When eight crops were discussed in the context of their eligibility as major sources of plant protein for Western Europe, considering their agronomic advantages, prospects for quick improvement, yield and quality of protein, technological aspects, functional and nutritional properties, lupine and pea were recognized as preferential over potato, triticale, alfalfa, etc. (Linnemann, Dijkstra, 2002; Dijkstra et al., 2003).

Beginning from the late 20th century, lupine seeds have been widely used as ingredients by food industries in a number of European countries, Canada, the U.S., Chile, Australia, and to a much lesser extent in Russia and Belarus. Each year Europe consumes about 500,000 tons of lupine-containing food products, including lupine flour, lupine bran, lupine curd (tofu), etc. used as ingredients of bread, pastry, pasta, dressings, milk substitutes, soybean substitutes in sausages, etc. Traditional for Southern Europe is a popular 'Lupini' snack, looking and tasting like popcorn or cornflakes (Yáñez, 1990).

Lupine products are regarded as functional food. They contain little fat and starch. Their glycemic index is low, which is taken into account by nutrition strategies to control obesity, diabetes and cardiovascular diseases. Besides, they are gluten-free, which is important for celiac patients, so they are a valuable reserve to widen the range of foodstuffs for this category of the population (Krasilnikov et al., 2010; Pankina, Borisova, 2015). Lupine proteins possess emulsifying and foaming capacities, which allow them to substitute butter and eggs in cookery (Kohajdorová et al., 2011). Lupine seeds are rich in ferritin, an iron-storing protein (Lucas et al., 2015).

Besides, the grain of lupine owes its functional value in human nutrition to dietary fibers whose content reach 41.5 % (Lakhmotkina, 2011; Lucas et al., 2015). Favorable properties to food products are rendered by lupine oil, with its well-balanced fatty acid composition and an optimal ratio of omega-3 and omega-6 acids – from 1 : 1.7 to 1 : 10.8 (Sedláková et al., 2016).

Phenolic components and flavonoids in narrow-leaved lupine demonstrate antioxidant activity (Martínez-Villaluenga et al., 2009), reduce the risk of cardiovascular diseases through their protective effect on blood vessels (Oomah et al., 2006), and deter the development of some types of cancer, specifically the rectal cancer (Lima et al., 2016). Unlike soybean, lupine contains small amounts of phytoestrogens and less antinutrients, such as phytic acid, oligosaccharides, trypsin inhibitors, lectins, tannins and saponins, than other legumes (Martínez-Villaluenga et al., 2009).

Lupine seeds contain lutein and zeaxanthin – compounds known for their ability to hinder retinal degradation (Fryirs et al., 2008; Wang et al., 2008).

The most popular lupine-based product with food industry is lupine flour. It is rich in lysine but poor in sulfur-containing amino acids, such as methionine and cysteine, therefore it can serve as a good supplement to lysine-deficient wheat flour (Dervas et al., 1999). Adding 10 % of lupine flour to bread, pasta or bakery products will not only increase their functional value but also improve their texture, flavor and color, concurrently extending their shelf life (Pollard et al., 2002).

In Russia, technologies have been proposed to make pastas and fillings from lupine grain (Pankina, Borisova, 2015). Properties and effects of dietary fibers from lupine hulls are being studied in the context of their use as functional ingredients in some meat products, such as intermediate minced poultry meat (Lakhmotkina, 2011), etc.

Utilization of lupine for food purposes is growing worldwide. In Australia it is called the 'superfood' of the 21st century. It is expected that in the nearest future major international markets of nutriceutics (in the European Union, United States and Japan) will rise due to the onset of chronic cardiovascular diseases, nervous disorders, and type 2 diabetes. A potentially huge market demand for lupine-based products also exists among vegetarians, vegans, and people intolerant to gluten, soy, milk or eggs as well as in the growing sector of those who favor healthy diets (Lucas et al., 2015).

Alkaloids in narrow-leaved lupine

Composition, variability and toxicity

Alkaloids are products of secondary metabolism. Unlike primary metabolites, their functional significance is not on the level of a cell but on the level of a whole plant. Most often these compounds perform 'ecological' functions, i. e., protect the plant from various pests and pathogens, ensure interactions among plants and between plants and other organisms within an ecosystem, etc. (Borisova et al., 2020).

Different *Lupinus* species possess a unique alkaloid profile. Usually it consists of 4–5 major alkaloids and several minor ones. The alkaloid composition of a plant is handy for taxonomic purposes (Frick et al., 2017).

Polymorphism of the narrow-leaved lupine gene pool in the content of alkaloids in seeds was uncovered by Polish researchers who studied 329 accessions from the lupine collection: 0.0005–2.8752 % (Kamel et al., 2016). A common feature of all species is a high alkaloid content in seeds (up to 4 %) and a lower content in green biomass (up to 1.5 %). In flowers up to 2.5 % is observed, while in roots their amount is minimal (Lee et al., 2007).

Prevailing alkaloids in narrow-leaved lupine seeds are lupanine (65–75 % of the total alkaloid content), angustifoline (10–15 %) and 13-hydroxylupanine (10–15 %). Minor levels are demonstrated by sparteine and lupinine (Blaschek et al., 2016). These values may vary depending on the genotype and its locality. The concentration of alkaloids in plant organs and their correlation can change under the effect of different growing conditions (Cowling, Tarr, 2004). Even in low-alkaloids cultivars their content is prone to variations within quite an extensive range, exceeding threshold limit values (Romanchuk, Anokhina, 2018). Lupines growing at high latitudes were found to contain less alkaloids than those in southern areas (Gresta et al., 2017).

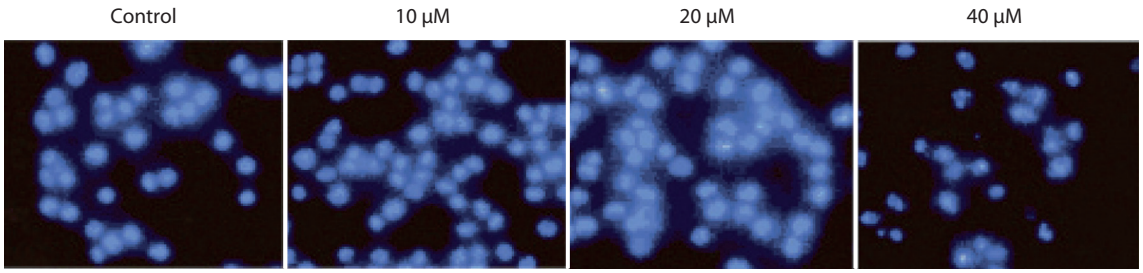

Fig. 2. The intensity of angustifoline-induced apoptosis in malignant tumor cell culture from the human large intestine grows with an increase of angustifoline concentration in the medium, according to (Ding et al., 2019).
Fluorescent microscopy with DAPI staining.

Accumulation of alkaloids in different plant organs is not simultaneous. In the branching phase, when photosynthesis is especially active, the highest alkaloid content is observed in leaves. In the flowering phase, an intensive efflux of alkaloids occurs from the vegetative organs of a plant to the generative ones, where their content reaches their maximum by the beginning of pod maturation. Each phase of plant development is characterized by its own qualitative composition of secondary metabolites, including alkaloids. Hydroxylupanine dominates at the start of branching, and lupanine at the time of flowering and pod maturation. By the seed ripening period, the alkaloid content in seeds is 5–10 times higher than in green biomass (Akritidou et al., 2015). Sparteine and lupanine are the most toxic, followed in descending order by lupinine, hydroxylupanine and angustifoline (Allen, 1998).

Interestingly enough, when a plant has been mechanically injured, the amount of alkaloids in it grows fourfold. The injury, in this case, mimics the bite of an insect, which may serve as an evidence of the protective functions performed by alkaloids. With this in view, lupanine inflicts the strongest toxic effect on sucking insects (Wink, 1983, 1992).

Metabolomic profiling may prove an effective approach to the assessment of alkaloid biosynthesis activity in the species' gene pool and the impact of diverse abiotic and biotic environmental factors on this process. Studying metabolomic profiles in wild lupine forms will help to identify or specify the role of individual alkaloids in the species' adaptation to changing environmental conditions (Romanchuk, Anokhina, 2018).

Practical importance of narrow-leaved lupine alkaloids

Since long ago alkaloids have been extensively applied in medicine, pharmacology, veterinary and other sectors. In the first place, they are used as effective agents in pharmaceuticals to provide complex treatment of many dangerous diseases, cancer included (Kruglov et al., 2015; Ding et al., 2019). They prevent the onset of various degenerative pathologies, binding free radicals and metal ions that activate enzymes of oxidative reactions. They also inhibit the growth and development of fungi, protozoa, bacteria, etc. (Ding et al., 2019).

Sparteine has the widest application. It decreases the level of glucose in an organism and initiates insulin secretion (Sgambato et al., 1986), exerts a mild analgesic effect, and acts as an anticonvulsant and antiepileptic (Villalpando-Vargas, Medina-Ceja, 2016). Together with lupanine and hydroxylupanine, it is included in the composition of antiarrhythmic drugs. Such antiarrhythmic effect weakens in the descending order of sparteine–lupanine–hydroxylupanine (Blaschek et al., 2016). Among the Class IA medicaments against tachyarrhythmia there is a combined drug, known as Pulsonorma, which incorporates in its composition ajmaline, sparteine, antazoline and phenobarbital (Ivashev et al., 2013).

Lupanine is a very active neurotransmitter for nAChR (nicotinic acetylcholine receptors) which play a decisive role in neuron signal transmission. The data were obtained on its ability to increase insulin secretion (Wiedemann et al., 2015). It may be used as source material for the synthesis of other alkaloids which are very difficult to produce artificially (Wink, 1987).

Angustifoline on the cell culture of a malignant tumor in the human large intestine (line COLO-205) induced autophagy in tumor cells, apoptosis processes and interruption of the cell cycle in the G2/M stage, so it may be regarded as an antineoplastic agent (Ding et al., 2019) (Fig. 2).

Lupinine demonstrates moderate antiglycation activity, without any cytotoxic effect (Abbas et al., 2017). It is also characterized by strong insecticidal activity (Campbell et al., 1933).

Allelopathic effects of lupine alkaloids are confirmed again and again (Wink, 1993), for example, by their antimicrobial activity in the culture of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* Mig., *Bacillus subtilis* Cohn., *Klebsiella pneumoniae* Trevis., in concentrations 3–4 orders lower than that of antibiotics (Erdemoglu et al., 2007).

An *in vivo* trial on lupine-fed goats showed moderate but credible activity of a lupine seed extract against the nematodes *Haemonchus contortus* and *Teladorsagia circumcincta* (Dubois et al., 2019).

There is a lot of evidence to the antifungal effect of alkaloids. *Fusarium* resistance of high-alkaloid lupine cultivars was shown to be higher than that in low-alkaloid ones, and

an increased alkaloid content in plant cells was reported in response to the infection by causative agents. Purified lupine alkaloids may be used for pre-sowing treatment of legume crop seeds to raise their resistance to anthracnose, *Fusarium*, and other fungal diseases. The advantage of alkaloids over synthetic fungicides is their biodegradation and lesser toxicity (Anokhina et al., 2008).

New breeding trends or old processing techniques?

There are five known genes reducing alkaloid content in narrow-leaved lupine seeds: *iuc* (*iucundus*), *es* (*esculentus*) (Hackbarth, 1957), *dep* (*depressus*) (Hackbarth, Troll, 1956), *a1*, *a2*, *a3* (*angustifolius*) (Mikolajczyk, 1966), and *tant* (*tantalus*) (Zachow, 1967). The *iuc* gene determines a reduction in alkaloid concentration approximately to 0.06 % dry weight, *dep* is responsible for very low content of alkaloids (ca. 0.01 %), while *es* governs their intermediate concentration (Hackbarth, Troll, 1956). Each stage in the synthesis of alkaloids is controlled by certain alleles, capable of independent mutations and recombinations. Non-allelic mutations are possible: they have a similar phenotypic effect, leading to a low content or absence of alkaloids (Anokhina, 1975). The discovery of complementary gene interactions made it possible to produce the first absolutely alkaloid-free forms by uniting the genes of two non-allelic recessive mutants in one genotype (Sengbusch, 1942). Thus, the absence or low content of alkaloids is a complex quantitative trait of polygenic nature with free complementation between its non-allelic complementary genes (Anokhina, 1975), which is a serious obstacle for the crop's breeding and seed production.

It was observed in the process of breeding sweet cultivars of narrow-leaved lupine that they were considerably less resistant to diseases and pests than bitter ones: their susceptibility to insect attacks increased as well as, accordingly, vulnerability to virus diseases carried by, for example, aphids (Berlandier, 1996; Adhikari et al., 2012). The end of the 20th century was marked by drastic onsets of *Fusarium* and anthracnose in all countries producing narrow-leaved lupine. The idea emerged to develop 'bitter/sweet' cultivars, combining the bitterness of green biomass as a means of defense against pests and low alkaloid content in seeds to make them usable as feed or food (Wink, 1990; Philippi et al., 2015). Such idea could not be implemented without the knowledge of the entire multistep way of alkaloid biosynthesis which starts within the chloroplasts of young lupine leaves (Wink, Hartmann, 1982; Bunsupa et al., 2012), from where they are transported through the phloem into the generative organs (Lee et al., 2007). Recent research on the expression of genes responsible for alkaloid biosynthesis has shown that such biosynthesis is completely or nearly absent in the seeds, which confirms the transport of alkaloids from other tissues (Otterbach et al., 2019).

Biosynthesis of quinolizidine alkaloids has been studied to a much lesser extent than that of some economically important alkaloids in other plants that represent the model species for better understanding of this process (*Nicotiana* spp., *Papaver somniferum*, etc.). That is why the attempts have been made to gain an insight into the ways of lupine alkaloid synthesis and transport, using the knowledge of the synthesis of other alkaloids and the searches for homologous genes (see the reviews by Bunsupa et al., 2012; Kamel et al., 2016; Frick et al., 2017; Romanchuk, Anokhina, 2018).

Along with approaching these breeding tasks, which prospectively can be solved through the use of new reverse breeding or genome editing technologies, they are trying to modernize the centuries-old experience in the removal of bitterness from lupine seeds and green biomass. They develop the techniques of alkaloid extraction from large amounts of raw plant produce yielded by bitter cultivars, thus making it fit for animal feed. In 2013, for example, Russian researchers developed and patented the cost-effective biotechnology of profound lupine grain processing in a milk serum medium. Such line may be installed into the technological process of any compound feed producing factory (Korol, Lakhmotkina, 2016b). Thermal seed treatment with alkaline solutions could reduce alkaloid concentrations in seeds to 0.003 % (Jiménez-Martínez et al., 2001). In Portugal, at the enterprises that extract alkaloids from large bulks of lupine and consume lots of water, a trial was conducted to test the technology of discharged water detoxification by nanofiltration and binding of 99 % of lupanine contained in it, so that the latter could be used as raw material for the pharmaceutical industry (Barbeitos, 2016).

Thus, at present there are two ways to obtain alkaloid-free raw produce of narrow-leaved lupine for food and feed purposes: lengthy and intricate development of low-alkaloid cultivars by conventional breeding techniques, and novel technological lines of alkaloid removal/extraction. The solution for lupine breeding is seen in the genome-based biotechnologies, as their certain prospects for narrow-leaved lupine are quite obvious.

Genetic and genomic resources of narrow-leaved lupine

Intensification of breeding practice requires rich and diverse source material. A number of the world's genebanks maintain the global diversity of *L. angustifolius*. The largest collections are in Australia, Poland, Portugal, and the Russian Federation. The Australian collection includes mostly wild lupine forms, recombinant inbred lines, mutant populations, and interspecies hybrids. These resources are used to study the genetic and molecular control over the key traits, and this work is expected to be reinforced by the ongoing research into *L. angustifolius* genome sequencing. The main objective of Australian researchers is to expand the genetic base of the species, including the involvement of wild lupine forms. Marker-based introgression of the desired traits is proposed (Berger et al., 2013). The marker-assisted selection has already become an integral

element of Australian breeding programs and accelerated the development of new cultivars (Rychel et al., 2015).

Genetic maps have been produced for narrow-leaved lupine (Yang et al., 2013; Kamphuis et al., 2015) as well as vast libraries of genomic insertions (Gao et al., 2011). Genes responsible for the expression of economically useful traits, alkaloid content included, have been discovered and mapped (Boersma et al., 2005; Bunsupa et al., 2011).

The only one recessive *iuc* gene, out of the five known ones that determine the alkaloid content in narrow-leaved lupine, is used in breeding programs. The gene's molecular functions have not yet been identified. Markers have been found for the locus *iuc*, and the denser cartographic resources and genome annotation have narrowed the region of the *iucundus* candidate gene (Li et al., 2011; Hane et al., 2016). The NGS (next generation sequence) technology is applied for more rapid development of markers for breeding (Yang et al., 2015).

The paths of alkaloid synthesis are partially known. However, their genetic base still remains poorly studied. Transcriptome sequencing (RNA-seq) and analysis of differentially expressed genes in a sample containing bitter and sweet narrow-leaved lupine accessions helped to detect 13 genes presumably involved in the synthesis of quinolizidine alkaloids (Kamel et al., 2016). The identified alkaloid biosynthesis genes were mapped, but only one transcriptomic factor from the RAP2 family of factors regulating secondary metabolism was closely linked with the *iuc* gene (Kroc et al., 2019). Investigating the mapping populations with the technique of massive analysis for cDNA ends (MACE) confirmed the idea that the *ETHYLENE-RESPONSIVE TRANSCRIPTION RAC2-7* gene factor could control the low-alkaloid phenotype in narrow-leaved lupine (Plewiński et al., 2019).

Conclusion

The gene pool of narrow-leaved lupine should become the target of more intense research on the phenotypic and genotypic levels, so that its diversity would be more obvious and available to plant breeders. This will help to optimize the development of cultivars with the desired properties. With this in view, it seems rational to make green manure cultivars high-alkaloid, but those intended for food and feed must not lose their adaptability-related traits, including pathogen resistance, at the expense of eliminated alkaloids. In this regard, a productive idea is to produce 'bitter/sweet' cultivars, combining high alkaloid content in their green biomass with a low alkaloid level in seeds. Its implementation depends on the knowledge of alkaloid biosynthesis and transport pathways in a plant and the possibility of their regulation, which seems a task for the nearest future, considering the currently available genomic resources. However, at the present moment it is not expedient to discard routine technologies of raw plant produce processing, using alkaloid extraction techniques with concurrent isolation of valuable ingredients for the pharmaceutical industry. Genetic resources–phenotyping–metabolomics–conventional breeding practice; genomic resources–marker-assisted and genomic selection/genome editing; and cost-effective technologies of alkaloid extraction from the raw produce of bitter cultivars – these approaches are, in our opinion, the best to improve the economic potential of this valuable pulse crop and make use of it in the present-day situation and in future.

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ORCID ID

M.A. Vishnyakova orcid.org/0000-0003-2808-7745
A.V. Kushnareva orcid.org/0000-0002-5709-7961
T.V. Shelenga orcid.org/0000-0003-3992-5353
G.P. Egorova orcid.org/0000-0002-8595-1336

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Regions enriched for DNA repeats in chromosomes of *Macrostomum mirumnovem*, a species with a recent Whole Genome Duplication

K.S. Zadesenets, N.B. Rubtsov ✉

Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
✉ e-mail: rubt@bionet.nsc.ru

Abstract. The free-living flatworm *Macrostomum mirumnovem* is a neopolyploid species whose genome underwent a recent Whole Genome Duplication (WGD). In the result of chromosome fusions of the ancient haploid chromosome set, large metacentric chromosomes were formed. In addition to three pairs of small metacentrics, the current karyotype of *M. mirumnovem* contains two pairs of large metacentric chromosomes, MMI1 and MMI2. The generation of microdissected DNA libraries enriched for DNA repeats followed by DNA probe preparation and fluorescent *in situ* hybridization (FISH) were performed. The DNA probes obtained marked chromosome regions enriched for different DNA repeats in the *M. mirumnovem* chromosomes. The size and localization of these regions varied in different copies of large chromosomes. They varied even in homologous chromosomes, suggesting their divergence due to genome re-diploidization after a WGD. Besides the newly formed chromosome regions enriched for DNA repeats, B chromosomes were found in the karyotypes of the studied specimens of *M. mirumnovem*. These B chromosomes varied in size and morphology. FISH with microdissected DNA probes revealed that some Bs had a distinct DNA content. FISH could paint differently B chromosomes in different worms and even in the same sample. B chromosomes could carry a bright specific fluorescent signal or could show no fluorescent signal at all. In latter cases, the specific FISH signal could be absent even in the pericentromeric region of the B chromosome. Possible mechanisms of B chromosome formation and their further evolution are discussed. The results obtained indicate an important role that repetitive DNAs play in genome re-diploidization initiating a rapid differentiation of large chromosome copies. Taking together, karyotype peculiarities (a high level of intraspecific karyotypic diversity associated with chromosome number variation, structural chromosomal rearrangements, and the formation of new regions enriched for DNA repeats) and some phenotypic features of *M. mirumnovem* (small body size, short life-cycle, easy maintenance in the laboratory) make this species a perspective model in the studies of genomic and karyotypic evolution in species passed through a recent WGD event.
Key words: metaphase chromosome microdissection; DNA probes; repetitive DNA; mobile element transposition; FISH; DNA amplification; B chromosomes.

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Районы, обогащенные повторенными последовательностями ДНК, в хромосомах *Macrostomum mirumnovem* – вида, недавно прошедшего полногеномную дупликацию

К.С. Задесенец, Н.Б. Рубцов ✉

Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
✉ e-mail: rubt@bionet.nsc.ru

Аннотация. Свободноживущий плоский червь *Macrostomum mirumnovem* – неополиплоидный вид, его геном претерпел недавнюю полногеномную дупликацию (Whole Genome Duplication, WGD). В результате слияния гаплоидного хромосомного набора в его кариотипе произошло формирование двух новых крупных хромосом, MMI1 и MMI2. Создание микродиссекционных ДНК-библиотек, обогащенных повторенными последовательностями ДНК, и их последующая гибридизация *in situ* с метафазными хромосомами *M. mirumnovem* выявили в этих хромосомах районы, обогащенные повторенными последовательностями ДНК. Разные ДНК-пробы устанавливали в хромосомах *M. mirumnovem* районы, обогащенные разными повторенными последовательностями. Локализация и размер этих районов варьировали в разных копиях крупных хромосом, это предполагало их дивергенцию и снижение уровня гомологии, что может после полной дупликации генома приводить к его редиплоидизации. Помимо возникших *de novo* районов хромосом основного набора, обогащенных повторенными последовательностями, в кариотипе у большинства исследованных особей

обнаружены B-хромосомы, которые варьировали по размеру и морфологии. Различия в составе ДНК у этих B-хромосом были показаны с помощью флуоресцентной гибридизации *in situ* (FISH) с полученными микродиссекционными ДНК-пробами на хромосомном материале, взятом от разных животных. Флуоресцентная гибридизация *in situ* этих ДНК-проб по-разному окрашивала B-хромосомы, содержащиеся в кариотипах у разных особей *M. mirumnovem*. Часть B-хромосом интенсивно окрашивалась при проведении FISH, тогда как на других B-хромосомах гибридизационных сигналов не было. Специфический FISH-сигнал отсутствовал даже в прицентромерных районах таких B-хромосом. В настоящей статье обсуждаются возможные механизмы возникновения и последующей эволюции B-хромосом у *M. mirumnovem*. Полученные результаты указывают на важную роль повторенных последовательностей, которую они могут играть в процессе реорганизации генома, приводя к быстрой дифференциации дуплицированных копий хромосом. Высокий уровень внутривидового кариотипического разнообразия по численным и структурным хромосомным перестройкам и по формированию новых хромосомных районов, обогащенных повторенными последовательностями, а также небольшой размер тела (~2 мм) и простота поддержания лабораторных культур *M. mirumnovem* делают этот вид перспективной моделью в исследованиях геномной и кариотипической эволюции видов, недавно прошедших полногеномную дупликацию.

Ключевые слова: микродиссекция метафазных хромосом; ДНК-пробы; повторенные последовательности ДНК; транспозиция мобильных элементов; FISH; амплификация ДНК; B-хромосомы.

Introduction

Comparative genomics opened up new possibilities for studies of mechanisms of whole-genome duplication (WGD), as well as its consequences after the large-scale changes of genome involving almost all of the genes. The most of the existing species of plants and animals were resulted from at least one round of WGD, more often a few WGD events (Wendel, 2000; Panopoulou et al., 2003; Dehal, Boore, 2005). In different phylogenetic lineages, these WGDs took place hundreds of MYA. Hence, the genomes of modern species are paleoploids, and they contain only traces of WGDs (Dehal, Boore, 2005). Therefore, comparative analysis of their genomes can provide only limited knowledge about mechanisms of polyploid formation and further reorganization of the duplicated genome. There are two main mechanisms of WGD: autopolyploidy resulted in autotetraploid genome formation, and allopolyploidization, i. e. doubling of hybrid genome after interspecific hybridization. In the latter case, a new allopolyploid genome contains both parental genomes, and it can retain many traits of its hybrid ancestor. The key role of WGD in genome evolution in animals was considered in the recent review (Zadesenets, Rubtsov, 2018).

WGD events occurred in genomes of different phylogenetic lineages (yeasts, plants, animals). Many studies performed by different research groups have uncovered that the number of WGDs can distinguish for different phylogenetic lineages: for instance, in rotifer – 1 WGD, in the vast majority of vertebrates – 2, in many plants – 3, in salmonids – 4 (Dehal, Boore, 2005; Glasauer, Neuhauss, 2014; Kenny et al., 2018). The genome evolution of *Brassica rapa* contained alternating rounds of multiplication events, including both whole-genome duplication and even triplication (Moghe et al., 2014). Some researchers suggest that WGD creates conditions for following large evolutionary transformations, despite other scientists take a different view, suggesting post-WGD species are mostly evolutionary dead-end (Mayrose et al., 2011; Soltis et al., 2014). For many years the most of studies were devoted to the analysis of polyploidy and WGD in plants that was linked with the limitations of the available methodological possibilities. Development of new methods in molecular genetics, improvement of NGS technologies have fundamentally changed the WGD studies in animals (Zadesenets, Rubtsov, 2017b). In a result, comparative genomics has arisen, and more and more animal species continue to be involved in these studies (Comparative Genomics, 2000). The limited number of species underwent a recent WGD is a challenge in researches devoted to WGD and early-stage genome reorganization after a WGD in animals (Zadesenets, Rubtsov, 2018).

It should be noted that a large number of polyploid variants are likely to be evolutionary dead-ends (Mayrose et al., 2011; Barker et al., 2016). They represent new, but less competitive variants in comparison with their diploid ancestors. Nevertheless, traces of WGDs revealed in genomes of many existing species indicate to a significant contribution of WGD in the formation of new mechanisms of environmental adaptation (Glasauer, Neuhauss, 2014; Fisher et al., 2018). Although WGD can be considered as a key event in the formation of new phylogenetic lineages, the possibilities of studying a WGD and the processes it triggers in the followed stages of genome evolution are limited. The rapid progress in the development of new methods of molecular genetics and genomics has opened up new opportunities and perspectives in the evolutionary studies. However, the number of perspective model organisms for studying WGD remains extremely low. The involvement in studies of new species, whose genomes passed through a recent WGD, is one of the most urgent tasks. The group of species of free-living flatworms of the genus *Macrostomum* may turn out to be one of such promising model species.

Molecular cytogenetic analysis of the *M. lignano* chromosomes uncovered that the main traits of its genome and karyotype organization differ from that of the other *Macrostomum* species, and these peculiarities are linked with a recent WGD event in its genome evolution (Zadesenets et al., 2017a, c). The karyotypes of many *Macrostomum* species consist of a low number of small metacentric chromosomes (Egger, Ishida, 2005; Zadesenets et al., 2016; Schärer et al., 2020). It was suggested that a basal chromosome number was $2n = 6$, but due to the WGD, the doubled chromosome number led to the increased karyotype $2n = 12$ in some *Macrostomum* species. Chromosome fusions of all chromosomes of one haploid

chromosome set of ancestor have resulted in the decreased chromosome number (from 12 to eight chromosomes) in the *M. lignano* karyotype (Egger, Ishida, 2005; Zadesenets et al., 2016). Moreover, paralogous regions revealed in the *M. lignano* chromosomes accumulated some differences that more likely arose after a WGD event (Zadesenets et al., 2017a, c). The uncovered karyotype instability in *M. lignano* is considered to be a consequence of the ongoing rediploidization processes in its genome. The karyotyping of new *Macrostomum* species led us to explore a new species, namely *M. mirumnovem* arose through a recent independent round of WGD. The genome evolution of this species accompanied by intensive chromosome reshuffling resulted in chromosome fusions, structural chromosome rearrangements, formation of new repeat-enriched regions, and B chromosome formation (Zadesenets et al., 2020). Based on the cytogenetic analysis of the *M. mirumnovem* chromosomes, we concluded that expansion and amplification of repetitive DNA play a significant role in the reorganization of its karyotype. By generation of microdissected DNA probes enriched with DNA repetitive sequences, we studied the distribution of these DNA repeats in the *M. mirumnovem* chromosomes.

Material and methods

Laboratory outbred culture of the free-living flatworm *M. mirumnovem*. The laboratory outbred culture of *M. mirumnovem*, kindly provided by Professor Lukas Schärer (Zoological Institute of the University of Basel, Switzerland), was used in the current study. The culture of *M. mirumnovem* has been cultivated under the laboratory conditions for three years. The first karyotyping was performed three months after sampling the *M. mirumnovem* worms from natural populations (Zadesenets et al., 2020). Already at this stage, a high level of karyotypic diversity was revealed. The most common karyotype consisted of nine chromosomes: the largest unpaired metacentric (MMI1), a pair of large metacentrics (MMI2) a bit smaller in size than the MMI1, and three pairs of small metacentric chromosomes MMI3–MMI5 (Schärer et al., 2020; Zadesenets et al., 2020).

In the chromosomes thus formed, the active expansion of transposable elements (TEs) and amplification of DNA sequences probably led to the formation of numerous regions enriched for repetitive sequences (Zadesenets, Rubtsov, 2020). Single-worm karyotyping, carried out before microdissection of small metacentrics, showed an increase in the karyotypic diversity among the worms of the laboratory culture, as well as the formation of B chromosomes in it. In the current study, we used the metaphase plates with the $2n = 9$ karyotype to generate the microdissected DNA probes. For fluorescence *in situ* hybridization (FISH) metaphase chromosome slides obtained from individual worms with different karyotype variants were used.

Preparation of metaphase chromosome plates. Regular karyotyping of individual worms of the laboratory culture of *M. mirumnovem* was done as was previously described (Zadesenets et al., 2016). We checked at least ten metaphase spreads per each specimen. For microdissection, we prepared chromosome slides using a cell suspension technique that provides a better preservation of DNA in metaphase chromosomes. To make a suspension of fixed mitotic cells, we used 100 mature worms, as was earlier described (Zadesenets et al., 2016). Metaphase plates were spread on a wet surface of cold coverslips (60 × 24 × 0.17 mm). Preparation of metaphase spreads on coverslips allowed us to use a maximum magnification of the inverted microscope AXIOVERT10 (ZEISS, Germany) during microdissection.

Staining of metaphase chromosomes. For routine karyotyping the metaphase chromosomes were stained with a fluorescent dye DAPI (4′,6-diamidino-2-phenylindole solution) dissolved in the VECTASHIELD® mounting medium containing antifade (Vector Laboratories Inc., Burlingame, CA, USA). Metaphase chromosome slides prepared for microdissection were stained in a 2 % Giemsa solution (in 1xPBS, pH = 7.2, PanEco, Moscow, Russia) (Zadesenets, Rubtsov, 2020).

Microscopic analysis of chromosome preparations was performed at the Center for the collective use of microscopic analysis of biological objects of the SB RAS at the Institute of Cytology and Genetics SB RAS (Novosibirsk, Russia) using an Axioplan 2 luminescent microscope (ZEISS) equipped with a CCD camera and a set of filters # 49, # 10 and # 15 (ZEISS). Registration and subsequent processing of microscopic images of chromosomes was performed using the ISIS4 software (METASystems GmbH, Germany).

Microdissection of metaphase chromosomes and generation of microdissected DNA probes. Microdissection of metaphase chromosomes was used for the generation of the DNA probes from the *M. mirumnovem* chromosomes as was described earlier (Zadesenets et al., 2016). To amplify the DNA of the dissected chromosomal material, we used a standard variant of sequence-independent polymerase chain reaction (PCR) (Zadesenets et al., 2017a, c). The procedure of microdissection from the selection of the metaphase plate to the transfer of dissected material into a PCR tube with a reaction mixture were described in detail (Zadesenets, Rubtsov, 2020). In the current study, we collected two single copies of small chromosomes.

The difference in the preparation of DNA probes from the standard protocol consisted in modifying the composition of the collection buffer, into which the dissected chromosome material was directly collected (~40 nl in the drawn-out silicone tip of the Pasteur pipette). The collection drop contained a proteinase and DNA fragmentation buffer (commercial Whole Genome Amplification 4 (WGA4) kit, Sigma-Aldrich, USA) and 0.1 % non-ionic detergent Triton X-100 (VWR Life Science AMRESCO, USA). All subsequent stages of DNA preparation for amplification and directly DNA amplification were carried out according to the previously described protocol (Zadesenets et al., 2016, 2017a, c) with an increased number of PCR cycles (up to 35 cycles) at the last stage of the preparation of a microdissected DNA library.

Generation of microdissected DNA libraries involves two steps: (1) the generation of DNA fragments (from the dissected chromosomal material) flanked by the corresponding sequences (WGA4 kit, Sigma-Aldrich, USA) and (2) the amplification of such DNA fragments in PCR. Depending on the efficiency of obtaining the flanked DNA fragments in the

first stage, there are two different variants for the DNA content of the obtained DNA libraries. In the first one, the sequences repeated many times in the genome and a significant part of the unique and low repetitive sequences are involved in amplification. In the second one, the part of genomic DNA sequences involved in amplification was reduced and mostly highly repeated sequences appeared to be amplified. The representation of unique DNA sequences in the obtained microdissected DNA libraries is significantly reduced with a decrease in the efficiency of generation of DNA fragments capable of amplification at the first stage. In a result, the generated DNA libraries are enriched with repetitive sequences.

An increase in the number of PCR cycles at the second stage of generation of microdissected DNA library allows to obtain the required amount of DNA product. The modification of the collection buffer used in the first stage of generation DNA libraries led to the production of DNA libraries that were enriched in highly repeated sequences. The presence mostly of highly repetitive DNA in the obtained DNA libraries was confirmed by the complete absence of a signal in most euchromatin regions of chromosomes after FISH with DNA probes obtained on their basis.

The resulting PCR product was labelled in 20 additional PCR cycles in the presence of fluorochrome-conjugated nucleotides: Flu-12-dUTP [fluorescein-5(6)-carboxamidocaproyl-[5(3-aminoallyl)2′-deoxyuridine-5′-triphosphate] (Biosan, Novosibirsk, RF) or TAMRA-5-dUTP [tetramethylrhodamine-5(6)-(5-[3-carboxamidoallyl]-2′-deoxyuridine 5′-triphosphate)] (Biosan) (Zadesenets et al., 2017a, c). GenomePlex® Whole Genome Amplification Reamplification kit (WGA3) (Sigma-Aldrich, USA) was used for labelling.

Fluorescent *in situ* hybridization on metaphase chromosomes of DNA probes with metaphase chromosomes of *M. mirumnovem* was performed according to the previously described protocol (Zadesenets et al., 2017a, c), without suppression of the repetitive DNA hybridization. During FISH, chromosome slides prepared from individual worms of *M. mirumnovem* were analyzed. When FISH was carried out with separate microdissected DNA probes (S3 or S4), as well as when two-colour FISH with DNA probes S3 and S4, was performed ten or more specimens of *M. mirumnovem* were studied.

Results and discussion

Generation of DNA probes from chromosomes of *M. mirumnovem* and FISH of the obtained DNA probes with metaphase chromosomes. The S3 and S4 DNA probes were obtained from single copies of two small metacentrics. FISH with the DNA probes on metaphase chromosomes of *M. mirumnovem* revealed chromosome regions enriched for repetitive sequences homologous to DNA in the DNA probes S3 and S4 (Fig. 1, 2). The regions painted by the DNA probes S3 or S4 will be called S3D- or S4D-regions, respectively, i. e. the regions detected by FISH using the DNA probes S3 and S4. In our study, the definition of precise localization of these regions in metaphase chromosomes of *M. mirumnovem* posed a certain problem of the description based on their morphology and differential staining.

Fluorescent *in situ* hybridization with the S4 DNA probe on the large metacentric chromosomes, MMI1 and MMI2, gave bright fluorescence exactly in the region of their primary constriction, and additional signals in some regions of their chromosome arms (see Fig. 1, 2). In addition to FISH signals in large chromosomes, the DNA probe S4 gave an intense signal in the pericentromeric region of the chromosome MMI4. In some specimens, the distal region of q-arm of MMI4 was also intensively painted (see Fig. 1, *a*, *b*, 2, *c*). The weak signals (on the edge of being able to detect them) were also detected in the pericentromeric regions of the chromosomes MMI3 and MMI5.

Due to the small block of the pericentromeric structural heterochromatin in the *M. mirumnovem* chromosomes, it is hardly possible to conclude whether the S4D-regions were located directly in the pericentromeric regions or in adjacent euchromatin regions even in the large chromosomes MMI1 and MMI2. Localization of FISH signal on the condensed metaphase chromosomes MMI3–MMI5 often turned out to be even more complicated (see Fig. 1, *a*, *b*). However, it should be noted that on less condensed chromosomes MMI3–MMI5 with a pronounced primary constriction, the FISH signal of the DNA probe S4 was always localized in this region (see Fig. 2, *c*).

Thus, taking into account the small size of the pericentromeric heterochromatic regions, the question whether the S4D-regions are located in the pericentromeric heterochromatin of the chromosomes or the proximal euchromatin of p- or q-arms, remains open. For simplicity, a further description of the obtained results, we will define these regions pericentromeric, recognizing some incorrectness of the use of this term.

Given the specificity in the description of FISH results, we can conclude that the DNA probe S4 painted the pericentromeric regions of all A-chromosomes. The intensity of FISH signals in different chromosomes varied significantly, changing from the intense signal in the region of the primary constriction of some copies of chromosomes MMI1, MMI2, and MMI4 to the weak separate signals on both chromatids of small metacentrics MMI3 and MMI5 (see Fig. 1, *a*, *b*). The variation in fluorescence intensity of the signal of the DNA probe S4 in regions of different chromosomes, including different copies of homologous chromosomes, was probably derived from the different copy number of the corresponding repeats in these regions. However, it cannot be ruled out that in some cases such differences may be based on the differences in homology level between repetitive sequences in these regions and the DNA of the S4 probe. It should be noted that additionally to specific signals in the regions of A-chromosomes, the DNA probe S4 weakly painted some B-chromosomes (see Fig. 1, *b*, 2, *a*, *b*).

Intense FISH signals of the DNA probe S3 localized only in small chromosome MMI4 and in large metacentric chromosomes (MMI1 and MMI2). Furthermore, two-colour FISH with the DNA probes S3 and S4 showed that the large chromosomes are enriched for different highly repeated sequences. Moreover, the revealed S3D- and S4D-regions were not co-localized (see Fig. 2, *a*–*c*). Even in the proximal region of MMI4, where the S3D- and S4D-regions were localized close

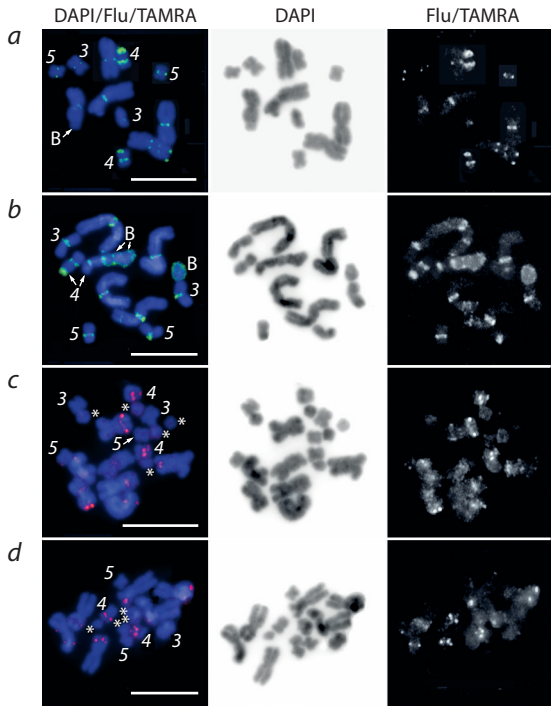

Fig. 1. Fluorescent *in situ* hybridization of the microdissected DNA probes S3 (red signal) and S4 (green signal) with metaphase chromosomes obtained from different specimens of *M. mirumnovem*. The chromosomes differ in the size of the regions enriched for repetitive DNA sequences (*a–d*). The inverted image of DAPI-staining and separate channels for fluorochromes Flu/TAMRA are presented.

The chromosomes MMI3–MMI5 are indicated by numbers (*3–5*, correspondently) and B chromosome(s) is/are marked by the letter "B". The suggested B chromosomes are shown with asterisks (small chromosomes having the size and morphology distinct from that of the chromosomes MMI3–MMI5). Scale bar 10 μm.

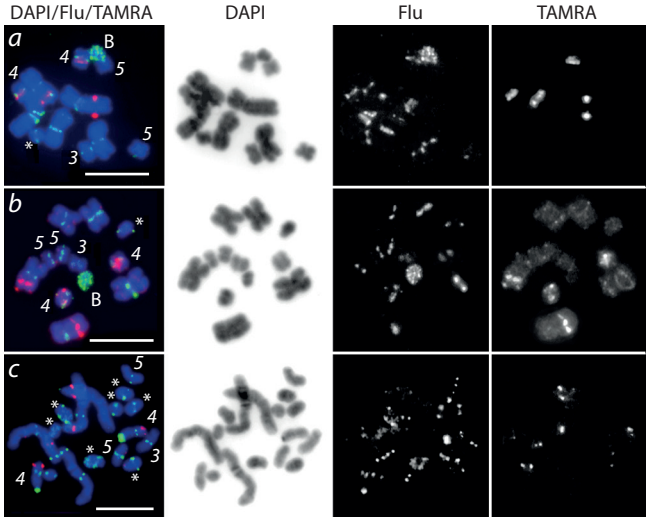

Fig. 2. Two-colour fluorescent *in situ* hybridization of the microdissected DNA probes S3 (red signal) and S4 (green signal) with metaphase chromosomes of high (*a–b*) and low (*c*) condensation level. Metaphase spreads were obtained from various samples of *M. mirumnovem*. The chromosomes differ in the size of the regions enriched in repetitive DNA sequences (*a–c*). The inverted image of DAPI-staining and separate channels for fluorochromes Flu/TAMRA are presented.

For trait designations see Fig. 1.

to each other, no co-localized FISH signals of the DNA probes S3 and S4 were observed.

In one specimen, a transition of the S3D-region to the distal region of the p-arm of MMI4 was revealed, which is visible on low condensed chromosomes (see Fig. 2, *c*). It is likely that this transition could be resulted from an inversion involving one of the breakpoints located between the S3D and S4D regions. The obtained FISH results indicated that different repetitive sequences belonging to MMI4 were involved in DNA amplification during generation of the DNA probes S3 and S4.

The low-intense FISH signals of the DNA probe S3 (on the edge of the possibility of their detection) were revealed in the regions of small chromosomes MMI3 and MMI5. Either the revealed regions probably contained a relatively low copy number of DNA repeats homologous to that of the DNA probe S3, or DNA repeats in them had a low homology level with the DNA of the probe S3. We should note that the DNA probe S3 intensively painted the regions in the arms of some copies of large metacentrics (see Fig. 1, *a–d*, 2, *a–c*). Some of these regions were painted even more intensively than the proximal part of MMI4.

Summarizing data on FISH with the DNA probes S3 and S4 it is possible to state that two different types of large chromosomes were revealed in the karyotypes of studied specimens of *M. mirumnovem.* One of them was characterized by the presence of strong hybridization signals in their arms, while another showed the level of specific FISH signal intensity comparable to registered ones in the regions of small chromosomes MMI3 and MMI5 (see Fig. 1, 2).

Possible mechanisms for the formation of regions enriched for repetitive sequences and their following evolution. The question of the molecular mechanisms providing the variability of the regions enriched for DNA repeats in size and location remains open. However, it is necessary to take into account that chromosomes MMI1 and MMI2 contain extended regions homologous to the euchromatic regions of MMI4 (Zadesenets et al., 2020). Besides variable S3D- and S4D-regions (in terms of localization and size) observed in the MMI1 and MMI2, the S3D- and S4D-regions were also detected in the chromosome MMI4, but only their traces were found in the MMI3 and MMI5. Probably, the fusions of ancestral chromosomes that formed the large metacentrics were accompanied by amplification of DNA repeats derived from MMI4. Further expansion and amplification of repetitive DNA sequences could lead to the divergence of the formed large metacentrics. The distribution of such regions within the chromosome could also occur as a result of inversions, as it was shown in case of transition of the S3D-region in the chromosome MMI4 in one of the analyzed specimens of *M. mirumnovem* (see Fig. 2, *c*).

Based on the results obtained in our study, it is not possible to estimate what kind of repetitive sequences are present in the microdissected DNA probes. In future, the answer to this question can be obtained by cloning DNA fragments from the generated DNA libraries and subsequent FISH with DNA probes prepared from these DNA fragments. Previously, this approach was successfully applied to analyze the DNA composition of the B chromosome of the locust species, *Po-*

disma kanoi (Bugrov et al., 2007). An alternative way for studying the DNA composition of the microdissected DNA libraries is their NGS sequencing. Earlier, this approach was applied to determine the DNA composition of Bs of the Korean field mouse *Apodemus peninsulae* from East Asia, the yellow-necked mouse *Apodemus flavicolis*, and a small supernumerary marker chromosome in humans (Makunin et al., 2018). However, the latter experimental design implies following verification of the results of NGS sequencing using DNA probes generated based on the obtained sequences, since a certain amount of DNA always contaminate microdissected DNA libraries. Additionally, for a successful and efficient interpretation of the data derived from the microdissected library sequencing, it is desirable to have a reference genome assembly or at least genome draft of the studied species (Zadesenets et al., 2017b). In the case of *M. mirumnovem*, it is problematic due to the prominent karyotype and genome instability, and therefore it requires consideration of alternative approaches.

B chromosomes of *M. mirumnovem*. In addition to the regions of A chromosomes, the DNA probe S4 gave specific signal in some Bs (see Fig. 1, *b*, 2, *a–c*). These results allow us to propose one of the scenarios for the formation of such Bs. They could be originated from the pericentromeric region of one of the A chromosomes as a result of chromosome breaks, leading to the arising of a small additional chromosome. At the beginning, this chromosome (or proto-B chromosome) consisted of the pericentromeric region of the ancestral A chromosome, and the subsequent amplification of repetitive DNA sequences homologous to DNA of the microdissected DNA probes and other different DNA repeats. The uneven and not intense specific fluorescence from the DNA probe S4 on the B indicates the presence of such different DNA repeats in the B chromosome. It is worth noting that these DNA repeats can derived both from the pericentromeric region of the ancestral A chromosome and from different chromosome regions. For instance, some Bs gave no specific signal after FISH with the DNA probe S4. Moreover, specific FISH signal was absent even in the pericentromeric regions of such B chromosomes (see Fig. 1, *a*), which indicates the amplification of different types of DNA repeats during the formation of the Bs, and, probably, their different origin.

Conclusion

Using microdissected DNA probes generated by the modified protocol and containing predominantly highly repeated DNA sequences, we identified the regions enriched in repetitive DNA sequences in the *M. mirumnovem* chromosomes. We have shown that enrichment of such chromosomal regions for DNA repeats could vary substantially. The formation and following changes of these regions in large metacentrics MMI1 and MMI2 could lead to differentiation of the copies of homologous chromosomes. Additionally, the obtained results indicate that the DNA content in different copies of B chromosomes could differ among individuals of *M. mirumnovem*. The repetitive DNA sequences homologous to DNA repeats from the proximal regions of A chromosomes took part in the formation of at least some B chromosomes.

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Характеристика активной субстанции препарата дрожжей *Saccharomyces cerevisiae*, обладающей радиопротекторными свойствами

Г.С. Риттер[1], В.П. Николин[1], Н.А. Попова[1, 2], А.С. Проскурина[1], П.Э. Кисаретова[1], О.С. Таранов[3], Т.Д. Дубатолова[4], Е.В. Долгова[1], Е.А. Поттер[1], С.С. Кирикович[1], Я.Р. Ефремов[1, 2], С.И. Байбородин[1, 2], М.В. Романенко[2], М.И. Мещанинова[5], А.Г. Веньяминова[5], Н.А. Колчанов[1], С.С. Богачев[1] ✉

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
[3] Государственный научный центр вирусологии и биотехнологии «Вектор» Роспотребнадзора, р. п. Кольцово, Новосибирская область, Россия
[4] Институт молекулярной и клеточной биологии Сибирского отделения Российской академии наук, Новосибирск, Россия
[5] Институт химической биологии и фундаментальной медицины Сибирского отделения Российской академии наук, Новосибирск, Россия
✉ e-mail: labmolbiol@mail.ru

Аннотация. В работе охарактеризованы некоторые биологические особенности радиопротекторного действия препарата двуцепочечной РНК. Обнаружено, что препарат дрожжевой РНК обладает пролонгированным радиопротекторным действием при облучении животных летальной дозой в 9.4 Гр. При облучении через 1 ч и на 4-е сутки после введения 7 мг препарата РНК выживает 100 % животных на 70-е сутки наблюдения, при облучении на 8-е и 12-е сутки – 60 % животных. Были оценены временные параметры процесса репарации двуцепочечных разрывов, индуцированных γ-лучами. Выявлено, что введение препарата РНК в момент максимального количества двуцепочечных разрывов, через 1 ч после облучения, снижает эффективность радиопротекторного действия по сравнению с введением за 1 ч до облучения и через 4 ч после облучения. Проведено сравнение эффективности радиозащитного действия штатного радиопротектора Б-190 и препарата РНК в одном эксперименте. Установлено, что препарат суммарной РНК не уступает по эффективности препарату Б-190. Выживаемость на 40-е сутки после облучения для группы мышей, получавших препарат РНК, составила 78 %, для Б-190 – 67 % животных. В ходе аналитических исследований препарата суммарной РНК дрожжей обнаружилось, что препарат представляет собой смесь одноцепочечной и двуцепочечной РНК. Радиопротекторными свойствами обладает только двуцепочечная РНК. При введении 160 мкг препарата двуцепочечной РНК выживает 100 % подопытных животных после абсолютно летальной дозы гамма-радиации 9.4 Гр. Установлено, что радиозащитный эффект двуцепочечной РНК зависит не от последовательности, а от ее двуцепочечной формы, причем для осуществления радиопротекторного действия двуцепочечная РНК должна иметь «открытые» концы молекулы. Предполагается, что радиозащитное действие препарата двуцепочечной РНК связано с участием молекул РНК в корректном восстановлении поврежденного облучением хроматина в стволовых клетках крови. Сохранившие жизнеспособность стволовые гемопоэтические клетки мигрируют на периферию и достигают селезенки, где активно пролиферируют. Вновь образовавшаяся клеточная популяция восстанавливает кроветворную и иммунную системы, что определяет выживание летально облученных животных.
Ключевые слова: двуцепочечная РНК; Б-190; селезеночные колонии; двуцепочечные разрывы.

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Characteristic of the active substance of the *Saccharomyces cerevisiae* preparation having radioprotective properties

G.S. Ritter[1], V.P. Nikolin[1], N.A. Popova[1, 2], A.S. Proskurina[1], P.E. Kisaretova[1], O.S. Taranov[3], T.D. Dubatolova[4], E.V. Dolgova[1], E.A. Potter[1], S.S. Kirikovich[1], Y.R. Efremov[1, 2], S.I. Bayborodin[1, 2], M.V. Romanenko[2], M.I. Meschaninova[5], A.G. Venyaminova[5], N.A. Kolchanov[1], S.S. Bogachev[1] ✉

[1] Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
[3] State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk region, Russia
[4] Institute of Molecular and Cellular Biology of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[5] Institute of Chemical Biology and Fundamental Medicine of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
✉ e-mail: labmolbiol@mail.ru

Abstract. The paper describes some biological features of the radioprotective effect of double-stranded RNA preparation. It was found that yeast RNA preparation has a prolonged radioprotective effect after irradiation by a lethal dose of 9.4 Gy. 100 % of animals survive on the 70th day of observation when irradiated 1 hour or 4 days after 7 mg RNA preparation injec-

tion, 60 % animals survive when irradiated on day 8 or 12. Time parameters of repair of double-stranded breaks induced by gamma rays were estimated. It was found that the injection of the RNA preparation at the time of maximum number of double-stranded breaks, 1 hour after irradiation, reduces the efficacy of radioprotective action compared with the injection 1 hour before irradiation and 4 hours after irradiation. A comparison of the radioprotective effect of the standard radioprotector B-190 and the RNA preparation was made in one experiment. It has been established that the total RNA preparation is more efficacious than B-190. Survival on the 40th day after irradiation was 78 % for the group of mice treated with the RNA preparation and 67 % for those treated with B-190. In the course of analytical studies of the total yeast RNA preparation, it was found that the preparation is a mixture of single-stranded and double-stranded RNA. It was shown that only double-stranded RNA has radioprotective properties. Injection of 160 μg double-stranded RNA protects 100 % of the experimental animals from an absolutely lethal dose of gamma radiation, 9.4 Gy. It was established that the radioprotective effect of double-stranded RNA does not depend on sequence, but depends on its double-stranded form and the presence of "open" ends of the molecule. It is supposed that the radioprotective effect of double-stranded RNA is associated with the participation of RNA molecules in the correct repair of radiation-damaged chromatin in blood stem cells. The hematopoietic pluripotent cells that have survived migrate to the periphery, reach the spleen and actively proliferate. The newly formed cell population restores the hematopoietic and immune systems, which determines the survival of lethally irradiated animals.

Key words: double-stranded RNA; B-190; spleen colonies; double-stranded breaks.

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Введение

Ионизирующее излучение воздействует на живой организм таким образом, что приводит к повреждению его функциональных систем и гибели. В настоящее время считается, что ионизирующее излучение оказывает наибольшее воздействие на мембранные структуры и ядро клетки. Лизис мембран приводит к разрушению структуры клетки, а дефекты в ядерной ДНК ведут к нарушению интегральной функциональной целостности хроматина, атипическому течению клеточного деления, появлению хромосомных аберраций и апоптозу (Dent et al., 2003). Основными клетками-мишенями для гамма-лучей являются низкодифференцированные клетки костного мозга, зародышевые клетки семенников, кишечный и кожный эпителий (Bergonié, Tribondeau, 2003; Vogin, Foray, 2013). Радиочувствительность всего организма у млекопитающих приравнивается к радиочувствительности кроветворных клеток, так как их аплазия, возникающая после общего облучения минимальной абсолютно смертельной дозой, приводит к гибели организма.

Под радиозащитным или радиопротекторным эффектом понимают снижение частоты и тяжести постлучевых повреждений биомолекул и(или) стимуляцию процессов их пострадиационной репарации. Наиболее эффективные радиопротекторы относятся к двум классам химических соединений (Patt et al., 1949; Fridovich, 1995). Это серосодержащие радиозащитные вещества (аминотиолы), выполняющие функцию «молекулярных ловушек» свободных радикалов, и производные индолилалкиламинов: агонисты биологически активных аминов, способные через специфические клеточные рецепторы вызывать острую гипоксию и угнетение метаболизма в радиочувствительных тканях (Ward, 1988; Dent et al., 2003; Wang et al., 2013).

Как было сказано ранее, наиболее губительное воздействие ионизирующее излучение оказывает на молекулу ДНК ядерного хроматина. Повреждения хромосом, следующий за этим аберрантный митоз и гибель клетки – это еще один механизм циторедуцирующего действия ионизирующего излучения. При воздействии активных метаболитов на ДНК хроматина возникают все возможные из описанных в литературе повреждений этих молекул. Наиболее фатальными считаются двуцепочечные разрывы (ДЦР). Если в клетке нарушены системы репарации таких повреждений, то клетка запускает механизмы самоуничтожения.

В настоящем исследовании описывается новый принцип радиопротекторного действия, не связанный с протекцией от непосредственно γ-кванта и с ограничением воздействия оксидативного стресса, вызываемого вторичными радикалами, а характеризующийся успешным пострадиационным восстановлением стволовых гемопоэтических предшественников, обусловленным участием в репаративном процессе фрагментов экстраклеточной двуцепочечной нуклеиновой кислоты. Такое введение в репаративный процесс внешнего «корректора» в конечном итоге определяет восстановление кроветворной и иммунной системы и сохранение жизнеспособности облученного организма.

Материалы и методы

Животные. В работе были использованы трехмесячные мыши линий CBA/Lac, C57BL и CC57BR (самцы и самки, 18–22 г) разведения вивария Института цитологии и генетики (ИЦиГ) СО РАН. Животные содержались в группах по 6–10 мышей на клетку со свободным доступом к пище и воде.

Облучение экспериментальных животных проводили на γ-установке (источник Cs^{137} ИГО 1, Россия) дозой 9.4 Гр при мощности дозы 0.74–1.4 Гр/мин. Подопытных и контрольных мышей облучали группами по 9–10 животных в контейнере размером 20 × 20 × 40 см. Радиопротекторное действие препарата суммарной РНК дрожжей (НПО «Биолар», Россия) оценивали по гибели экспериментальных животных в промежуток времени до 30–90 сут. Препарат суммарной РНК дрожжей и двуцепочечная РНК вводились мышам однократно внутривенно

до облучения в количестве, отдельно указанном для каждого эксперимента. Оценка количества селезеночных колоний после фиксации органа в 4 % параформальдегиде проводилась на 9–12-е сутки после облучения.

Выделение фракций препарата РНК. Хроматографию препарата РНК выполняли на колонке объемом 10 мл, диаметром 1 см. Сухой гидроксиапатит (ГАП) подвергали набуханию в 10 мл воды, после чего заполняли колонку. Колонку промывали 30 мл 0.01 М PBS. Наносили на колонку раствор РНК и промывали 30 мл 0.01 М PBS. Элюировали РНК 0.15 М PBS, затем промывали 0.18 М PBS и повторно элюировали нуклеиновые кислоты 0.25 М PBS. Полученный при хроматографии раствор РНК в PBS диализовали против ТЕ-буфера (H_2O, обработанная DEPC; 10 мМ Tris-HCl, 10 мМ ЭДТА, pH 7.4) при +4 °С в течение суток с двумя сменами буфера. Электрофорез препаратов нуклеиновых кислот проводился в 1 % или 1.5 % агарозном геле с содержанием 2 мкг/мл бромистого этидия в трис-ацетатном буфере.

Выделение клеток костного мозга. Клетки костного мозга мышей вымывали из трубчатых костей средой RPMI-1640, тщательно ресуспендировали. Суспензию аккуратно наслаивали на 3 мл смеси фикол-урографин (15 % урографин, 7 % фиколл, ρ = 1.119), центрифугировали при 400 g, 4 °С в течение 40 мин. После центрифугирования вся клеточная масса разделялась на клетки, составляющие интерфазное кольцо (мононуклеары) и осадок. Мононуклеары отбирали в новую пробирку, промывали 4 мл RPMI-1640 и осаждали центрифугированием при 400 g, 4 °С в течение 5 мин.

Анализ репаративного цикла в клетках костного мозга. Через 30, 60 и 120 мин после облучения мышей линии СВА абсолютно летальной дозой 9.4 Гр из трубчатых костей выделяли клетки костного мозга. Анализ репаративного цикла по количеству ДЦР осуществляли методом «кометных хвостов» или при помощи антител к гистону γ-H2aX, как описано в работе (Dolgova et al., 2014). Длину «кометных хвостов» (TM – tail moment) оценивали в программе CASP и ImageJ. Образцы, окрашенные антителами к гистону γ-H2aX, анализировали при помощи проточного цитофлуориметра BD FACSAria в ЦКП проточной цитофлуорометрии ИЦиГ СО РАН. Значения, полученные после анализа 50–100 кометных хвостов, были нормированы к показателям, определенным в 30-й минуте, и усреднены.

Патоморфологический анализ органов. Органы фиксировались в 4 % формальдегиде и заливались в парафиновые блоки. Парафиновые срезы проводились через серию спиртов и окрашивались гематоксилином-эозином.

Сравнение эффективности радиопротекторного действия препарата суммарной РНК дрожжей и штатного радиопротектора Б-190. В качестве препарата сравнения использовали радиопротектор Б-190 (ФГУП НПЦ «Фармзащита» ФМБА России). Препарат Б-190 вводили мышам за 20 мин до облучения перорально в количестве 2.5 мг/мышь в объеме 0.25 мл. Препарат суммарной РНК вводили мышам за 60 мин до облучения внутривенно в количестве 7 мг/мышь в объеме 0.5 мл (0.15 М PBS). Сравнивали выживаемость животных после облучения дозой 9.4 Гр, делали патоморфологический анализ органов.

Качественная реакция на ДНК (реакция Дише). Метод основан на способности дезоксирибозы образовывать соединение синего цвета с дифениламином при нагревании в среде, содержащей смесь ледяной уксусной и концентрированной серной кислот (Dische, 1957). С рибозой РНК аналогичная реакция дает зеленое окрашивание. Дифениламиновый реактив представляет собой 1 % (W/V) раствор дифениламина в смеси ледяной уксусной кислоты и 2.75 % (W/V) концентрированной серной кислоты (p_{20} = 1.836). К осадку нуклеиновых кислот добавляют 0.5 мл раствора едкого натра (0.1 М) и приливают равный объем дифениламинового реактива. Раствор нагревают в течение 15–20 мин на кипящей водяной бане. Появляется характерное для субстрата окрашивание.

Клонирование кДНК копий молекул РНК, элюирующихся с ГАП 0.25 М PBS. Для получения кДНК с РНК использовали систему ревертазного синтеза и набор DOP-PCR master kit («Медиген»). кДНК копии клонировали в плазмидном векторе Bluescript (НПО «Вектор») после «полировки» концов фрагментов с помощью Pfu полимеразы и трансформировали в электрокомпетентные клетки XL1-Blue MRF. Штамм *E. coli* XL1-Blue MRF любезно предоставлен лабораторией иммуногенетики Института молекулярной и клеточной биологии СО РАН. После анализа электрофоретической подвижности ДНК, выделенной из полученных трансформантов, отобранные клоны были секвенированы с использованием протокола фирмы Applied Biosystems (США) при помощи автоматического ДНК секвенатора Applied Biosystems 3500 Genetic Analyzer с 8-канальным капиллярным блоком. Секвенированные клоны анализировались в программе Vector NTI. Последовательности были выравнены и собраны в группы гомологии. Контекстный анализ проводили на сайте http://genome.ucsc.edu, используя инструмент Blat.

Результаты

Радиопротекторное действие суммарной дрожжевой РНК

Было проанализировано 10 различных серийных препаратов дрожжевой суммарной РНК на ее способность защищать животных от летальной дозы γ-радиации. Обнаружено, что радиозащитный эффект препарата прямо не связан с процентным содержанием РНК и белка в препарате. Оценена длительность радиозащитного действия препарата. Для этого очищенная стерильная РНК дрожжей в количестве 7 мг вводилась экспериментальным мышам линии C57BL за час, за сутки, за 4, 8 и 12 сут до облучения летальной дозой радиации 9.4 Гр. Оказалось, при облучении через 1 ч и на 4-е сутки от введения препарата РНК выживает 100 % животных на 70-е сутки наблюдения, при облучении на 8-е и 12-е сутки – 60 % животных (Риттер и др., 2018).

Состояние экспериментальных животных, выживших после летальных доз γ-радиации в отдаленные сроки после проведенного облучения, свидетельствовало о значительных нарушениях в клетках, формирующих кожный покров мышей. В течение 50–150 дней после обработки мыши прогрессивно седели (рис. 1, *а*). Анализ развития селезеночных колоний после облучения, проведенного на

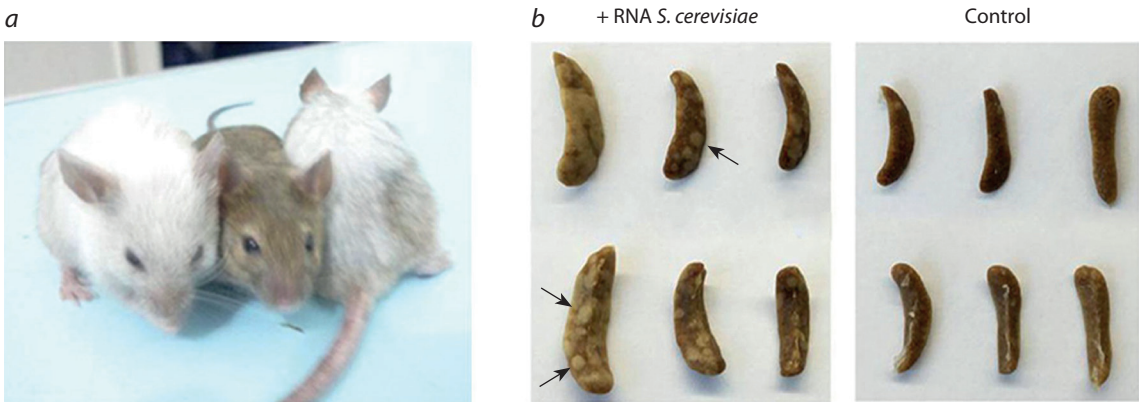

Fig. 1. Characterization of the radioprotective effect of RNA after irradiation of mice with the absolute lethal radiation dose 9.4 Gy.
a – phenotypic comparison of CBA mice irradiated one hour after injection of RNA preparation (left and right) and an intact mouse (center) 150 days after irradiation; *b* – Spleen colonies detected on days 9–12 after irradiation in animals treated with the RNA preparation and in control animals. Arrows indicate leukocyte colonies, which are the criterion for the survival of experimental mice.

9–12-е сутки после воздействия, предполагал, что главной мишенью воздействия препарата РНК являлись стволовые клетки крови костного мозга (см. рис. 1, *б*). Спасенные от разрушения ионизирующим облучением гемопоэтические предшественники способны выходить на периферию и заполнять опустошенные вследствие обработки радиацией иммунокомпетентные органы, например селезенку. В результате мобилизации спасенных стволовых клеток крови в белой пульпе селезенки формируются селезеночные колонии, из которых развивается новая иммунная и кроветворная системы организма взамен разрушенных радиацией.

Радиотерапевтическое действие препарата суммарной РНК дрожжей, ориентированное на временные параметры цикла репарации ДЦР в клетках костного мозга мышей, индуцированных гамма радиацией

Известно, что γ-радиация индуцирует разрушение хроматина в клетках костного мозга и в том числе в стволовых клетках крови, что и приводит к развитию лучевой болезни и гибели организма (Goodhead, 1994; Belli et al., 2002; Morgan, 2003a, b; Shemetun, Pilinska, 2019). Основным повреждением хромосом являются ДЦР, некорректное восстановление которых приводит к аберрантному митозу и апоптозу. В этой связи в начальных экспериментах были оценены временные параметры процесса репарации ДЦР, индуцированных γ-лучами. Мыши подвергались воздействию летальной дозы облучения 9.4 Гр, клетки костного мозга вымывались через 30, 60 и 120 мин после облучения. Количество ДЦР оценивали «методом комет» или по свечению специфических антител к гистону γH2AX (Rogakou et al., 1998, 1999; Maréchal, Zou, 2013). Результаты измерений суммированы в графике на рис. 2, *а*.

Установлено, что основной пик накопления ДЦР приходится на 60 мин от получения дозы радиации 9.4 Гр. К 120 мин и в более поздние сроки наблюдается практически полное восстановление целостности хроматина, тем не менее кривая не опускается до значений, полученных до облучения (данные не приводятся). Этот факт позволяет предполагать, что в указанный отрезок времени детектируются ДЦР, являющиеся интермедиатами репарации, идущей по механизму гомологичной рекомбинации в клетках, находившихся на момент облучения в фазе S клеточного цикла. По результатам проведенных исследований для оценки радиотерапевтического действия препарата РНК были выбраны временные точки 1 и 4 ч. Идея выбора состояла в том, чтобы воздействовать на клетки препаратом РНК во время наиболее интенсивного хода репаративного процесса по механизму негомологичного объединения концов в клетках, находившихся в момент облучения в фазе G1, и во время, когда возможно продолжение репарации по механизму гомологичной рекомбинации в клетках, находившихся в момент облучения в фазе S клеточного цикла (см. рис. 2, *б*). Установлено, что препарат РНК обладает определенным радиотерапевтическим эффектом при его введении в определенный момент времени после завершения репаративного процесса по механизму негомологичного объединения концов. Введение препарата РНК во время идущей репарации негомологичного объединения концов приводит к гибели мышей экспериментальной группы в стандартные временные параметры, показанные для контроля (11–14-е сутки после обработки радиацией).

Параллельно был проведен патоморфологический анализ селезенок контрольных и экспериментальных мышей (см. рис. 2, *в*). Как показали более ранние эксперименты, основное действие препарат РНК оказывает на стволовые клетки крови, которые, пережив радиацию, мигрируют в селезенку, где формируют селезеночные колонии. Предполагалось, что в селезенках мышей, входящих в группы с высокой долей выживаемости, будут обнаружены центры размножения лимфоцитов, потомков выживших и мигрировавших в селезенку стволовых клеток крови.

Патоморфологический анализ свидетельствует о следующем. В селезенках мышей контрольной группы лимфатические фолликулы достаточно многочисленные, однако резко сокращены в размерах до периферической зоны фолликулярной (центральной) артерии. У мышей, которым ввели препарат РНК за час до облучения (группа «–1 ч»), большая часть паренхимы занята сплошной

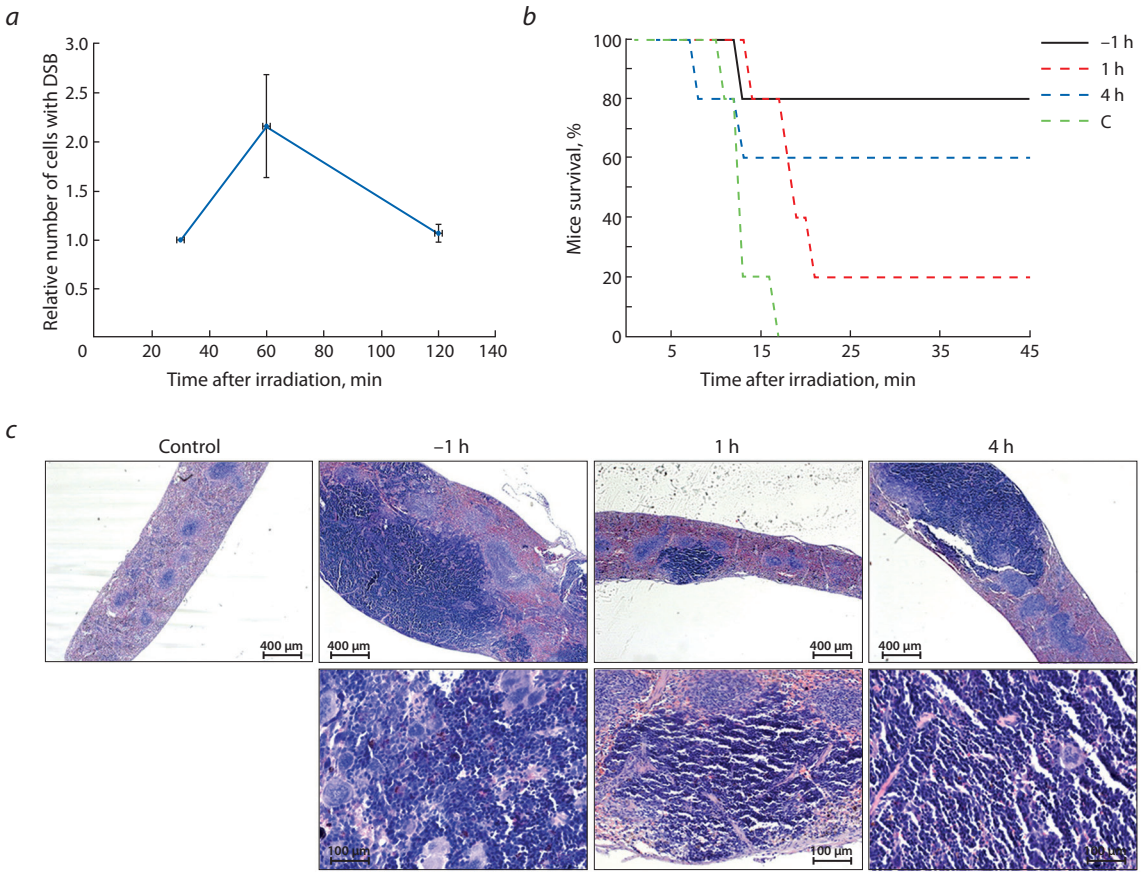

Fig. 2. Analysis of the radioprotective effect of the RNA preparation versus the duration of double-strand break (DSB) repair.

a – the duration of the DSB repair cycle in the bone marrow cells of mice after exposure to the lethal dose of γ-radiation 9.4 Gy. The graph shows the relative number of bone marrow cells in mice in which DSBs are detected; *b* – survival graph of experimental animals treated with the RNA preparation at critical points of the repair process, established in the analysis of the repair cycle. Mice were administered 7 mg of RNA 1 hour before irradiation (–1 h), 1 hour after irradiation (1 h), or 4 hours after irradiation (4 h), C is the control group, not treated with RNA; *c* – histological slides (stained with hematoxylin-eosin) of the spleen of mice on day 11 after irradiation. Control: the spleen is mostly devastated; lymphatic follicles are quite abundant but sharply reduced in size and confined to the peripheral zone of the central artery. –1 h – the major part of the organ parenchyma is occupied by a large aggregate of proliferating lymphocytes. 1 h – one large focus of lymphocytic proliferation is detected with the general decrease in the numbers of red and white pulp cells. 4 h – at least half of the section area is occupied by large foci of proliferation of lymphoid elements.

массой пролиферирующих лимфоцитов. У группы «1 ч», которой препарат РНК ввели через час после облучения, обнаруживается один крупный очаг пролиферации лимфоцитов, занимающий менее 1/10 объема паренхимы. У группы «4 ч», получившей препарат РНК через 4 ч после облучения, по меньшей мере половину объема стромы занимают крупные очаги пролиферации лимфоидных элементов.

Полученный результат говорит об активной пролиферации клеточных элементов в паренхиме селезенок мышей, обработанных до облучения и через 4 ч после экспозиции к γ-лучам.

Сравнение эффективности радиопротекторного действия препарата суммарной РНК дрожжей и штатного радиопротектора Б-190

При сравнении эффективности радиозащитного действия радиопротектора Б-190 и препарата РНК в одном эксперименте (рис. 3, *а*) установлено, что препарат суммарной РНК обладает ярко выраженным радиопротекторным действием, не уступающим по эффективности штатному препарату Б-190. Выживаемость на 40-е сутки после облучения для группы мышей, получавших препарат РНК, составила 78 %, для Б-190 – 67 % животных.

Был проведен патоморфологический анализ селезенок и трех отделов кишечника мышей, взятых из групп суммарной РНК и Б-190 (см. рис. 3, *б*). Ткани и органы забирались на 11-е сутки после проведенного облучения в абсолютно летальной дозе 9.4 Гр. Значимых патоморфологических изменений в эпителии кишечника экспериментальных мышей не обнаружено.

В селезенке мышей контрольной группы наблюдались только отдельные бластные клетки, лежащие небольшими островками среди сохранившихся клеток стромы. У животных обеих опытных групп, суммарной РНК и Б-190, в селезенке отмечено большое количество эритроидных клеток как в просвете сосудов, так и в паренхиме, при этом значительную их часть составляли молодые клетки кроветворной ткани, располагавшиеся в виде различного размера колоний. Таким образом, в опытных группах в

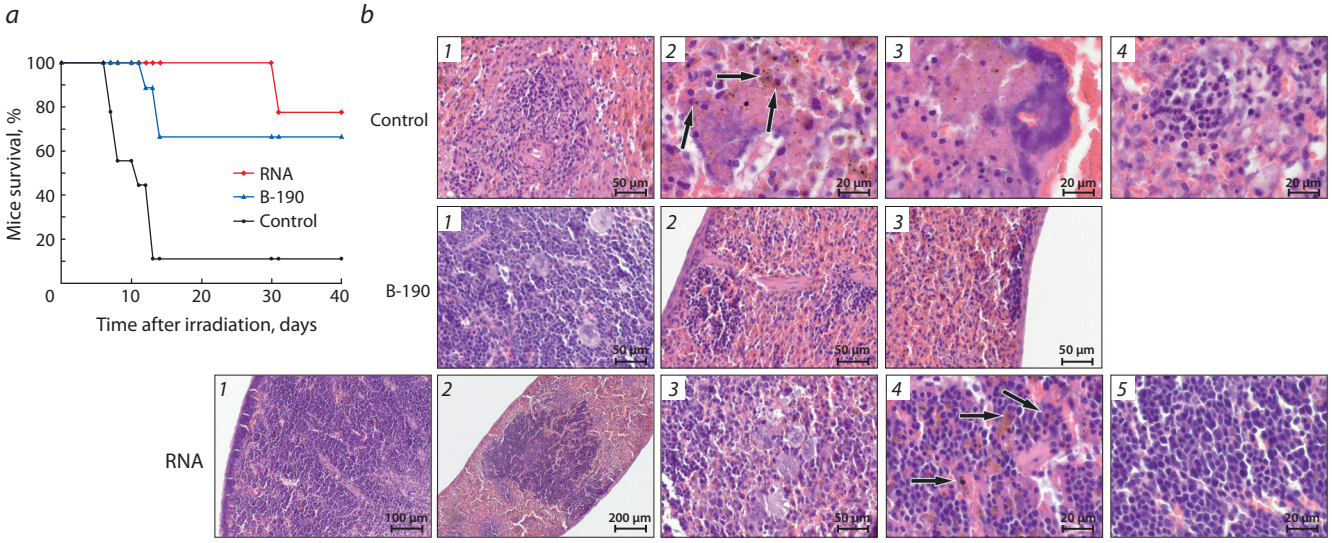

Fig. 3. Comparison of the radioprotective effects of B-190 and the total RNA preparation.

a – survival of animals after irradiation with a dose of 9.4 Gy.
b – light microscopy. Control: *1* – The lymphatic follicle is reduced to the size of the periarterial zone, *2* – numerous siderophages (arrows) against the background of lysed red blood cells, *3* – aggregate of bacterial cells, *4* – hematopoietic islet in the red pulp. B-190: *1* – The spleen parenchyma is densely filled with hematopoietic blast elements, lymphoid follicles are absent, megakaryocytes are present in abundance, *2* – subcapsular location of the hematopoietic islet, *3* – accumulation of blast hematopoietic elements under the spleen capsule. RNA: *1* – Pronounced reduction of white pulp follicles, subcapsular concentration of blast hematopoietic cells in the dense layer along the contour of the left side, *2* – a large aggregate of blast elements in the central part of the parenchyma, *3* – a group of megakaryocytes, *4* – numerous siderophages among blast elements (arrows), *5* – young lymphopoietic cells.

селезенке наблюдалась картина экстрамедуллярного гемопоэза с образованием колоний кроветворных клеток, большинство из которых являлись предшественниками эритропоэза.

В отличие от мышей, профилактически получавших препарат Б-190, в группе животных, пролеченных препаратом суммарной РНК, отмечены выраженная пролиферация клеток лимфоцитарного ростка и отдельные клетки-предшественники или небольшие колонии клеток других ростков гемопоэза. В селезенке мышей, получавших препарат суммарной РНК, присутствовали бластные клетки-предшественники миело- и лимфопоэза, многочисленные мегакариоциты. Предшественники лимфоцитов были преобладающим типом клеток в большей части полей наблюдения при ТЭМ исследовании. Кроме того, среди мезенхимальных клеток идентифицированы мелкие группы клеток-предшественников гранулопоэза. Одновременно наблюдалась стимуляция фагоцитоза клетками стромы и увеличение васкуляризации органа.

Полученные результаты в большей мере предполагают, что два препарата обладают различными механизмами радиозащитного действия. В случае Б-190 защищаются клетки эритроидного ростка кроветворения. В случае препарата РНК и эритроидный, и лимфоидный росток кроветворения сохраняют свой функциональный потенциал.

Радиопротекторное действие двух фракций дрожжевой РНК

Аналитическое исследование препарата суммарной РНК дрожжей свидетельствовало, что в препарате присутствуют две четко разграниченные фракции, одна из которых элюируется с ГАП как одноцепочечная РНК при элюции 0.15 M PBS. Вторая фракция элюируется в условиях, характерных для двуцепочечных нуклеиновых кислот, при 0.25 M PBS. Размер элюирующихся нуклеиновых кислот находился в пределах 50–400 п. н. (рис. 4, *а*, *б*). Проведенные эксперименты по радиопротекции обеих фракций свидетельствовали, что при равных количествах радиопротекторные свойства характерны только для фракции, элюирующейся в 0.25 M PBS. При этом количество вводимого препарата, необходимое для радиопротекторного действия, многократно сокращалось. Если для достижения 80–100 % радиозащитного эффекта требуется 7–10 мг препарата РНК на мышь, то при использовании фракции, элюирующейся в 0.25 M PBS, количество препарата, равное 160 мкг на мышь, полностью защищает животное от абсолютно летальной дозы γ-облучения (см. рис. 4, *в*).

Одним из интригующих вопросов, касающихся фракции дрожжевой РНК, обладающей радиопротекторным действием и элюирующейся в условиях, характерных для двуцепочечных структур ДНК или РНК (0.25 M PBS), был вопрос о типе нуклеиновых кислот этой фракции. В этой связи нами проведены эксперименты по характеристике молекулярного состава данной фракции суммарной РНК дрожжей. Для исследования, как и для экспериментов по радиопротекции, фракционирование нуклеиновых кислот препарата дрожжевой РНК осуществляли методом адсорбционной хроматографии на колонке с ГАП. Определено, что в препарате суммарной РНК присутствует ~1–3 % нуклеиновых кислот в двуцепочечной форме.

Для определения типа нуклеиновых кислот фракций были выполнены различные эксперименты с использованием обработки нуклеазами (ДНКаза I, SI нуклеаза) после

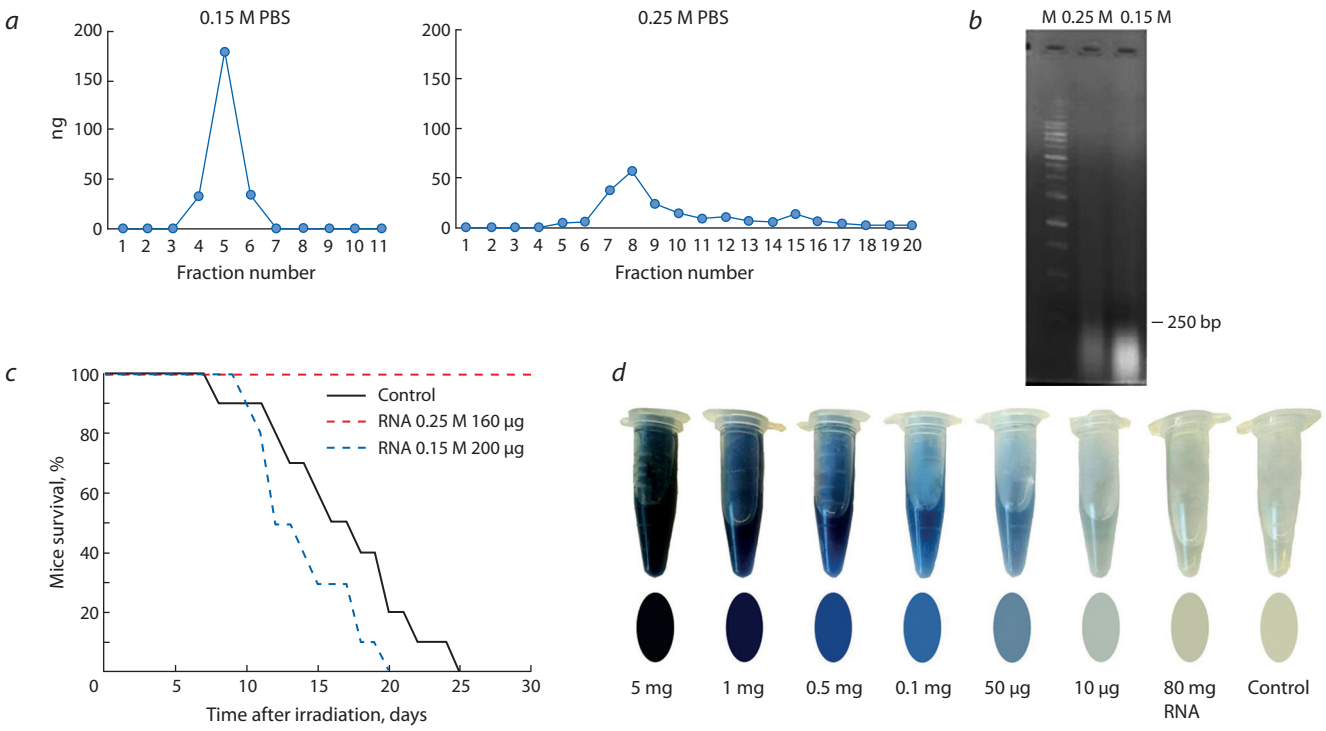

Fig. 4. Identification of the fraction determining the radioprotective effect of total yeast RNA.

a – Chromatography of total yeast RNA on hydroxyapatite. The graphs show the elution profiles of nucleic acids in 0.15 M and 0.25 M PBS; *b* – Electrophoretic analysis of the 0.15 M and 0.25 M nucleic acid fractions in 1% agarose gel, ethidium bromide staining, M, 1-kb molecular weight ladder; *c* – Radioprotective effect of the total RNA preparation fraction obtained by elution from hydroxyapatite with 0.25 M PBS. CBA mice were treated with RNA preparations 40 minutes before irradiation; *d* – Assessment of DNA content in the preparation of total yeast RNA by the Dische assay. The figure shows the results of the color reaction with various amounts (10–5000 μg) of DNA preparations compared to a nucleic acid preparation obtained by hydrolysis of 80 mg of total yeast RNA. The upper part of the figure shows color images of the samples after the Dische reaction. The lower part of the figure shows the colors of the samples obtained in comparison with colors of the Pantone scale. The reaction buffer was used as reference.

денатурации или щелочью, или кипячением, или без таковой, которые не дали однозначно трактуемых результатов. В результате, чтобы установить принадлежность анализируемой фракции к тому или иному типу нуклеиновых кислот, был выбран метод анализа нуклеиновых кислот с использованием дифениламина и специфической цветной реакции на дезоксирибозу. Предполагалось, что если в исходной РНК присутствует ~1–3 % двуцепочечной формы нуклеиновых кислот, то при выделении из большого количества исходного препарата РНК (50–100 мг) будет получена уверенная, однозначно трактуемая цветная реакция. Исходный препарат РНК в количестве 80 мг гидролизовали 24 ч слабой щелочью. После гидролиза проводилось осаждение полимерной формы нуклеиновых кислот. Полученные результаты свидетельствуют, что фракция препарата суммарной РНК, элюирующаяся в 0.25 М фосфатном буфере, является двуцепочечной формой РНК (см. рис. 4, *г*), и, таким образом, можно полагать, что радиопротекторный эффект обусловлен молекулами двуцепочечной РНК.

Анализ нуклеотидных последовательностей фрагментов двуцепочечной РНК фракции 0.25 М

Для понимания происхождения РНК фракции 0.25 М было необходимо определить принадлежность составляющих двуцепочечных РНК фрагментов к генетическому локусу хромосом дрожжей. Фрагменты двуцепочечной РНК фракции, элюирующейся 0.25 М PBS, клонировали и секвенировали. Последовательности были объединены в группы гомологий (рис. 5). Анализ последовательностей секвенированных клонов свидетельствует, что в популяции выделяемых молекул присутствуют различные типы РНК, относящиеся к РНК рибосомального кластера или к транскриптам, кодирующим белки, ассоциированные с рибосомами. Приведенные данные предполагают, что для радиозащитного действия нуклеотидные последовательности фрагментов РНК не имеют значения.

С помощью программы https://eu.idtdna.com/calc/analyzer была проанализирована возможность секвенированных РНК образовывать шпилечные структуры. Для этого были выбраны наиболее протяженные последовательности из каждой группы гомологий. В результате обнаружено, что все проанализированные последовательности могут формировать шпилечные структуры. Для многих вариантов энергия образования шпилек (энергия Гиббса) имеет высокое значение, что предполагает преимущественное формирование таких структур (данные не приводятся).

Обсуждение

В нашей ранней работе (Likhacheva et al., 2007) было показано, что фрагментированная ДНК (препарат «Панаген», ЛСР № 004429/08 от 09.06.2008, ДНК мыши), введенная в организм смертельно облученных мышей, обладает вы-

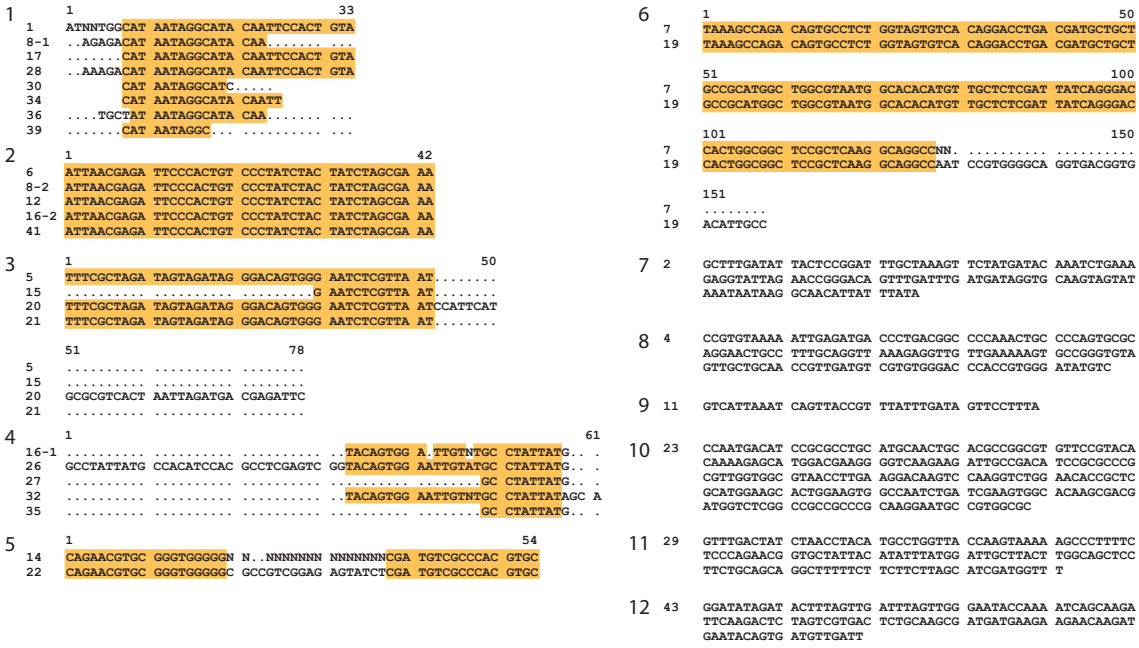

Fig. 5. Determination of the double-stranded RNA structure. Homology groups and species affiliation of sequences: 1, 4–8, 10, 12 – homology groups or species are not defined; 2, 3 – *S. cerevisiae* ribosomal RNA genes RDN25-1 and RDN37-1; 9 – *S. cerevisiae* ribosomal RNA genes RDN18-1 and RDN37-1; 11 – *S. cerevisiae* TMA22 (YJR014W) gene (ribosome-associated protein).

раженным радиопротекторным действием – при ЛД100/30 выживаемость животных составляет 70–90 %. При этом радиопротекторный эффект фрагментов двуцепочечной ДНК коррелирует с развитием селезеночных колоний. Кроме того, при внутривенном введении экстраклеточная двуцепочечная ДНК доставляется в клетки костного мозга, в том числе в CD34+ стволовые гемопоэтические клетки мыши, где может депонироваться и обнаруживается в течение 14 дней после введения (Dolgova et al., 2013a, b). Именно эти два факта легли в основу предположения, что фрагменты двуцепочечной ДНК спасают стволовые гемопоэтические клетки, которые мигрируют на периферию, стабилизируются в селезенке и дают начало новой кроветворной и иммунной системам мышиного организма, разрушенным высокодозовой γ-радиацией.

Поскольку было показано, что двуцепочечная форма нуклеиновых кислот (ДНК) отвечает за радиопротекторный эффект, детектируемый в проведенных экспериментах, нами сделано предположение, что за радиопротекторный эффект препарата суммарной РНК *S. cerevisiae* отвечает присутствующая в нем двуцепочечная фракция нуклеиновых кислот.

Хроматографией на гидроксиапатите была выделена фракция препарата РНК, элюирующаяся с колонки как двуцепочечная форма нуклеиновых кислот. Биологические тесты на радиопротекторные свойства этой фракции однозначно свидетельствовали, что за радиопротекторный эффект препаратов РНК дрожжей отвечает двуцепочечная форма нуклеиновых кислот, составляющая ~1–3 % от суммарной РНК препарата, находящегося в работе. При этом эффективная доза ЛД100/30 для суммарного препарата составляла 7–10 мг/мышь, в то время как для двуцепочечной формы – 160 мкг/мышь, что в ~60 раз меньше. В многократных экспериментах показано, что введение препарата двуцепочечной нуклеиновой кислоты за 60–30 мин до облучения полностью купирует радиационное действие γ-потока. Выживает 80–100 % экспериментальных мышей.

С использованием метода дифференцированного гидролиза щелочью и кислотой и специфического качественного окрашивания на присутствие ДНК было установлено, что двуцепочечная форма, выделяемая в составе препаратов РНК и обладающая радиопротекторными свойствами, представляет собой двуцепочечную РНК. Фрагменты двуцепочечной РНК, переведенные в форму кДНК, были клонированы и секвенированы. Определено, что смесь фрагментов двуцепочечной РНК гетерогенна по первичной структуре и, по-видимому, для осуществления радиопротекторного действия не требуется специфической последовательности.

При анализе радиозащитного действия двуцепочечной РНК установлено, что так же, как и в случае с препаратами двуцепочечной ДНК, в селезенках экспериментальных животных формируются селезеночные колонии. Колонии состоят из пролиферирующих клеточных элементов, которые, как предполагается, представляют собой потомков, спасенных стволовых гемопоэтических предшественников, дающих начало новой кроветворной и иммунной системе, которые были разрушены облучением.

Введение препарата в точку максимально активной репарации ДЦР, идущей по механизму негомологического объединения концов, через 1 ч после получения полной летальной дозы 9.4 Гр (см. рис. 2, *б*) не защищает мышей от гибели от облучения. При этом инъекции препарата через 4 ч после облучения, т. е. тогда, когда активная фаза процесса репарации негомологичного объединения концов завершена, эффективно (до 60 %) спасают мышей от

гибели. У таких животных полностью восстанавливаются кроветворные ростки костного мозга и детектируется выраженное колониеобразование в селезенках.

Предполагается, что фрагменты двуцепочечной РНК, доставленные в клетку в момент идущего репаративного процесса, интерферируют процесс репарации негомологичного объединения концов, причем эта интерференция может быть обусловлена различными механизмами (конкурентное связывание репаративных комплексов, индукция конфликтного репаративного процесса иной природы, блокада квазиматрицей субстратных двуцепочечных концов).

Облучение с указанной дозой 9.4 Гр и мощностью 0.74–1.4 Гр/мин является острым облучением, для которого появление и накопление ДЦР, оцененное методом фокусов к гистону γH2X, происходит к 40–60-й минуте после окончания облучения (Peitzsch et al., 2013; Озеров, Осипов, 2015). Такой результат близок к данным, полученным в настоящем исследовании.

Известно, что при остром облучении помимо простых ДЦР формируются «сложные», образующиеся в результате индукции других повреждений хроматина и активации иных репаративных процессов (Озеров, Осипов, 2015). Авторы цитируемой работы сообщают, что до 20 % ДЦР при γ-облучении относятся к «сложным повреждениям» и репарируются значительно позже, чем ДЦР, индуцированные непосредственным разрывом хроматина. Возможно, обнаруженный терапевтический эффект связан с репарацией хроматина в стволовых клетках костного мозга по типу гомологичной рекомбинации с участием внешней РНК матрицы. Этот тип репарации активируется значительно позже по сравнению с аварийным негомологичным объединением концов. Тот факт, что на графиках, полученных при оценке числа ДЦР, показатели в последней анализируемой точке (2 ч) ни в одном из приведенных экспериментов не опускались до значения исходной нулевой отметки, согласуется с высказанным выше предположением.

В литературе известны варианты репаративных процессов с использованием РНК и ДНК матрицы. Для РНК описаны модели, в которых основным лейтмотивом является построение кДНК копии и вовлечение двуцепочечной формы этой нуклеиновой кислоты в репаративный процесс (Storici et al., 2007; Meers et al., 2016). Для двуцепочечной ДНК также известны различные модели репарации с привлечением внешней двуцепочечной матрицы (Leung et al., 1997; Bärtsch et al., 2000; Li et al., 2001; Symington, 2005). Характерным для участия таких нуклеиновых кислот в идущем репаративном процессе является внедрение процессированного 3′OH конца разорванного хроматина между цепей внешней матрицы и формирование интермедиата репарации. Далее могут осуществляться различные описанные варианты достраивания цепей и восстановления целостности хроматина. Можно предположить, что репарация в присутствии экстраклеточных двуцепочечных РНК идет именно по такому общему молекулярному сценарию. О важной роли двуцепочечной формы нуклеиновых кислот при осуществлении репаративного процесса свидетельствуют данные, полученные в работе (Storici et al., 2007), где показано, что дуплекс РНК/ДНК повышает эффективность репарации по сравнению с одноцепочечной РНК на два-три порядка.

Полное отсутствие радиозащитного действия у препарата одноцепочечной РНК в дозах, сопоставимых с радиопротекторными дозами двуцепочечной РНК, предполагает, что радиопротекторное действие двуцепочечной РНК связано с появившейся в клеточном пространстве стволовых гемопоэтических клеток внехромосомной двуцепочечной матрицы. Внедрение между цепями такой двуцепочечной РНК матрицы процессированного филамента ДНК ДЦР может быть главным событием, определяющим дальнейшие фазы репарации фатального повреждения, индуцированного γ-радиацией.

Заключение

Таким образом, данные молекулярно-биологических исследований, экспериментов с использованием клеточных технологий и биологические тесты свидетельствуют, что субстанцией, определяющей радиопротекторное действие фракции «0.25 М» РНК дрожжей *S. cerevisiae*, является двуцепочечная форма молекул РНК.

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ORCID ID

G.S. Ritter orcid.org/0000-0003-1573-3795
A.S. Proskurina orcid.org/0000-0002-7650-4331
O.S. Taranov orcid.org/0000-0002-6746-8092
T.D. Dubatolova orcid.org/0000-0003-2807-774X
E.V. Dolgova orcid.org/0000-0002-5543-248X
E.A. Potter orcid.org/0000-0003-1558-8655
S.S. Kirikovich orcid.org/0000-0002-3426-4501
Ya.R. Efremov orcid.org/0000-0002-0649-7543
M.V. Romanenko orcid.org/0000-0003-2184-8919
M.I. Meschaninova orcid.org/0000-0003-0045-2413
A.G. Venyaminova orcid.org/0000-0002-7858-6932
N.A. Kolchanov orcid.org/0000-0001-6800-8787
S.S. Bogachev orcid.org/0000-0002-2019-9382

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Phosphate-modified CpG oligonucleotides induce *in vitro* maturation of human myeloid dendritic cells

A.A. Ostanin[1] ✉, O.Y. Leplina[1], E.A. Burakova[2, 3], T.V. Tyrinova[1, 2], A.A. Fokina[2, 3], A.S. Proskurina[3], S.S. Bogachev[3], D.A. Stetsenko[2, 3], E.R. Chernykh[1]

[1] Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
[3] Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
✉ e-mail: ostanin62@mail.ru; ct_lab@mail.ru

Abstract. Myeloid dendritic cells (DCs) play an important role in the immune response; therefore, the search for compounds that can effectively activate DCs is a needful goal. This study was aimed to investigate the effect of synthetic CpG oligodeoxynucleotides (CpG-ODN) on the maturation and allostimulatory activity of myeloid DCs in comparison with other PAMP and DAMP molecules. For the research, we synthesized known CpG-ODN class C (SD-101 and D-SL03) containing thiophosphate internucleotide groups, and their original phosphate-modified analogues (SD-101M and D-SL03M) with mesylphosphoramide internucleotide groups (M = μ-modification). The effects of CpG-ODN and other activators were evaluated on DCs generated from blood monocytes in the presence of GM-CSF and IFN-α (IFN-DC) or IL-4 (IL4-DC). Evaluation of the intracellular TLR-9 expression showed that both types of DCs (IFN-DC and IL4-DC) contained on average 52 and 80 % of TLR-9-positive cells, respectively. The CpG-ODNs studied enhanced the allostimulatory activity of IFN-DCs, and the effect of μ-modified CpG-ODNs was higher than that of CpG-ODNs with thiophosphate groups. The stimulating effect of CpG-ODN at a dose of 1.0 μg/ml was comparable (for D-SL03, D-SL03M, SD-101) with or exceeded (for SD-101M) the effect of LPS at a dose of 10 μg/ml. At the same time, IFN-DCs were characterized by greater sensitivity to the action of CpG-ODNs than IL4-DCs. The enhancement of DC allostimulatory activity in the presence of CpG-ODNs was associated with the induction of final DC maturation, which was confirmed by a significant decrease in the number of $CD14^+$DC, an increase in mature $CD83^+$DC and a trend towards an increase in $CD86^+$DC. Interestingly, the characteristic ability of LPS to enhance the expression of the co-stimulatory molecule OX40L on DCs was revealed only for the μ-analogue SD-101M. In addition, CpG-ODNs (SD-101 and SD-101M) had a stimulatory effect on IFN-γ production comparable to the action of LPS. The data obtained indicate a stimulating effect of CpG-ODN on the maturation and allostimulatory activity of human myeloid DCs, which is more pronounced for μ-modified analogs.
Key words: monocytes; dendritic cells; differentiation; maturation; PAMP- and DAMP-activators; allo-MLR; CpG-oligonucleotide.

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CpG олигонуклеотиды с модифицированными фосфатными группами индуцируют созревание миелоидных дендритных клеток человека *in vitro*

А.А. Останин[1] ✉, О.Ю. Леплина[1], Е.А. Буракова[2, 3], Т.В. Тыринова[1, 2], А.А. Фокина[2, 3], А.С. Проскурина[3], С.С. Богачев[3], Д.А. Стеценко[2, 3], Е.Р. Черных[1]

[1] Научно-исследовательский институт фундаментальной и клинической иммунологии, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
[3] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
✉ e-mail: ostanin62@mail.ru; ct_lab@mail.ru

Аннотация. Миелоидные дендритные клетки (ДК) играют важную роль в иммунном ответе, поэтому актуальной задачей является поиск соединений, способных эффективно активировать ДК. Целью настоящей работы было изучение влияния синтетических CpG олигодезоксинуклеотидов (CpG-ODN) на созревание и аллостимуляторную активность миелоидных ДК в сравнении с другими PAMP и DAMP молекулами. Для исследований были синтезированы CpG-ODN класса С (SD-101 и D-SL03), содержащие тиофосфатные межнуклеотидные группы, а также получены их оригинальные фосфат-модифицированные аналоги (SD-101M и D-SL03M) с мезилфосфорамидными межнуклеотидными группами (M = μ-модификация). Эффекты CpG-ODN и других активаторов оценивали в культурах ДК, генерированных из моноцитов крови в присутствии GM-CSF и IFN-α (IFN-ДК) или IL-4 (IL4-ДК).

First three authors (AAO, OYL and EAB) contributed equally to this work.

Оценка внутриклеточной экспрессии TLR-9 показала, что оба типа ДК (IFN-ДК и IL4-ДК) содержали в среднем 52 и 80 % TLR-9-позитивных клеток соответственно. Исследуемые CpG-ODN усиливали аллостимуляторную активность IFN-ДК, причем эффект μ-модифицированных CpG-ODN был выше, чем тиофосфатных CpG-ODN. Стимулирующий эффект CpG-ODN в дозе 1.0 мкг/мл был сопоставим (для D-SL03, D-SL03M, SD-101) или превышал (для SD-101M) действие липополисахарида (LPS) в дозе 10 мкг/мл. При этом IFN-ДК характеризовались большей чувствительностью к действию CpG-ODN, чем IL4-ДК. Усиление аллостимуляторной активности ДК в присутствии CpG-ODN было связано с индукцией конечного созревания клеток, что подтверждалось значимым снижением количества $CD14^+$ ДК, увеличением доли зрелых $CD83^+$ ДК и тенденцией к возрастанию $CD86^+$ ДК. Интересно, что характерная для LPS способность усиливать экспрессию костимуляторной молекулы OX40L на ДК была выявлена только для μ-аналога SD-101M. Кроме того, CpG-ODN (SD-101 и SD-101M) оказывали стимулирующий эффект на продукцию IFN-γ, сопоставимый с действием LPS. Полученные в целом данные свидетельствуют о стимулирующем действии CpG-ODN на созревание и аллостимуляторную активность миелоидных ДК человека, которое более выражено для μ-модифицированных аналогов.
Ключевые слова: моноциты; дендритные клетки; дифференцировка; созревание; PAMP- и DAMP-активаторы; алло-СКЛ; CpG-олигонуклеотид.

Introduction

Dendritic cells (DC) play an important role in immune responses, thus justifying their application as cellular targets and potential cellular modality for developing novel anti-cancer immunotherapies. Taking into account that only mature DC with high antigen-presenting and co-stimulatory molecule expression possess immunostimulatory activity (Banchereau et al., 2000), R&D efforts toward discovery of novel molecular candidates capable of effectively activating DC and induce their maturation are clearly very topical.

Pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) released upon autologous cell damage belong to natural DC activators. PAMP-dependent effects are mediated via pattern-recognition receptors, while DAMP molecules are recognised by intracellular sensors and activate DC via secondary messengers, such as tumour-necrosis factor α (TNF-α) (Jounai et al., 2013; Kawasaki, Kawai, 2014). The effects of various compounds on human DC are usually assessed *in vitro* in monocyte-derived DC cell cultures generated in the presence of GM-CSF/IL4 or GM-CSF/IFN-α (Cehim, Chies, 2019) cytokine combinations. In these settings, a Toll-like receptor 4 (TLR-4)-specific ligand, bacterial lipopolysaccharide (LPS), serves as a standard cell activator. However, LPS-associated pyrogenicity effectively prevents its clinical application.

Bacterial and viral DNA species are also capable of activating terminal DC maturation. This activity is accounted for by the presence of unmethylated CpG-dinucleotides in their structure, which could be imitated by synthetic CpG oligodeoxynucleotides (CpG-ODN) that mediate downstream signalling via TLR-9 (Polovinkina, Markov, 2010). Synthetic CpG-ODN derivatives have demonstrated pronounced immunostimulatory and anti-cancer effects *in vivo,* and therefore these compounds are currently considered as perspective adjuvants in anti-cancer immunotherapy (Scheiermann, Klinman, 2014; Shirota, Klinman, 2014; Shirota et al., 2015). CpG-ODNs along with plasmacytoid DC have been shown to exert stimulatory effects on bone marrow-derived DC in murine experimental systems (Behboudi et al., 2000). Meanwhile, CpG-ODN-dependent sensitivity in humans is attributed mainly to plasmacytoid DC, while CpG-ODN-mediated effects on myeloid DC were addressed in just a few studies that produced rather contradictory results (Behboudi et al., 2000; Hoene et al., 2006).

This study aimed to assess the effects of CpG-ODN on maturation and allostimulatory activity of myeloid DC generated from blood monocytes in the presence of GM-CSF and IFN-α (IFN-DC) or IL-4 (IL4-DC) in comparison with other PAMP (LPS) and DAMP activators. Specifically, we assessed the effects of CpG-ODN class C derivatives SD-101 (Levy et al., 2016) and D-SL03 (Yang et al., 2013) with thiophosphate internucleotide groups, as well as original phosphate-modified analogues (SD-101M, D-SL03M) with mesyl-phosphoramidate internucleotide groups (M = μ-modification) (Chelobanov et al., 2017).

In addition, we analysed the effects of DAMP activators: double-stranded DNA (dsDNA) and a synthetic polycationic adjuvant azoximer bromide (AB) (Kabanov, 2004; Powell et al., 2015), which was shown to enhance antigen-presenting DC function via activating pro-inflammatory signalling pathways (Dyakonova et al., 2004).

Materials and methods

In this study the following compounds were synthesised, purified and characterised: CpG-ODN class C: SD-101 and D-SL03 containing thiophosphate (phosphorothioate) internucleotide groups, and original modified analogues (SD-101M, D-SL03M) with mesyl-phosphoramidate (μ) internucleotide linkages. CpG-ODN sequences used in this study are shown in Table 1.

DC were obtained from peripheral blood mononuclear cells (PBMC) isolated from heparinised venous blood of healthy donors using Ficoll-Verografin gradient centrifugation. IFN-DC were generated by cultivating an adherent PBMC fraction in Falcon (BD Biosciences, UK) flasks in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.3 mg/ml L-glutamine, 5 mM HEPES-buffer, 100 μg/ml gentamycin and 5 % foetal bovine serum (FBS) (BioloT, St. Petersburg, Russia) in the presence of GM-CSF (Sigma-Aldrich, 40 ng/ml) and IFN-alpha (1000 U/ml, Roferon®-A, Hoffmann-La Roche Ltd, Basel, Switzerland,) for 3 days with a subsequent maturation step in the presence of LPS (LPS *E. coli* 0114:B4, Sigma-Aldrich, 10 μg/ml) for 48 h.

IL4-DC were generated from an adherent PBMC fraction after incubation in full culture medium in the presence of GM-CSF (40 ng/ml, Sigma-Aldrich), IL-4 (40 ng/ml, Sigma-Aldrich) and 5 % FBS for 5 days followed by an additional maturation step in the presence of LPS for 48 h. In addition to

Table 1. List of CpG-oligonucleotides (CpG-ODN)

Name	Sequence (5′–3′)	Size (bp)	Internucleotide group	CpG (yes/no)
SD-101	t**cg**aa**cg**tt**cg**aa**cg**tt**cg**aa**c:g**tt**cg**aat	30	PS	CpG
SD-101M	t**cg**aa**cg**tt**cg**aa**cg**tt**cg**aa**c:g**tt**cg**aat	30	μ	CpG
D-SL03	t**cgcg**aa**cg**tt**cg**c**cgcg**tt**c:g**aa**cgcg**g	29	PS	CpG
D-SL03M	t**cgcg**aa**cg**tt**cg**c**cgcg**tt**c:g**aa**cgcg**g	29	μ	CpG
ODNcontrol	tgcaagcttgcaagcttgcaag:cttgcaat	30	μ	no

Note. CpG dinucleotides are shown in bold; palindromic sequences are underlined; a middle of the palindromic sequence is indicated with the colon. PS – thiophosphate group; μ – mesylphosphoramide group.

different concentrations of CpG-ODNs, terminal DC maturation was also achieved by incubation with other activators azoximer bromide (AB, NPO Petrovaks Farm, Moscow, Russia) at 2 ng/ml, and double-stranded DNA (dsDNA) at 5 μg/ml.

Intracellular TLR-9 expression in immature DC was assessed in IFN-DC and IL4-DC populations on days 3 and 5, respectively. To this end, cells were permeabilised using a commercially available Fixation/Permeabilization Solution Kit (BD Cytofix/Cytoperm™, San Jose, CA, USA), according to the manufacturer's instructions. Cells were stained with allophycocyanin (APC)-labelled anti-TLR-9 antibodies (BD PharMingen, San Jose, CA, USA). Matching isotype antibodies labelled with an appropriate fluorochrome were used as negative controls. Percentages of TLR-9-positive DC were calculated based on 10000 events collected during flow cytometric analysis for each sample.

Stimulatory DC activity was assessed in allogeneic mixed leukocyte reactions (allo-MLR) using donor PBMC as responder cells cultivated in round-bottomed 96-well plates (0.1×10^6/well) in RPMI-1640 medium containing 10 % inactivated AB (group IV) donor serum at 37 °C in a CO_2-incubator. DC used as stimulator cells were added at a ratio of PBMC : DC = 10 : 1. To assess proliferation, cells were incubated for 4 days, followed by pulse-labelling with 1.0 μCi/well of [3H]thymidine for the last 18 h.

To perform immunophenotyping of IFN-DC, cells were stained with phycoerythrin (PE)-labelled anti-CD14 (Sorbent, Moscow) or anti-OX40L (anti-CD252, BioLegend, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-labelled anti-CD83, anti-CD86 (BD PharMingen) and anti-HLA-DR (Sorbent), and analysed by flow cytometry (FACSCalibur, Becton Dickinson).

Cytokine (TNF-α, IFN-γ) concentrations were measured in 5-day culture supernatants of IFN-DC by ELISA using commercially available kits (Vector-Best, Novosibirsk), according to the manufacturer's instructions.

Statistical analysis was performed using an analytics software portfolio Statistica 6.0 for Windows (StatSoft, USA). Data is presented as Median (Me) with the interquartile range (IQR, 25–75 % quartiles). Related samples were compared using a nonparametric paired difference test (Wilcoxon signed-rank test), and independent samples were analysed using Mann–Whitney U test; $p < 0.05$ was considered statistically significant.

Results

The assessment of intracellular TLR-9 expression in freshly isolated blood monocytes derived from healthy donors and immature IFN-DC/IL4-DC generated after 3- and 5-days culture, respectively, showed that the proportion of TLR-9-positive cells in monocyte precursors and IL4-DC was at about 80 % level (Fig. 1). In contrast, relative content of TLR-9$^+$ cells in IFN-DC population was significantly lower ranging from 40 to 56 % (Me 52.5 %, $p < 0.05$). Nevertheless, the data obtained implies potential sensitivity of DC generated both in the presence of IL-4 and IFN-alpha to the stimulatory effects of CpG-ODN delivering maturation signals.

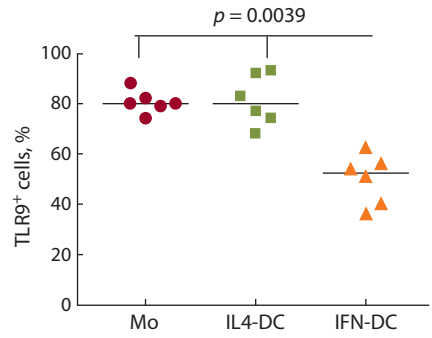

Fig. 1. Intracellular TLR-9 expression in monocytes and immature IL4-DC and IFN-DC of healthy donors ($n = 6$).

The data are presented as individual and median values; p – Mann–Whitney U-test.

Therefore, in our next set of experiments we applied dilution series (from 0.5 to 5.0 μg/ml) of CpG-ODN derivatives synthesised here to assess their effect on IFN-DC-mediated ability to stimulate T cell proliferative responses in allo-MLR. Indeed, allostimulatory DC activity is a distinct integral marker of an overall DC activity, being associated with the degree of DC maturation, HLA/co-stimulatory molecule expression, as well as the spectrum and levels of cytokines produced by DC. A "classical" PAMP-activator LPS (at 10 μg/ml) was used as a positive control, while ODN with μ-modifications, but lacking CpG-dinucleotides (at 1 μg/ml), was used as a negative control. Table 2 shows that LPS treatment caused nearly a 3-fold enhancement of allostimulatory DC activity. All CpG-ODN tested induced IFN-DC maturation, which manifested itself in a statistically significant enhancement of allostimulatory DC activity. In control experiments,

Table 2. T cell proliferative response in allo-MLR and allostimulatory activity (FI) of IFN-DC generated with CpG-ODNs

Types of allo-MLR		Control	CpG-ODN			
			0.5 μg/mL	1.0 μg/mL	2.5 μg/mL	5.0 μg/mL
PBMC + immature DC	cpm	2310 (1630–2600)				
PBMC + $DC_{ODNcontrol}$	cpm	2350 (2100–2500)				
	FI	1.03 (0.99–1.39)				
PBMC + DC_{LPS}	cpm	7730 (5630–9620)				
	FI	2.9 (2.5–4.6)				
PBMC + DC_{SD-101}	cpm		5520 (4650–11 890)	6550 (3510–9650)	7520 (4950–10 380)	6960 (5830–10 130)
	FI		3.0 (2.4–5.0)	3.1 (1.8–4.6)	3.6 (3.1–4.9)	3.0 (2.6–4.8)
PBMC +$DC_{SD-101M}$	cpm		7680 (6450–14 170)	9030 (8010–12 100)	7520 (6030–13 710)	6530 (6030–13 700)
	FI		4.6 (3.0–6.7)**	5.0 (3.7–6.0)**	4.5 (2.8–6.5)*	4.2 (2.7–6.1)
PBMC + DC_{D-SL03}	cpm		3060 (2290–10 080)	4660 (3550–12 960)	4260 (3200–11 140)	5180 (3210–11 460)
	FI		1.6 (1.2–4.8)	2.4 (1.5–5.3)	2.4 (1.5–5.3)	2.5 (2.0–4.8)
PBMC + $DC_{D-SL03M}$	cpm		5930 (3380–11 420)	7210 (5080–16 060)	6670 (4700–17 100)	6000 (3760–14 150)
	FI		2.5 (1.6–5.4)**	4.0 (2.1–6.7)**	3.7 (2.0–7.7)**	2.7 (2.1–6.1)

Note. The data of two independent experiments (n = 8) are presented as median and interquartile range (in brackets); cpm – count per minute; FI – index of influence. * $p < 0.05$; ** $p < 0.01$ – significant difference between μ-CpG-ODN and PS-CpG-ODN (Wilcoxon's W test).

Table 3. T cell proliferative response in allo-MLR (cpm) and allostimulatory activity (FI) of IFN-DC and IL4-DC generated with different stimuli

Types of allo-MLR		IFN-DC	IL4-DC
PBMC + immature DC	cpm	2250 (1900–2600)	2720 (2180–3080)
PBMC + DC_{dsDNA}	cpm	5710 (4810–8510)	6070 (4950–7100)
	FI	2.6 (2.3–3.1)	2.4 (2.0–2.5)
PBMC + DC_{AB}	cpm	7350 (6040–8160)	4410 (3880–5410)
	FI	2.8 (2.4–3.7)	1.7 (1.5–2.0)
PBMC + $DC_{SD-101M}$	cpm	7900 (6090–9510)	7200 (5460–8470)
	FI	3.3 (2.9–5.0)	2.6 (2.5–2.8)
PBMC + $DC_{D-SL03M}$	cpm	7050 (5590–10 560)	5540 (4420–7650)
	FI	3.2 (2.6–4.6)	2.0 (1.8–2.7)

Note. The data of two independent experiments (n = 8) are presented as median and interquartile range (in brackets); cpm – count per minute; FI – index of influence. Stimuli: dsDNA 5 μg/ml; AB 2 ng/ml; SD-101M and D-SL03M 1 μg/ml.

ODN lacking CpG motif did not affect functional DC activity in allo-MLR [fold increase (FI) = 1.03; IQR 0.99–1.39].

Characteristically, treatment with CpG-ODN containing mesyl-phosphoramidate (μ-) groups (SD-101M and D-SL03M) at 0.5, 1.0 and 2.5 μg/ml was significantly more effective ($p < 0.01$) in inducing terminal IFN-DC maturation, as compared to analogous CpG-ODN that contained thiophosphate groups. Notably, SD-101M exerted the most pronounced effect in these experiments. Thus, SD-101M treatment at the minimal dose tested (0.5 μg/ml) induced clear DC maturation to a degree comparable to that observed in the presence of LPS at 10 μg/ml, while the effectiveness of μ-analogue SD-101 at 1.0 μg/ml was significantly higher than LPS ($p < 0.05$).

Next, we performed comparative studies of the effect of μ-analogues CpG-ODN (SD-101M and D-SL03M) and DAMP activators (dsDNA, AB) on allostimulatory activity of two DC subsets (IFN-DC and IL4-DC). Table 3 shows that stimulatory effect of CpG-ODN μ-analogues at 1 μg/ml on functional activity of IFN-DC and IL4-DC in allo-MLR was comparable to that displayed by dsDNA and AB. Furthermore, as compared to IL4-DC, IFN-DC were characterised by higher sensitivity to the chemical compounds studied here, such that all subsequent experiments with CpG-ODN were conducted using IFN-DC cell cultures.

To ascertain that the enhancement of allostimulatory DC activity was indeed attributable to terminal DC maturation,

Table 4. Phenotype of IFN-DCs generated with different stimuli

IFN-DC cultures	$CD14^+$, %	$CD83^+$, %	$CD86^+$, %	$HLA\text{-}DR^+$, %	$HLA\text{-}DR^+OX40L^+$, %
DC_0 (control)	41.5 (38–47)	11.5 (9 –16)	72.0 (44–87)	83.5 (76–87)	8.0 (5.5–17.0)
DC_{LPS}	31.5 (26–37) $\downarrow^{**}$	33.0 (16–44) $\uparrow^{*}$	80.5 (77–91)	86.5 (81–90)	15.0 (7.8–24.0) $\uparrow^{*}$
DC_{dsDNA}	30.0 (27–33) $\downarrow^{**}$	20.0 (11–38)	84.0 (81–88)	81.5 (79–91)	9.3 (5.4–24.0)
DC_{AB}	30.5 (23–34) $\downarrow^{**}$	22.5 (12–43) $\uparrow^{*}$	81.0 (79–85)	83.0 (76–88)	9.0 (6.3–20.0)
$DC_{SD\text{-}101}$	37.0 (32–38)	20.0 (14–35) $\uparrow^{*}$	82.5 (77–86)	81.0 (69–92)	7.0 (6.0–27.0)
$DC_{SD\text{-}101M}$	33.5 (32–36) $\downarrow^{**}$	18.5 (16–42) $\uparrow^{*}$	79.0 (67–81)	82.0 (76–91)	12.5 (7.3–27.0) $\uparrow^{*}$

Note. The data of three independent experiments (n = 7; % of positive IFN-DC) are presented as median and interquartile range (in brackets); Stimuli: LPS 10 μg/ml; dsDNA 5 μg/ml; AB 2 ng/ml; SD-101 and SD-101M 1 μg/ml. * $p < 0.05$; ** $p < 0.01$ – vs control (Wilcoxon's W test).

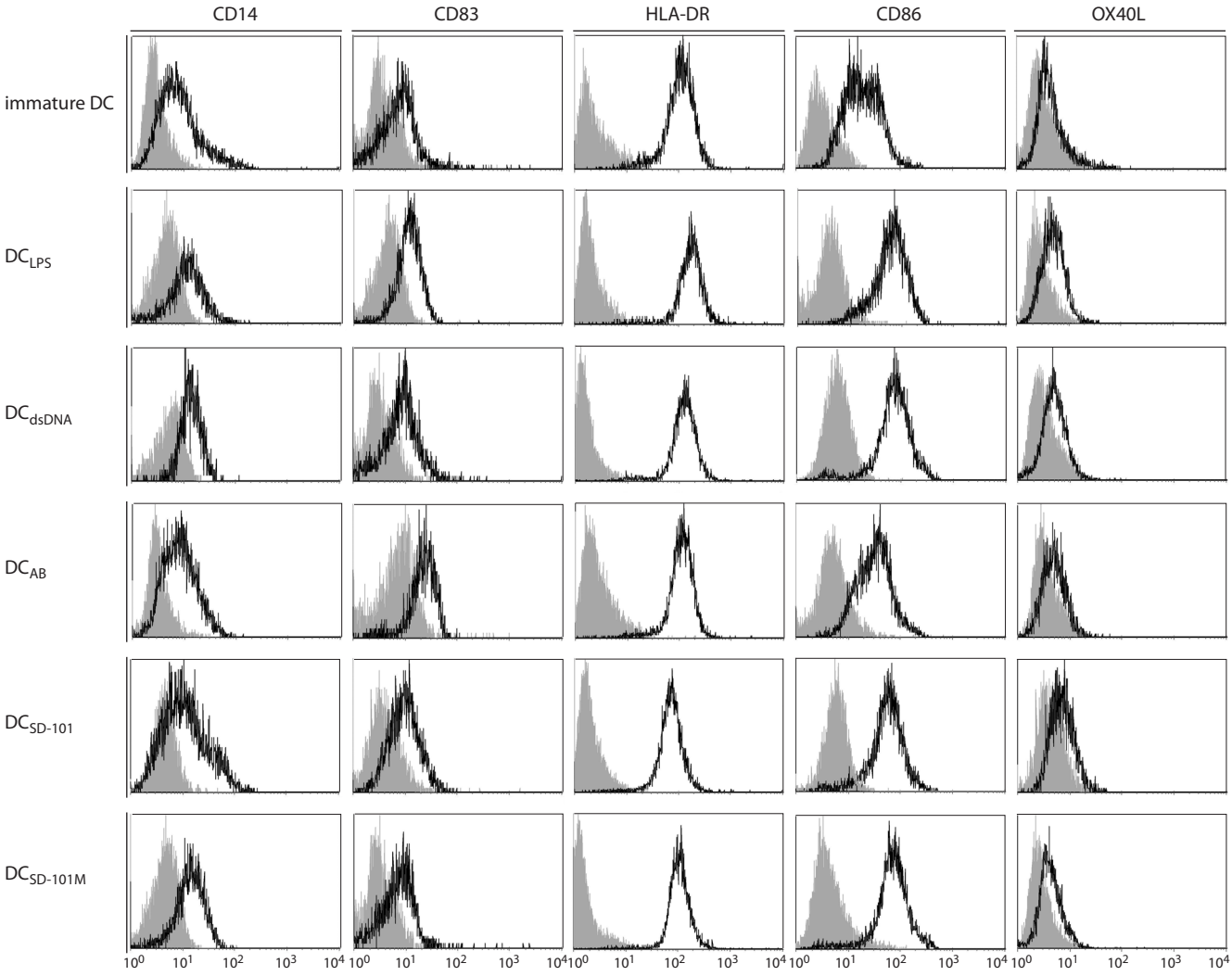

Fig. 2. Phenotypic analysis of IFN-DC generated *in vitro* with different stimuli.

Immature IFN-DC were cultured with different stimuli for 24 h followed by flow cytometry analysis of surface molecules CD14, CD83, HLA-DR, CD86, OX40L. Figures show flow cytometry histograms representing the expression of studied markers (bold-line histograms) and matched isotype controls (gray-filled histograms).

we studied immunophenotypic changes occurring in IFN-DC cultivated in the presence of CpG-ODN, as compared to other PAMP and DAMP activators. In this series of experiments, we selected CpG-ODN SD-101 and its μ-analogue SD-101M that showed most pronounced stimulatory activity in previous experiments. Table 4 and Figure 2 indicate that CpG-ODN derivatives studied exerted a clear maturation effect on DC analogous to that observed in the presence of LPS. This maturation effect manifested itself in: (i) reduced percentages of $CD14^+$ monocyte precursors, (ii) increased proportion of

Table 5. TNF-α and IFN-γ concentration in IFN-DC cultures generated with different stimuli

IFN-DC cultures		TNF-α	IFN-γ
Immature DC (control)	pg/mL	290 (206–500)	130 (90–190)
DC_{LPS}	pg/mL	1680 (840–1790)**	225 (145–385)**
	FI	3.7 (1.4–8.4)	1.6 (1.3–2.0)
DC_{dsDNA}	pg/mL	785 (264–1250)	250 (190–400)*
	FI	1.4 (0.9–2.7)	1.4 (1.1–2.0)
DC_{AB}	pg/mL	240 (145–670)	240 (185–460)*
	FI	0.9 (0.8–1.1)	1.2 (1.1–1.9)
DC_{SD-101}	pg/mL	220 (70–490)	230 (160–460)*
	FI	0.7 (0.3–1.9)	1.71 (1.5–2.1)
$DC_{SD-101M}$	pg/mL	215 (120–740)	230 (180–380)*
	FI	1.0 (0.6–1.9)	1.74 (1.3–2.1)

Note. The data of six independent experiments (n = 13) are presented as median and interquartile range (in brackets); * $p < 0.05$; ** $p < 0.01$ – vs control (Wilcoxon's W test).

mature $CD83^+$DC, and (iii) a clear upward trend for DC expressing a co-stimulatory molecule CD86 (p = 0.07–0.11). Similar effects were documented in the presence of dsDNA and AB. Interestingly, LPS treatment also significantly enhanced percentages of OX40L-positive DC from 8 to 15 %, which corresponds to 45 % cumulative gain in this particular cell population. The effect of CpG-ODN containing mesyl-phosphoramidate internucleotide linkages (SD-101M) on OX40L expression was comparable to that observed in the presence of LPS, with percentages of $OX40L^+$DC increasing by 39 % (up to 12.5 %, p = 0.0282). Meanwhile, the effects of dsDNA, AB and SD-101 with thiophosphate internucleotide groups on OX40L expression were less pronounced and did not reach statistically significant levels.

In the last experiments we assessed the effects of CpG-ODN SD-101 derivative and its μ-analogue SD-101M on TNF-α and IFN-γ production in 5-day IFN-DC cultures (Table 5) because DC maturation is known to be accompanied by the enhancement of cytokines with pro-inflammatory and Th1-stimulatory activity. As compared to immature cells, LPS-activated DC produced higher levels of TNF-α and IFN-γ ($p < 0.01$). CpG-ODN derivatives SD-101 and SD-101M, as well as AB did not affect TNF-α production, while dsDNA treatment increased TNF-α concentrations in DC cultures by 43 %, although this effect was not statistically significant.

Interestingly, SD-101 and SD-101M significantly enhanced the ability of IFN-DC to produce IFN-γ by 71 and 74 %, respectively ($p < 0.05$), which was commensurate with LPS-mediated effects. Other DAMP activators (dsDNA and AB) also enhanced IFN-γ production by DC.

Discussion

Data obtained in this study demonstrated intracellular TLR-9 expression in monocyte-derived IFN-DC or IL4-DC. Both DC populations studied here were characterised by sensitivity to CpG-ODN class C derivatives, which enhanced DC-dependent ability to stimulate T cell proliferation in allo-MLR via up-regulation of CD83 differentiation antigen and OX40L/CD86 co-stimulatory molecule expression, as well as increased IFN-γ production by DC. Interestingly, IFN-DC possessed higher sensitivity to the stimulatory effects of CpG-ODN, as compared to IL4-DC.

TLR-9 expression and CpG-ODN sensitivity have been shown to be characteristic of both plasmacytoid and myeloid DC in murine experimental systems (Behboudi et al., 2000; Iwasaki, Medzhitov, 2004). Early human studies were based on RT-PCR analysis and demonstrated constitutive TLR-9 mRNA expression only in plasmacytoid (but not myeloid or monocyte-derived) DC (Bauer et al., 2001; Krug et al., 2001; Rothenfusser et al., 2002). Nevertheless, in later studies H. Tada et al. discovered TLR-9 mRNA in monocyte-derived DC (mo-DC) and demonstrated enhancement of IL-12p70 and IFN-γ production in mo-DC cell cultures in response to CpG-ODN stimulation (Tada et al., 2005). In line with these observations, V. Hoene et al. identified TLR-9 protein in mo-DC, which notably was present in the same amounts as in plasmacytoid DC. These authors showed that CpG-ODN class A derivatives stimulated DC maturation and enhanced the ability of DC to stimulate proliferation of allogeneic T cells (Hoene et al., 2006). In this respect, our results constitute another confirmation of human myeloid DC sensitivity to the stimulatory effects exerted by CpG-ODN. In view of the fact that the majority of DC present in tumour microenvironment are of monocyte origin (Veglia, Gabrilovich, 2017), our results imply high clinical potential for CpG-ODN in DC activation protocols, such as those employed in anti-cancer immunotherapy.

Importantly, our study was also first to compare classical thiophosphate CpG-ODN class C derivatives (SD-101 and D-SL03) with their original analogues containing mesyl-phosphoramidate (μ-modified) internucleotide groups (SD-101M and D-SL03M). CpG-ODN class C derivatives combine immunomodulating properties characteristic of CpG-ODN class A and B derivatives (Marshall et al., 2005) and also possess pronounced immunostimulatory and anti-cancer effects (Li et al., 2020). For instance, L. Yang et al. demonstrated

distinct stimulatory activity of CpG-ODN D-SL03 derivative, which was able to: (i) activate human B cells, NK cells and T cells *in vitro*; (ii) intensity the expression of CD80, CD86 and HLA-DR in mononuclear cell cultures, and (iii) furnish anti-cancer effects in a murine model of breast cancer *in vivo* (Yang et al., 2013). As far as CpG-ODN SD-101 is concerned, this derivative demonstrated immunostimulatory and anti-cancer effects during local anti-cancer immunotherapy in patients (Levy et al., 2016; Li et al., 2020). Based on the aforementioned CpG-ODN derivatives, this study was first to synthesise modified analogues SD-101M and D-SL03M with mesyl-phosphoramidate internucleotide groups, which were shown in our previous reports to be more stable to enzymatic cleavage (Miroshnichenko et al., 2019).

A comparison analysis of CpG-ODN derivatives performed here showed that μ-modified analogues were superior in enhancing allostimulatory DC activity, as compared to CpG-ODN containing thiophosphate internucleotide groups. Moreover, it was the particular μ-type SD-101 derivative (SD-101M) that was found to enhance OX40L [an important co-stimulatory molecule regulating the intensity of T cell proliferation in allo-MLR (Ukyo et al., 2003)] expression in IFN-DC population, i.e. displayed properties characteristic of LPS.

It should be stressed that TLR-4-specific ligand LPS constitutes a powerful DC maturation activator, which is widely used in various *in vitro* settings as a positive control. TLR-4-mediated signalling is known to increase a downstream augmentation of co-stimulatory molecule expression, pro-inflammatory cytokine production, DC-mediated stimulatory activity with respect to allogeneic T cell proliferation, and Th1-dependent responses (Cehim, Chies, 2019), thus supporting full-fledged immunological functionality of activated DC. Interestingly, V. Hoene et al. showed that stimulatory effect of D19 (CpG-ODN class A compound) on maturation and allogeneic activity of DC was lower than LPS (Hoene et al., 2006). Meanwhile, this study showed that SD-101M activity was in fact higher than LPS, with SD-101, D-SL03 and D-SL03M-associated activity being comparable to that of LPS. Enhancement of IFN-γ production in response to CpG-ODN (SD-101 and SD-101M) treatments was also commensurate to LPS.

It should be mentioned that we compared CpG-ODN class C derivatives not only with LPS, but also with such DAMP activators as human dsDNA and azoximer bromide. Stimulatory effects of SD-101M on DC maturation and allostimulatory activity was found to be commensurate with dsDNA and AB, which complements our previous studies that showed stimulatory effects of dsDNA on DC maturation and allostimulatory activity (Alyamkina et al., 2010; Orishchenko et al., 2013). However, this study also described for the first time an ability of an AB-based polymeric adjuvant developed in Russia to stimulate *in vitro* maturation of DC derived from monocytes in the presence of IL-4 or IFN-α, as well as to enhance DC allostimulatory activity. These findings provide an important experimental support for the effective clinical application of this adjuvant in anti-viral vaccine formulations. Of note, SD-101M outcompeted for a number of parameters (for example, induction of OX40L expression and IFN-γ production) dsDNA and AB.

Conclusion

Taken together, data obtained in this study demonstrated pronounced stimulatory effects of CpG-ODN class C derivatives (SD-101 and D-SL03) on human myeloid DC, which were commensurable to that displayed by PAMP (LPS) and DAMP (dsDNA and AB) activators, while the effects of a mesyl-phosphoramidate (μ-) analogue SD-101M were even stronger. Further studies in murine experimental models are needed to analyze the efficacy of the μ-modified CpG-ODN (SD-101M with mesyl-phosphoramidate internucleotide groups) in anti-cancer immunotherapy.

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ORCID ID

A.A. Ostanin orcid.org/0000-0001-6895-938X
O.Y. Leplina orcid.org/0000-0003-3169-8643
E.A. Burakova orcid.org/000-0001-6644-5959
T.V. Tyrinova orcid.org/0000-0002-7987-2017
A.A. Fokina orcid.org/0000-0001-5692-2483
A.S. Proskurina orcid.org/0000-0002-7650-4331
S.S. Bogachev orcid.org/0000-0002-2019-9382
D.A. Stetsenko orcid.org/0000-0002-5215-5704
E.R. Chernykh orcid.org/0000-0003-2346-6279

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Picobirnaviruses: prevalence, genetic diversity, detection methods

A.Yu. Kashnikov ✉, N.V. Epifanova, N.A. Novikova

I.N. Blokhina Nizhny Novgorod Research Institute of Epidemiology and Microbiology, Nizhny Novgorod, Russia
✉ e-mail: a.kashn@yandex.ru

Abstract. This article presents a general overview of the prevalence, genetic diversity and detection methods of picobirnaviruses (PBVs), which are small, non-enveloped icosahedral viruses with a segmented double-stranded RNA genome consisting of two segments taxonomically related to the genus *Picobirnavirus* of the family *Picobirnaviridae*. This review of scientific papers published in 1988–2019 provides data on the PBV distribution in the nature and a broad host range. PBV infection is characterized as opportunistic, the lack of understanding of the etiological role of PBVs in diarrhea is emphasized, since these viruses are detected both in symptomatic and asymptomatic cases. The concept of PBV infection as a chronic disease caused by a long-lasting persistence of the virus in the host is considered. Such factors as stress syndrome, physiological conditions, immune status and host age at the time of primary PBV infection influence the virus detection rate in humans and animals. The possible zoonotic nature of human PBV infection is noted due to the capacity for interspecies PBV transmission acquired during evolution as a result of the reassortment of the genome segments of different viruses infecting the same host. Data providing evidence that PBVs belong to eukaryotes and a challenging hypothesis stating that PBVs are bacterial viruses are presented. The need to intensify work on PBV detection because of their wide distribution, despite the complexity due to the lack of the cultivation system, is emphasized. Two strategies of RT-PCR as main PBV detection methods are considered. The genomes of individual representatives of the genus isolated from different hosts are characterized. Emphasis is placed on the feasibility of developing primers with broader specificity for expanding the range of identifiable representatives of the genus PBV due to a huge variety of their genotypes. The importance of effective monitoring of PBV prevalence for studying the zoonotic and anthroponotic potential using metagenomic analysis is highlighted, and so is the possibility of using PBV as a marker for environmental monitoring.
Key words: picobirnavirus; genomic segment; specific genomic fragment; RT-PCR; primer; amplicon; sequencing.

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Пикобирнавирусы: распространенность, генетическое разнообразие, методы детекции

А.Ю. Кашников ✉, Н.В. Епифанова, Н.А. Новикова

Нижегородский научно-исследовательский институт эпидемиологии и микробиологии им. академика И.Н. Блохиной Роспотребнадзора, Нижний Новгород, Россия
✉ e-mail: a.kashn@yandex.ru

Аннотация. Обзор посвящен пикобирнавирусам (ПБВ) – мелким безоболочечным изометрическим вирусам, таксономически относящимся к роду *Picobirnavirus* (PBV) семейства *Picobirnaviridae*, с геномом, представленным двумя сегментами двуцепочечной РНК. На основании публикаций за 1988–2019 гг. представлена информация о распространенности пикобирнавирусов в природе, о широком спектре поражаемых хозяев. Раскрыт оппортунистический характер ПБВ инфекции и подчеркивается отсутствие ясной картины в понимании роли ПБВ в качестве этиологического агента диареи, поскольку эти вирусы выявляются и при отсутствии симптомов заболевания. Рассматривается концепция, обосновывающая представление о ПБВ инфекции как о хроническом заболевании, обусловленном длительной персистенцией вируса в организме хозяина. Причины высокой частоты выявления ПБВ у людей и животных объясняются влиянием таких факторов, как стрессовый синдром, физиологическое состояние, иммунный статус и возраст хозяина при первичном инфицировании. Отмечается возможный зоонозный характер ПБВ инфекции человека, природа которого объясняется способностью этих вирусов к межвидовой трансмиссии, приобретенной в ходе эволюции благодаря реассортации сегментов генома разных вирусов, инфицировавших одного хозяина. Приводятся данные, доказывающие принадлежность ПБВ к вирусам эукариот, а также ставящая эти факты под сомнение гипотеза о возможной принадлежности ПБВ к вирусам бактерий. Подчеркнута необходимость активизации работ по выявлению ПБВ в связи с их широким распространением, несмотря на сложность из-за отсутствия системы для их культивирования. В качестве основных способов их детекции рассмотрены две

стратегии ОТ-ПЦР ПБВ. Приведена характеристика геномов отдельных представителей рода, выделенных от разных хозяев. Акцент сделан на целесообразности разработки праймеров с более широкой специфичностью для увеличения диапазона выявляемых представителей рода ПБВ в связи с огромным разнообразием их генотипов. Подчеркивается важность эффективного мониторинга распространенности ПБВ для изучения их зоонозного и антропонозного потенциала с помощью метагеномного анализа, а также возможность использования этих вирусов в качестве перспективного маркера для мониторинга за чистотой окружающей среды.
Ключевые слова: пикобирнавирус; сегмент генома; фрагмент сегмента генома; ОТ-ПЦР; праймер; ампликон; секвенирование.

History of the PBV discovery

In 1988 in Brazil when human fecal samples collected during acute gastroenteritis outbreaks were subjected for detection of segmented rotavirus genomes by polyacrylamide gel electrophoresis (PAGE), two band profiles were revealed (Pereira et al., 1988a). Similar profiles were found when examining intestinal contents of rats (Pereira et al., 1988b). These segments were double stranded RNA (dsRNA). Their length was estimated by electrophoretic mobility at about 2.6 and 1.5 kbp for the slow- and fast-migrating segments, respectively. This RNA was cosedimented in the caesium chloride gradient at a density of 1.39–1.40 g/ml with uniform particles ~35 nm in diameter with an indistinct surface structure, detected by electron microscopic examination of samples. The authors proposed the name "picobirnaviruses" (from *pico* ('small'), *bi* ('two'), and *rna* ('RNA')) for new, previously undescribed small viruses with a bisegmented RNA genome, in contrast to the known larger birnaviruses that infect birds, fish, insects, and mollusks.

Follow-up studies showed a widespread prevalence of picobirnaviruses (PBVs) that were found in the feces of terrestrial and marine mammals, reptiles, birds (Ganesh et al., 2014; Malik et al., 2014; Conceicao-Neto et al., 2016; Navarro et al., 2018), in the respiratory tract of pigs (Smits et al., 2011) and humans (Smits et al., 2012), in fish, invertebrates (Delmas et al., 2019), fungi (Yinda et al., 2018), and, according to recent data, in bacteria (Krishnamurthy, Wang, 2018). The chronology of PBV detection in humans and animals according to data from 1988 to 2018, inclusive, is presented in the Table.

Taxonomy

Picobirnaviruses (family *Picobirnaviridae*) is a family of non-enveloped small spherical viruses, according to Baltimore's classification, belonging to the class III of viruses with a double-stranded RNA genome (https://viralzone.expasy.org). This new viral family is composed of only one viral genus, *Picobirnavirus*, uniting the viruses of five genetically variable clusters (genogroups) (Luo et al., 2018).

The two species under the genus are Human *Picobirnavirus* and Rabbit *Picobirnavirus*, where the former one is nominated as a type species and the latter one as designated species by the International Committee on Taxonomy of Viruses (ICTV) in 2008 (Delmas et al., 2019). Picobirnaviruses from other hosts have not yet been approved as type species and are considered unclassified (Malik et al., 2014; Takiuchi et al., 2016). In addition, it is not completely clear whether eukaryotes or bacteria are the natural hosts of PBVs (Krishnamurthy, Wang, 2018). Information about the taxonomy of *Picobirnaviridae* is available in the ICTV report summary by reference at the link www.ictv.global/report/picobirnaviridae. Taxonomically, the closest relatives of PBVs are viruses of the family *Partitiviridae*, that have a similar capsid structure and genome organization (Delmas et al., 2019). Natural hosts of partitiviruses are fungi and plants (Vainio et al., 2018).

Structural and molecular organization of PBV

Morphologically PBV virions are small non-enveloped particles 35–40 nm in diameter with indistinct surface structure (Fig. 1) (Rosen et al., 2000; Wakuda et al., 2005; Duquerroy et al., 2009; Collier et al., 2016). A capsid has a cubic (icosahedral) type of symmetry, has a 30-sided (triacontahedral) organization, and consists of 60 asymmetric subunits that are homodimers (Fig. 2). These subunits form 60 protrusions on the surface of the capsid. Since each of the subunits is a dimer, in total, the capsid consists of 120 protein molecules, which makes it possible to attribute PBVs to structures with a triangulation number "T = 2" when characterizing the virion symmetry. In the capsid there are channels connecting the internal cavity with the virion surface (Duquerroy et al., 2009).

Significantly different in capsid architecture from higher eukaryotic viruses with dsRNA (*Reoviridae*), PBVs are similar to dsRNA viruses of the family *Partitiviridae* (Ochoa et al., 2008). However, according to recent data, in contrast to partitiviruses, PBVs can infect prokaryotic cells in addition to fungal host cells (Knox et al., 2018).

PBV genome consists of two dsRNA segments whose sizes differ in viruses isolated from different animal species. In polyacrylamide gel electrophoresis (PAGE) these segments diverge relative to each other at a certain distance. In this case, two types of electrophoregrams are formed: with a larger (segments higher) genome profile (the segments 1 and 2 correspond to 2.7 kbp and 1.9 kbp, respectively) and with a shorter genome profile (segments lower, 2.2 kbp and 1.2 kbp) (Fig. 3) (Duquerroy et al., 2009).

The larger segment 1 of the PBV genome can consist of two or three open reading frames (ORF). It should be noted that in most studies, the segment 1 of the PBV genome is schematically represented as two ORFs, for example, in the PBV genome schemes of human (Rosen et al., 2000), pig (Carruyo et al., 2008), bull (Ghosh et al., 2009), sea lion (Woo et al., 2012), fox (Bodewes et al., 2013), tur-

History of the detection of PBV

Year of publication	PBV host	Country	Authors
1988	Human, Rat	Brazil	Pereira et al.
	Chicken		Alfieri et al.
1989	Pig		Gatti et al.
	Guinea pig		Pereira et al.
1990	Calf	Bulgaria	Vanopdenbosch, Wellemans
1991	Foal	Great Britain and Ireland	Browning et al.
1993	Rabbit	Great Britain	Gallimore et al.
1996	Goat kid, Lamb	Spain	Munoz et al.
1999	Hamster, Rat, Giant Anteater	Brazil	Haga et al.
2001	Dog		Volotäo et al.
2007	Donkey	Argentina	Masachessi et al.
	Monkey	USA	Wang et al.
2009	Snake	Brazil	Fregolente et al.
2010	Turkey	California, USA	Day et al.
2012	Ostrich	South America	Masachessi et al.
	Sea lion	Hong Kong, China	Woo et al.
2013	Red fox	Netherlands	Bodewes et al.
	Bat	Regions of China	Yang et al.
	Giant cats	Uruguay	Gillman et al.
2014	Camel	Hong Kong, China	Woo et al.
2015	Orangutan	South East Asia	Masachessi et al.
2016	Wolf	Portugal	Conceicao-Neto et al.
2017	Roe deer	Slovenia	Kuhar et al.
2018	Cattle	Brazil	Navarro et al.
2016–2018	Fish, invertebrates, molluscs, algae	China	Shi et al.

key (Verma et al., 2015), horse (Li et al., 2015), gorilla (Duraisamy et al., 2018), marmot (Luo et al., 2018). In a number of other works, the segment 1 consists of three ORFs, for example, in the genome schemes of rabbit, roe deer and chicken in studies of Green et al. (1999), Kuhar et al. (2017) and Boros et al. (2018), respectively.

In schemes where the segment 1 consists of three ORFs, the smallest ORF1 encodes a polypeptide comprising only a few tens of amino acids. For example, in the scheme of the human PBV genome (strain Hy005102) presented by King et al. (2012), ORF1, preceding two larger frames ORF2 and ORF3 encoding peptides of 224 and 552 amino acids, consists of only 39 codons (Fig. 4). In the PBV genome schemes of rabbit, roe deer and chicken in the studies of Green et al. (1999), Kuhar et al. (2017) and Boros et al. (2018) ORF1 is slightly larger and comprises 55, 63 and 188 codons, respectively. The functionality of ORF1 is unclear, and therefore its presence is not always mentioned by other researchers (Boros et al., 2018).

ORF2 in schemes with the segment 1, consisting of three ORFs, encodes the so-called hydrophilic peptide containing conserved repeating sequences, which is one of the main features of the PBV genome (Boros et al., 2018). There is an assumption that two frame shifts occur during translation to generate one long protein (Green et al., 1999).

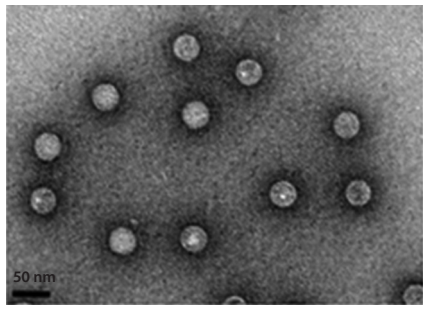

Fig. 1. Electron microscopic images of purified and concentrated particles of PBV (Collier et al., 2016). The hexagonal contour of the particles proves their icosahedral symmetry.

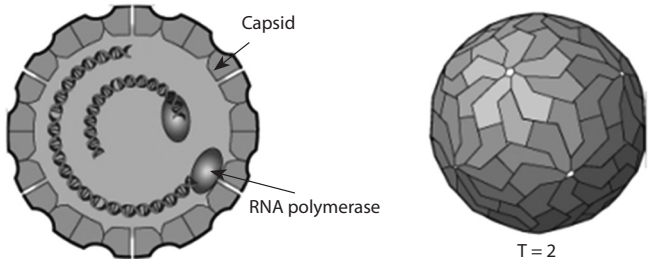

Fig. 2. Virion PBV (https://viralzone.expasy.org/by_species/740).

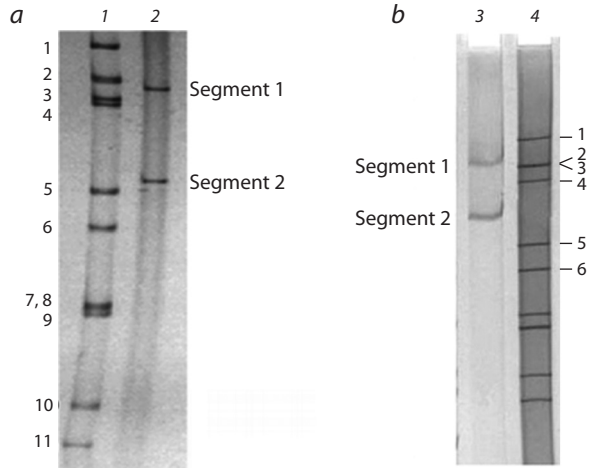

Fig. 3. "Large" (*a*) and "small" (*b*) migration profiles of PBV RNA segments in PAGE compared to the rotavirus RNA migration profile (Wakuda et al., 2005; Ghosh et al., 2009).

1 – monkey rotavirus SA-11; *2* – human PBV Hy005102; *3* – PBV cattle RUBV-P; *4* – human rotavirus DS-1.

The third, the largest reading frame (ORF3) in the PBV segment 1 encodes a virus capsid protein of 552–591 amino acids. Frames may overlap. In the scheme of King et al. (2012), the three ORFs overlap at eight (ORF1–ORF2 junction) and one (ORF2–ORF3 junction) nucleotides.

The shorter segment 2 of the PBV genome contains one ORF encoding the enzyme RNA-dependent RNA polymerase (RdRp) (see Fig. 4). Depending on differences in RdRp gene specificity in the segment 2 of the PBV genome, PBVs are divided into genogroups (Malik et al., 2014).

In addition to the "typical" PBVs described above, "atypical" PBVs were detected using PAGE, first in the feces of calves (Vanopdenbosch et al., 1989), and later in the human feces (Gallimore et al., 1995a; Khramtsov et al., 1997). "Atypical" PBVs have a smaller genome than "typical" ones (RNA segment sizes range from 1.75 to 1.79 kbp and from 1.37 to 1.55 kbp), and differ in the location of genes encoding functional proteins (Gallimore et al., 1995b; Khramtsov et al., 1997). If in "typical" PBVs, the segment 2 encodes the viral RNA polymerase, and the segment 1 encodes a capsid protein, then in "atypical" ones it is the opposite.

The PBV genome is usually segmented (Duquerroy et al., 2009; Delmas et al., 2019). However, several non-segmented PBV genomes belonging to different genetic clusters have recently been described, particularly PBV genomes of horses (Li et al., 2015), of Himalayan marmots (Luo et al., 2018), fish and invertebrates (Shi et al., 2016). It was found that there is an evolutionary relationship between PBVs with a segmented and non-segmented genome, which is due to the possibility of transition from one form to another. It turned out that the PBV genome of marmots can be both segmented and non-segmented. A non-segmented genome contains three ORFs – ORF1, ORF2, ORF3 encoding a hydrophilic protein, a capsid protein, and RdRp, respectively (Luo et al., 2018).

Sequencing studies of PBVs have shown that PVBs are extremely variable (Bányai et al., 2003, 2008; Carruyo et al., 2008; Smits et al., 2011; Ganesh et al., 2014; Malik et al., 2014; Li et al., 2015; Duraisamy et al., 2018; Luo et al., 2018). PBV genome variability is explained by characteristic genetic variability, caused not so much by a change in the primary structure as a result of mutations, but by the genome segment reassortment (Woo et al., 2019).

Until 2014, researchers identified two main PBV genogroups based on the study of short incomplete RdRp gene sequences (Malik et al., 2014). When analyzing the PBV sequences presented in the database GenBank (https://www.ncbi.nlm.nih.gov/nuccore), as of 17.07.2014 (Malik et al., 2014) it was noted that 83.11 % of the sequences belong to the PBV genogroup I (GI), and only 2.52 % – to the genogroup II (GII), which corresponds to the prevalence of representatives of these genogroups in nature.

However, further research results have shown that PBVs exhibit a high level of genetic diversity that is not reflected by just two genogroups. In 2014–2015, there were reports of the discovery of a new genetic PBV variant in human stool samples (Smits et al., 2014) and in the environment (Zhang S. et al., 2015). The new human PBV showed low similarity (19.4–26.1 %) in the sequence of amino acids in the RdRp gene product with the human PBV genogroups I and II, which allowed them to be assigned to the genogroup III (PBV GIII/Homo sapiens/VS6600008/2008/NL/KJ206569 in GenBank). In 2015 picobirnaviruses were identified in horses, which formed separate clusters and were assigned to two genogroups GIV and GV during

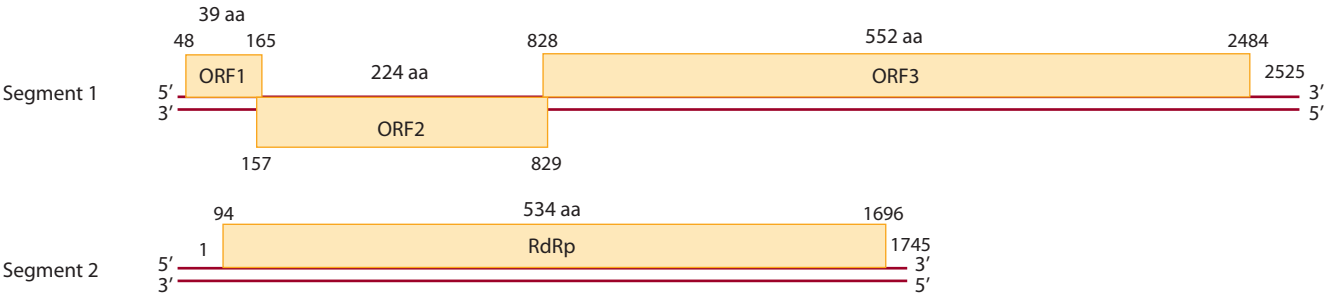

Fig. 4. Schematic representation of the location of genes in segments 1 and 2 of dsRNA strain Hy005102 of human picobirnavirus (King et al., 2012).

Numbers indicate nucleotide positions; aa – aminoacid.

phylogenetic analysis of 450 aa RdRp protein sequence (Li et al., 2015).

Thus, picobirnaviruses are currently classified into five genogroups GI–GV based on RdRp sequences. At the same time, the intra-genogroup amino acid identities range from 44.8 to 97.1 %, whilst the inter-genogroup amino acid identities range from 21.6 to 30.8 % (Li et al., 2015). In humans, four PBV genogroups – GI, GII, GIII (Smits et al., 2014) and GV (Ng et al., 2014) were identified. All five PBV genogroups were identified in the marmot (Luo et al., 2018). Genogroup GIII PBVs were found in diatoms and invertebrates (Shi et al., 2016, 2018; Delmas et al., 2019).

According to the currently accepted nomenclature of picobirnaviruses proposed by Fregolente et al. (2009), the name of the strain begins with the genogroup name (GI–GV), followed by the abbreviation PBV, the common name of the host species, the three-letter country code, the name of the strain and the year of isolation, separated by a slash. Using this nomenclature, PBV strains detected in humans and turkeys received the following designations – GI/PBV/human/BRA/PBV_RVH275/2013 (human PBV) и GI/PBV/turkey/USA/MN-1/2011 (turkey PBV).

Opportunistic character of PBV-infection. On PBV persistence

In spite of the fact that PBVs are often detected from humans and animals with diarrhea separately and co-infection with other pathogens (Ganesh et al., 2014), the role of PBVs as causative agents of intestinal disorders has not been established, since these viruses are detected in asymptomatic cases (Masachessi et al., 2007; Martínez et al., 2010; Verma et al., 2015). For example, Verma et al. (2015) showed that the excretion of PBVs in turkeys was not associated with the symptoms of diarrhea. Of the 80 fecal samples from poultry with diarrhea and 40 without diarrhea, 39 (48.8 %) and 23 (57.5 %) were positive for PBVs, respectively.

It is not shown, that PBVs might have etiological relation with diarrhea in humans and animals infected with other pathogens that cause gastroenteritis. The adaption of the virus to grow in the cell culture and experiments on gnotobiotic animals will be essential to establish the etiologic role of PBVs (Ganesh et al., 2014).

Since the end of the 90s of the last century, PBVs have been identified as opportunists for their ability to provoke diarrhea in animals infected with the primary pathogen or humans with weakened immunity and later to manifest themselves as a chronic disease with or without signs of diarrhea (Giordano et al., 1998; González et al., 1998; Martínez et al., 2003; Masachessi et al., 2007, 2012; Ghosh et al., 2009). There is a concept about the opportunistic (conditionally pathogenic) nature of PBV infection, which is presented in the review published by Ganesh et al. (2014) and other reports based on the results of examination of healthy animals for the presence of PBVs (Masachessi et al., 2007; Carruyo et al., 2008; Martínez et al., 2010). According to this concept, the chronic nature of PBV infection is explained by the long-term persistence of the virus in the host body (Ganesh et al., 2014). The persistent nature of PBV infection is manifested by periods of silence, in which the virus is not detected even using highly sensitive methods, intermingled with periods of viral activity. In this case, adult animals infected with PBVs could be asymptomatic PBV carriers or may be persistently infected and serve as reservoirs of the infection. In particular, research results obtained by Carruyo et al. (2008) shown that PBVs can infect piglets 7 to 56 days of age (the PBV prevalence among piglets has been estimated to be around 10 to 12 %).

The virus excretion level by virus carriers is affected by a number of factors such as stress syndrome, physiological status, age of the primary infected individual, immune status of the hosts, and environmental conditions (biotic and abiotic factors). These factors contribute to an increase in the viral load. When taking samples based on these factors, the detection of PBVs by the PAGE was observed with a higher frequency.

The dependence of the PBV excretion level on the stress caused by keeping animals captive was observed in African green monkeys from the Caribbean Islands in 2014–2015 (Gallagher et al., 2017). 270 fecal samples were collected from wild (160 individuals) and captive (110 individuals) monkeys subjected to PAGE for PBVs. 16 samples (14.5 %) from captive monkeys kept under stressful conditions were indicative for PBVs. None of the fecal samples from wild monkeys was tested positive for PBVs (Gallagher et al., 2017).

The dependence of the PBV excretion level on the physiological status of pigs, in particular during the reproduction period, was studied by researchers in Argentina (Martínez et al., 2010). Observations showed that PBVs persisted in the host as a permanent infection, with periods of low and high PBV excretion intermingled with periods of silence. Low PBV excretion levels were detected by reverse transcription followed by polymerase chain reaction (RT-PCR) throughout the study period. In case of sharp increase in the PBV excretion level in pigs, observed during farrowing and lactation periods, the virus was detected not only by OTP-PCR, but also by PAGE. PBV detection rate was highest in the group of sows sampled within the lactogenic period (38.02 % of samples collected from 71 sows), followed by pregnant sows at the final stage of gestation (15.09 % of samples collected from 53 pregnant sows) (Martínez et al., 2010).

The dependence of the PBV excretion level on the age of the animal is shown. From 289 fecal samples of individual calves between 5 and 60 days of age analyzed by PAGE the PBVs were detected in 24 (8.3 %) (Takiuchi et al., 2016.) In the study (Martínez et al., 2010), a high PBV excretion level in pigs was observed in young animals 2–5 months of age (18.42 %), while no excretion was observed in adult male pigs. Studies on turkeys also revealed a change in the frequency of PBV excretion (Verma et al., 2015). 39 of 80 (48.8 %) fecal samples from turkeys were PBV positive.

The maximum number of samples was positive in turkeys at 2 weeks of age (20 of 20) followed by 3 weeks of age (15 of 20). A sharp decrease in the number of positive samples (2/20 were positive at 8 weeks of age) indicated a decisive influence of age on this process.

The PBV excretion level is also affected by the host's immune status. It was found that in people with weakened immunity, PBVs are detected more often. For example, Giordano et al. (1998) when investigating 197 stool samples collected from HIV-infected and noninfected patients with and without diarrhea for the presence of PBVs by PAGE detected PBVs in 8.8 % of 57 HIV-infected patients with diarrhea, but detected it in neither those without diarrhea nor in the group of subjects uninfected with HIV. Further research by these authors reinforced their view of the relationship between PBV excretion levels and diarrhoea in HIV-infected individuals. In a study of 244 stool samples from HIV-infected and uninfected patients with and without diarrhea, PBVs were detected in 14.63 % of 82 HIV-infected patients with diarrhea and it was detected neither in those without diarrhea nor in the group of subjects uninfected with HIV (Giordano et al., 1999). At a certain PBV excretion level, PBVs are detected in HIV-infected patients without signs of diarrhea, for example, González et al. (1998) detected PBVs in 2.3 % of 125 HIV-infected patients without diarrhea (González et al., 1998).

The PBV excretion level may be influenced by biotic environmental factors such as primary pathogens. In some studies, for example, PBVs are most often isolated as co-infected agents with a number of diarrheal causes, such as rotaviruses (Kuhar et al., 2017), or noroviruses (Bányai et al., 2003). These studies indicated that PBVs might have played synergistic effect in association with the primary enteric causes (Malik et al., 2014; Kylla et al., 2019).

Climate factors such as sunlight (Masachessi et al., 2015), temperature, and humidity (Ribeiro et al., 2014) also affect the viral activity. These factors that affect the PBV excretion level should be taken into account when identifying them.

Zoonotic nature of PBV-infection. Capacity for interspecies transmission

Most human viral diseases are of zoonotic origin. The zoonotic character of the PBV infection is indicated by the detection of genetically related PBVs in humans and animals. In particular, from pigs in Hungary, Venezuela and Argentina genogroup I PBVs were detected which, when sequencing the genome, showed genetic similarity to the human genogroup I PBVs (Bányai et al., 2008; Carruyo et al., 2008; Giordano et al., 2011). There have been reports of PBV strains found in children in Calcutta that are genetically related to porcine PBV strains (Ganesh et al., 2010, 2011a). Equine PBV strains isolated from the faeces of foals in Calcutta (India) showed a genetic relationship with human strains from the same city (Ganesh et al., 2011b). The detection of genetically similar PBVs in humans and foxes (Lojkić et al., 2016), in humans and bats has been reported. For example, the report (Yinda et al., 2019) noted that the cause of zoonotic transmission of PBVs to humans from bats in Cameroon is the hunting and eating of bats (Yinda et al., 2019).

Zoonotic PBV transmission is one of the variants of interspecies transmission. The capacity for interspecies transmission was acquired by PBVs in the course of evolution due to the reassortment of segments of their genome while simultaneously infecting a single cell by PBVs of different species (McDonald et al., 2016). The genetic lability caused by reassortment could lead in the course of evolution either to genetic convergence of PBV strains belonging to different hosts or, conversely, to genetic divergence – genetic distance of PBV belonging to hosts of the same species (Lojkić et al., 2016).

The capacity for interspecies PBV transmission is confirmed both in the cases of zoonotic infections, and in cases of infection of animals with human PBVs, for example, young pigs (Carruyo et al., 2008) or horses (Ganesh et al., 2011b). The genomes of some porcine PBV strains were found to be identical to those of human genogroup I PBVs (Ganesh et al., 2014). In 2011, genetically similar PBVs were found in the respiratory tracts of pigs and humans (Smits et al., 2011, 2012).

Genetic divergence of related PBV strains was demonstrated by Zhang B. et al. (2014). Thus, three of four porcine PBVs identified in the study were genetically closer to human PBVs than to previously revealed porcine PBVs.

It is possible to transfer PBV strains from one host to another through fecal-contaminated raw sewage (Symonds et al., 2009). In an environmental study Symonds et al. (2009) showed that picobirnaviruses are potentially useful viral indicators of fecal pollution of naturally impounded bodies since they were found in 100 % of raw sewage samples and 33 % of final effluent samples.

Hypothesis about the phage nature of PBV

PBVs commonly found in animal fecal samples are currently thought to be animal viruses, but no animal model or cell culture for PBV propagation has yet been found. Recently, some Indian scientists hypothesized that PBVs are prokaryotic RNA viruses (Krishnamurthy, Wang, 2018). The hypothesis is based on the fact, that like prokaryotic viruses with the RNA-genome, in the PBV genome, there are conserved ribosomal binding site (RBS) sequences called Shine–Dalgarno sequences upstream of the three presumed ORFs of the segment 1 and a single presumed ORF of the segment 2. Such sites are 6-mers (AGGAGG) preceding codons that initiate the translation of the viral genome sequences. In bacterial viruses, these 6-mers are ribosomal binding sites and serve to enhance the translation efficiency of viral proteins. For example, such sites are present in the genome of some bacteriophages of the family *Cystoviridae* with a segmented dsRNA genome (Boros et al., 2018).

Findings obtained by Adriaenssens et al. (2018) support the hypothesis that PBVs are bacteriophages. The authors

demonstrated a high incidence of 6-mer motif AGGAGG in the PBV genome. In contrast, the different families of eukaryotic viruses analyzed in that study only showed a low incidence of SD-sequences, which were mostly 4-mers (AGGA, GGAG, GAGG). Findings supporting the bacteriophage-nature of PBVs were also obtained by Boros et al. (2018), who revealed in the chicken PBV genome the presence of conserved prokaryotic Shine–Dalgarno-like (SD-like) sequences upstream of the three presumed ORFs of the segment 1 and a single presumed ORF of the segment 2.

If we assume that PBVs are prokaryotic viruses, we can explain their widespread prevalence and a broad host range. Bacteriophages are widely distributed in nature. They are found in water, soil, food products, various excretions of humans and animals, that is, where bacteria are found.

The identification of PBV strains with genetically related genome sequences in different animal species can be explained by assuming that PBVs might actually infect bacteria that populate the enteric tract of vertebrates and invertebrates. At the same time, the authors of the phage hypothesis (Krishnamurthy, Wang, 2018) believe that PBVs replicate in bacteria of a certain type, in the genome of which there is a prokaryotic SD-sequence in most genes (more than 10 %). Such bacteria are *Firmicutes* in the genome of which there are more than 80 % of genes with prokaryotic SD-sequence (Omotajo et al., 2015).

The authors point out, that, even viral families that include species whose genomes are enriched for SD-sequences, are exclusively prokaryotic viral families. The acquisition of immunity against PBVs by infected animals also does not contradict the phage hypothesis, since it is established that immune responses can be raised against bacterial viruses (Dabrowska et al., 2005; Górski et al., 2006). It is possible that PBVs cause an immune response to infection not of human cells, but of the bacterial cells that make up its microbiome, which again does not exclude the possibility that PBV are prokaryotic viruses.

The possession of a capsid protein with perforation activity, which means the ability to translocate through the cell membrane (Duquerroy et al., 2009), as proof that it can infect animal cells, also does not contradict the phage hypothesis, since it is known that representatives of the bacterial RNA-virus family also have the ability to exit the cell (Reed et al., 2013). The process of interaction of human and animal PBVs with the host cell is similar to the process of interaction of a virulent phage with a bacterium, which proceeds in several stages – penetration into the bacterial cell, autonomous reproduction in it and lysis of the bacterium. Consequently, the perforation of liposomes by PBVs does not exclude that they may be prokaryotic RNA-viruses.

Summing up the arguments in favor of the phage hypothesis, we can conclude that, perhaps, PBVs belong to a new family of RNA-viruses that infect a certain type of bacteria with a genome with a high content of SD-sequences (more than 80 %). These bacteria populate the intestinal tract of animals and humans and they can be bacteria *Firmicutes*, containing most genes with SD-sequences.

These arguments strongly suggest that PBVs can actually infect prokaryotes, not eukaryotes. And if PBVs actually infect bacteria, then it is necessary to change the approach to their study – to direct efforts towards finding a host for their replication among prokaryotic cells, rather than eukaryotic ones. Separation prokaryotic and eukaryotic virus families by the frequency of the presence of SD-sequences in the genome allows identifying new prokaryotic virus families.

Thus, the final proof of the phage nature of PBVs requires the selection of host cells for its replication. In all likelihood, until successful cultivation of PBVs in specific bacterial cultures is achieved, the phage nature of PBVs remains hypothetical. Given the fact that gut microbiome consists of several hundreds of mostly uncultivable bacteria the identification of true bacterial or archeal host(s) of PBVs (if any) will be challenging (Boros et al., 2018).

RT-PCR-amplification strategies for PBV sequencing and genotyping

Before the use of nucleic acid amplification methods, the detection rate of PBVs remained extremely low, because of low sensitivity of the method of genomic RNA PAGE (Masachessi et al., 2007). In addition, PBVs are very labile agents. It is shown that samples tested PBV positive by PAGE, become negative after several freezing–thawing procedures (Gallimore et al., 1995a).

The low frequency of detection of PBVs by PAGE is evidenced by the work of Argentine virologists (Giordano et al., 2008). These authors collected 2224 stool samples from children with diarrhea over a 25-year period from January 1977 to December 2002. Only two samples (0.09 %) were tested PBV positive by PAGE. Similar results were obtained in our studies on the detection of rotaviruses by PAGE, in stool samples from children under 14 years of age with acute intestinal infection, who were admitted to infectious hospitals in the city of Nizhny Novgorod, Nizhny Novgorod region. For the period 1994–2001 PBVs were found in only 3 of 4535 samples tested (Novikova et al., 2003). Subsequently, from July 2006 to January 2010, PBVs were detected in 0.08 % of 3645 stool samples from children with gastroenteritis (Epifanova et al., 2010).

The use of PAGE limited the frequency of PBV detection, since at the low viral load virus excretion was undetectable in most clinical samples (Gallimore et al., 1995a; Giordano et al., 1998). A low frequency of PBV detection by PAGE was reported by Cascio et al. (1996), Pereira et al. (1993) and Ludert, Liprandi (1993), in a study of sporadic gastroenteritis cases in children in Italy (0.43 %), Brazil (0.5 %) and Venezuela (0.5 %), respectively. However, in outbreaks of gastroenteritis, when the virus excretion level was high, the virus was detected by PAGE with a significantly higher frequency. For example, Pereira et al. (1988a) reported a frequency of human PBV detection in outbreaks of gastroenteritis in Brazil up to 20 %.

The introduction of RT-PCR amplification and sequencing technologies has contributed to an increase in the PBV detection rate in various wildlife objects. For example, a total 60 % (87/144) of the fecal samples from newborn piglets tested were found to be PBV positive by RT-PCR (versus 27 % by PAGE) (Carruyo et al., 2008). The information presented in the paper on the prevalence of porcine PBVs in Argentina (Martínez et al., 2010) also demonstrates the significantly greater capabilities of the RT-PCR method compared to PAGE. If the PAGE method in this study detected PBVs only at high PBV excretion level conditioned by age (primary infection), and host physiological status, low PBV excretion levels were detected by RT-PCR throughout the entire study period.

The application of these methods allowed to establish that PBVs are more widespread in nature than it was previously discovered (Boros et al., 2018). In particular, a group of researchers from the Netherlands when testing 83 stool samples from patients with diarrhea by RT-PCR confirmed 17 samples positive for genogroup I PBV sequences (20 %) (van Leeuwen et al., 2010). By the same method, a high proportion of PCR positive samples (23.4 %) was detected in a total of 77 bovine fecal samples from different Brazilian regions analyzed (Navarro et al., 2018). The high frequency of infection in the sheep flock in Brazil evaluated by RT-PCR was established where 62 % of the analyzed fecal samples were PBV-positive (Kunz et al., 2018).

Most researchers use two specific amplification strategies to detect PBVs: the single and double primer PCR amplification strategies. The first amplification strategy is based on the ligation of a viral RNA as a matrix with an oligonucleotide as an adapter, followed by the synthesis of cDNA on this matrix using a complementary adapter primer. This method developed by Lambden et al. (1992) to carry out amplification of the viruses with segmented dsRNA genomes was further used by researchers for the amplification of the PBV genome (Wakuda et al., 2005; Ghosh et al., 2009; Wang et al., 2012; Boros et al., 2018). In particular, Wakuda et al. (2005) applied the modified single primer strategy to prepare full-length cDNAs corresponding to RNA segments 1 and 2 of a human picobirnavirus (strain Hy005102).

The single primer PCR amplification strategy is usually applied to characterize full-length PBV genome segments. For PBV genotyping based on short genome specific fragments to characterize the strain with the definition of its genogroup, the second strategy of specific amplification is used, which involves the use of a pair of primers flanking the selected viral genome fragment. Reverse transcription-PCR detection assays were developed with primers targeted to the genome segment of 2 PBV strains – 4-GA-9 and 1-CHN-97 isolated in the United States and China, respectively (Rosen et al., 2000).

In the case of human PBVs, the primers are targeted to specific conserved sites (motifs) in the PBV genome segment 2 encoding RNA-dependent RNA polymerase. For detection of the genome group I, direct and reverse primers PicoB25 and PicoB43 are used, which flank the 201 bp (in positions 665–679 and 850–865 of the segment 2) fragment of the RdRp gene. To detect the genome group II, a pair of primers PicoB23 and PicoB24 flanking the 369 bp (in positions 685–699 and 1039–1053) fragment of the RdRp gene is used.

These primers can be used not only for human PBV genotyping (Rosen et al., 2000), but also for the genetic characterization of PBVs in some animals, in particular pigs (Bányai et al., 2008). However, they are not able to recognize the full range of PBV strains circulating among humans, pigs, and other hosts due to their narrow specificity (Bányai et al., 2008; Carruyo et al., 2008; Ganesh et al., 2010, 2011a; Martínez et al., 2010). In this regard, primers flanking other degenerated parts of the RdRp gene (Carruyo et al., 2008; van Leeuwen et al., 2010; Verma et al., 2015; Wilburn et al., 2016; Woo et al., 2019), as well as primers with a wider range of detection of representatives of the genus *Picobirnavirus*, were later developed to expand the specificity in detecting sequences of segments of the PBV genome (Malik et al., 2017; Ghosh et al., 2018; Kleymann et al., 2020).

In particular, a RNA polymerase gene based RT-PCR diagnostic assay was developed for detecting PBVs in early stages of infection in a wide range of hosts including animals and humans (Malik et al., 2017). Through RdRp gene nucleotide sequences alignment analysis, the conserved regions of PBV were used for generating primers universal for the genus. The best results for RT-PCR specificity and sensitivity were given by a primer set with sense primer PBV-7F (position 754–771 of the segment 2), and antisense primer PBV-7R (position 1011–1028) flanking the 275 bp amplicon. The developed assay made it possible to effectively amplify this fragment of the RdRp gene in all tested PBVs infecting different host species, and did not give false positive results when tested on other viruses.

The use of primers with broad specificity, in addition to expanding the range of detection of representatives of the genus *Picobirnavirus*, allows amplifying sequences of greater length. In particular, Ghosh et al. (2018) using a wide-specific terminal primer, amplified a region of the RdRp gene overlapping several conserved sites and as a result were able to characterize the complete genome segment 2 of rat PBVs, providing important information on genetic diversity and evolution of PBVs in rats.

Recently, Kleymann et al. (2020) to detect PBVs from different mongoose species, have designed a pair of widely specific primers PBV 1.2FP and PBV 1.2RP that amplify a significant part of the RdRp gene (1229 bp from ~1700 bp). This pair of primers allows detecting PBVs isolated from different hosts and having differences in primary structure (Kleymann et al., 2020). To identify genovariants belonging to the genogroup I among positive samples of mongoose PBVs the authors used a combination of the known reverse primer PicoB43 (Rosen et al., 2000) and the forward primer PBV-7F, which allowed amplifying the 390 bp fragment of the segment 2.

In recent years, metagenomic analysis has become widely used in the diagnosis of viral infections (Adriaenssens et al., 2018; Boros et al., 2018; Duraisamy et al., 2018; Yinda et al., 2019; Wille et al., 2019). The method of metagenomic analysis is based on next-generation sequencing (NGS) technology that allows identifying all the genetic material present in an environmental sample, consisting of the genomes of many individual organisms – metagenome. In contrast to PCR technologies that require reference sequences that can only detect known viruses, the metagenomic analysis method can detect viruses with a new genotype (Adriaenssens et al., 2018). Moreover, new genotypes can be detected more often than known ones (Duraisamy et al., 2018; Wille et al., 2019).

The metagenomic analysis characterizes both the viral diversity and the frequency of occurrence of individual metagenome viruses. For example, according to Adriaenssens et al. (2018), the raw sewage metagenome contained representatives of the genera *Astroviridae*, *Caliciviridae*, *Picobirnaviridae*, *Picornaviridae*, with only PBVs being detected in 100 % of raw sewage samples in this study. In the publication Boros et al. (2018) reported that using the method of metagenomic analysis of the total number of read sequences (13016) present in the metagenome, which is a fecal sample of a chicken, in 516 reads were identified PBVs. In a study Yinda et al. (2019) using metagenomic analysis of fecal samples from Cameroonian residents with and without signs of gastroenteritis (after contact with bats), found that up to 28 out of the 63 pools contained reads annotated as *Picobirnaviridae* with most of the positive pools from individuals in age groups above 20. These facts indicate a fairly high level of PBV occurrence in wildlife.

Conclusion

Summarizing the information presented in the review, obtained from publications on PBVs for the period under study, we can conclude:

- PBVs are characterized by a broad host range and ubiquitous distribution.
- The role of PBVs as causes of gastroenteritis is still not fully understood due to the lack of cell culture or animal models for their cultivation. This significantly impedes virus isolation and clinical and pathological studies.
- The frequency of PBV detection varies in different studies, but it is found that it is associated with the physiological status and environmental conditions.
- There is a hypothetical explanation for the spread of PBV infection based on the idea of PBVs as conditionally pathogenic viruses, according to which adult infected hosts with normal immune status can be PBV carriers and serve as reservoirs of viruses without symptoms of diarrhea.
- The detection of PBV strains with genetically related genome sequences in different animals indicates the possible zoonotic nature of the infection for humans and the ability of PBVs to transmit effectively.
- Being the most common in raw sewage, PBVs correlate better than other viruses with the presence of pathogenic viruses dangerous for humans in water bodies and are potentially useful viral indicators of fecal pollution of these water bodies.
- PBVs are significantly different genetically. To date, 5 PBV genogroups have been identified (GI–GV).
- It has been suggested that PBVs can infect prokaryotes, being not mammalian viruses, but a new family of RNA bacteriophages. In support of this assumption, the authors provide convincing arguments showing that PBVs can actually infect prokaryotes, and not eukaryotes, in particular, bacteria *Firmicutes*. However, until a host is found for PBV propagation, this assumption remains hypothetical.

The presented information allows characterizing PBVs as viruses that are genetically variable with a broad host range, quickly evolving and easily spreading. However, for a more complete study of PBV biology, etiological role in the occurrence of diseases, and pathogenic potential, experiments on gnotobiotic animals are required. Molecular characterization of new PBV strains from different hosts will provide valuable information about the origin, transmission, distribution, and genetic diversity of these quickly evolving dsRNA viruses to study their zoonotic and anthroponic potential, and to use as a potentially promising marker for environmental monitoring.

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ORCID ID

A.Yu. Kashnikov orcid.org/0000-0003-1033-7347
N.V. Epifanova orcid.org/0000-0001-7679-8029
N.A. Novikova orcid.org/0000-0002-3710-6648

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Wolbachia, *Spiroplasma*, and *Rickettsia* symbiotic bacteria in aphids (Aphidoidea)

D.A. Romanov[1, 2], I.A. Zakharov[1], E.V. Shaikevich[1, 3]

[1] Vavilov Institute of General Genetics of the Russian Academy of Sciences, Moscow, Russia
[2] Moscow Region State University, Mytishi, Moscow region, Russia
[3] Martsinovsky Institute of Medical Parasitology, Tropical and Vector Borne Diseases, Sechenov First Moscow State Medical University, Moscow, Russia
e-mail: elenashaikevich@mail.ru

Abstract. Aphids are a diverse family of crop pests. Aphids formed a complex relationship with intracellular bacteria. Depending on the region of study, the species composition of both aphids and their facultative endosymbionts varies. The aim of the work was to determine the occurrence and genetic diversity of *Wolbachia*, *Spiroplasma* and *Rickettsia* symbionts in aphids collected in 2018–2019 in Moscow. For these purposes, 578 aphids from 32 collection sites were tested by PCR using specific primers. At least 21 species of aphids from 14 genera and four families were identified by barcoding method, of which 11 species were infected with endosymbionts. *Rickettsia* was found in six species, *Wolbachia* in two species, *Spiroplasma* in one species. The presence of *Rickettsia* in *Impatientinum asiaticum*, *Myzus cerasi*, *Hyalopterus pruni*, *Eucallipterus tiliae*, *Chaitophorus tremulae* and *Wolbachia* in *Aphis pomi* and *C. tremulae* has been described for the first time. A double infection with *Rickettsia* and *Spiroplasma* was detected in a half of pea aphid (*Acyrthosiphon pisum*) individuals. For the first time was found that six species of aphids are infected with *Rickettsia* that are genetically different from previously known. It was first discovered that *A. pomi* is infected with two *Wolbachia* strains, one of which belongs to supergroup B and is genetically close to *Wolbachia* from *C. tremulae*. The second *Wolbachia* strain from *A. pomi* belongs to the supergroup M, recently described in aphid species. *Spiroplasma*, which we observed in *A. pisum*, is genetically close to male killing *Spiroplasma* from aphids, ladybirds and moths. Both maternal inheritance and horizontal transmission are the pathways for the distribution of facultative endosymbiotic bacteria in aphids.
Key words: aphids; endosymbionts; PCR; plant pests; mutualism.

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Симбиотические бактерии *Wolbachia*, *Spiroplasma* и *Rickettsia* среди тлей (Aphidoidea)

Д.А. Романов[1, 2], И.А. Захаров[1], Е.В. Шайкевич[1, 3]

[1] Институт общей генетики им. Н.И. Вавилова Российской академии наук, Москва, Россия
[2] Московский государственный областной университет, Мытищи, Московская область, Россия
[3] Институт медицинской паразитологии, тропических и трансмиссивных заболеваний им. Е.И. Марциновского Первого Московского государственного медицинского университета им. И.М. Сеченова (Сеченовский университет) Министерства здравоохранения РФ, Москва, Россия
e-mail: elenashaikevich@mail.ru

Аннотация. Тли – разнообразное семейство вредителей сельскохозяйственных культур. Тли сформировали сложную взаимосвязь с внутриклеточными бактериями, известными как эндосимбионты, которые оказывают как положительное, так и отрицательное влияние на хозяина, что может иметь практическое значение. В разных регионах мира состав факультативных симбионтов в популяциях тлей варьирует. Задачей работы было установить распространение и генетическое разнообразие симбионтов *Wolbachia*, *Spiroplasma* и *Rickettsia* в тлях, собранных в 2018–2019 гг. в Москве и Подмосковье. Для этого 578 тлей из 32 мест сбора тестировали методом ПЦР, используя специфические праймеры для мтДНК тлей, *Wolbachia*, *Spiroplasma* и *Rickettsia*. Методом молекулярно-генетического анализа определено не менее 21 вида тлей из 14 родов и четырех семейств. Одиннадцать видов оказались инфицированы эндосимбионтами, а именно: у шести видов обнаружены *Rickettsia*, у двух видов – *Wolbachia*, у одного – *Spiroplasma*. Впервые выявлено заражение бактерией *Rickettsia* у *Impatientinum asiaticum*, *Myzus cerasi*, *Hyalopterus pruni*, *Eucallipterus tiliae*, *Chaitophorus tremulae* и бактерией *Wolbachia* у *Aphis pomi* и *C. tremulae*. У половины особей гороховой тли *Acyrthosiphon pisum* установлено двойное заражение *Rickettsia* и *Spiroplasma*. Впервые выявлены риккетсии у шести видов тлей, которые генетически отличаются от известных ранее. Впервые обнаружено заражение яблонной тли *A. pomi* двумя штаммами

Wolbachia, причем один из штаммов относится к супергруппе B и генетически близок с *Wolbachia* из осиновой тли *C. tremulae*, а второй штамм относится к супергруппе M, недавно описанной у видов тлей. *Spiroplasma*, найденная нами у *A. pisum*, генетически близка *Spiroplasma*, вызывающей андроцид у тлей, божьих коровок и молей, и кластеризуется с *S. ixodetis*. Разнообразие ДНК симбионтов убедительно свидетельствует о том, что как материнское наследование, так и горизонтальный перенос являются путями распространения факультативных бактерий у тлей.
Ключевые слова: тля; эндосимбионты; ПЦР; вредители растений; мутуализм.

Introduction

Aphids (Aphidoidea) are an insect superfamily from the order Hemiptera, which includes about 10 families or subfamilies and about 5000 species. Aphids are widespread sap-feeding plant pests and can transmit at least 30 % of plant viruses (Augustinos et al., 2011). A complex interplay with intracellular bacteria also known as endosymbionts is commonly observed in aphids. Obligate associations with *Buchnera aphidicola* in aphids provide insect hosts with essential amino acids absent in sap (Douglas, 1998). Apart from that, there are nine species of facultative symbionts of aphids (see the review by Guo et al., 2017) coexisting with *Buchnera*, having both positive and negative effects on the hosts.

Depending on the species, facultative intracellular symbiotic bacteria may increase the resistance of aphids against heat stress, parasitoid wasps, and fungal infections, participate in the synthesis of essential nutrients for the host with the obligate symbiont, and facilitate the interaction of aphids with plants that they feed on (Guo et al., 2017). However, it has been shown that the *Rickettsia* bacteria have a negative effect on the fitness of the pea aphid *Acyrthosiphon pisum* and suppress the activity of *Buchnera aphidicola* (Sakurai et al., 2005). *Spiroplasma* bacteria in *A. pisum* shorten the life span of aphids and inhibit their reproduction (Simon et al., 2007, 2011), albeit have a (minor) protective effect against the parasitoid wasp *Aphidius ervi* (Mathé-Hubert et al., 2019). The role of *Wolbachia* in aphids is not yet fully understood (De Clerck et al., 2015; Manzano-Marín, 2019), but *Wolbachia* endosymbionts in the Asian citrus psyllid *Diaphorina citri* repress holin promoter activity in the alpha-proteobacteria '*Candidatus* Liberibacter asiaticus', a citrus disease agent, thereby killing the bacteria and preventing the disease from spreading (Jain et al., 2017).

The present paper is dedicated to studying facultative endosymbionts *Wolbachia*, *Spiroplasma*, and *Rickettsia* in aphids. *Wolbachia* is the most common genus of symbiotic bacteria in insects, aphids not being an exception. *Wolbachia* infection has been recorded in 82 aphid species (Zytynska, Weisser, 2016). Two genera of symbionts, *Rickettsia* (Sakurai et al., 2005) and *Spiroplasma* (Fukatsu et al., 2001), were recorded in *A. pisum*. Both *Spiroplasma* and *Rickettsia* were recorded in the cowpea (*Vigna sinensis*) aphid *Aphis craccivora* (Brady et al., 2014) and the bean aphid *Aphis fabae* (Zytynska et al., 2016). *Spiroplasma* were also recorded in tropical aphids, specifically the citrus aphid *Aphis citricidus* and polyphagous *Aphis aurantii* (Guidolin, Cônsoli, 2018). *Rickettsia* were recorded in the blackberry aphid *Amphorophora rubi* (Haynes et al., 2003) and the cotton aphid *Aphis gossypii* (Jones et al., 2011). The symbiont composition varies in aphid populations around the world (Augustinos et al., 2011; Zytynska, Weisser, 2016; Guo et al., 2017; Guidolin, Cônsoli, 2018). For instance, *Wolbachia*, *Spiroplasma*, and *Rickettsia* were not recorded among facultative aphid symbionts in the Saratov Region, Russia (Malyshina et al., 2014).

Symbiont infection research in aphids is of practical value since such studies are instrumental in developing new strategies of controlling pathogen transmission in plants (Heck, 2018). Depending on the symbiont type, the information on infection may be used for symbiont elimination or transinfection with a specific strain of bacteria to inhibit the vector's ability for pathogen transmission.

The goal of the present study was to investigate the incidence and genetic diversity of the *Wolbachia*, *Spiroplasma*, and *Rickettsia* symbionts in the samples of aphids collected in Moscow and the cities of Zvenigorod and Lyubertsy in the Moscow Region. Thus, 578 aphids from 32 collection sites were PCR-tested using specific primers for *Wolbachia*, *Spiroplasma*, and *Rickettsia*. The taxonomic positions of aphid hosts and their symbionts were identified based on the sequences of aphid and bacterial genes.

Materials and methods

Aphids were collected in July–September 2018 and in May 2019 in Moscow, Zvenigorod, and Lyubertsy (Table 1). A total of 32 aphid samples were collected from 17 plant species. The collected adult aphids were preserved in 96 % ethanol.

Phenol-chloroform extraction was used to isolate total DNA from individual specimens (Sambrook et al., 1989). Amplification reactions were performed in a 25-μl volume using the universal Encyclo Plus PCR kit (Evrogen, Moscow) following the manufacturer's protocol. All the reactions were performed using the MiniAmp Plus thermocycler (Applied Biosystems). Aphid species were identified based on PCR using universal primers LCO1490 and HCO2198 complementary to the 5′ end of the mitochondrial cytochrome c oxidase subunit I gene (*COI*), as described earlier (Folmer et al., 1994).

Symbionts were identified using specific primers as follows: RicF141 and RicR548 for the *Rickettsia gltA* gene (Goryacheva et al., 2017), spi_f1 and spi_r3 for *Spiroplasma* 16S rRNA (Sanada-Morimura et al., 2013), ftsZ-F1 and ftsZ-R1 for the *Wolbachia ftsZ* gene (Baldo et al., 2006). The amplification procedure consisted of initial denaturation for 4 min 30 sec at 94 °C followed by 36 amplification cycles as follows: denaturation for 30 sec at 94 °C, annealing for 30 sec at 59 °C (53 °C for spi_f1 and spi_r3; and 56 °C for *ftsZ*), and extension for 40 sec (1 min for spi_f1 and spi_r3) at 72 °C. The PCR was finalized by an extension for 5 min at 72 °C.

The PCR results were visualized by agarose gel electrophoresis (1.5 %). DNA fragments were eluted from gel using the

Table 1. Aphid and plant species and their dates and sites of collection

Aphid species (sample)		Collection site	Date	Plant species
Acyrthosiphon caraganae		55°41′37″ N, 37°51′55″ E	May, 2019	*Caragana arborescens*
Acyrthosiphon pisum		55°41′59″ N, 36°43′19″ E	July, 2018	*Pisum sativum*
Anoecia sp.	(1)	55°42′46″ N, 37°35′02″ E	September, 2019	*Cornus alba*
	(2)	55°42′51″ N, 37°35′14″ E		*Cornus sanguinea*
Anuraphis subterranea		55°41′22″ N, 37°51′34″ E	May, 2019	*Pyrus calleryana*
Aphis fabae		55°41′15″ N, 37°51′27″ E	May, 2019	*Philadelphus* sp.
Aphis pomi	(1)	55°42′17″ N, 37°31′30″ E	May, 2019	*Malus domestica*
	(2)	55°35′25″ N, 37°22′03″ E		*Cotoneaster* sp.
	(3)	55°41′37″ N, 37°34′15″ E		
Chaitophorus tremulae		55°35′13″ N, 37°22′20″ E	May, 2019	*Populus tremula*
Corylobium avellanae		55°41′49″ N, 37°33′54″ E	May, 2019	*Corylus avellana*
Dysaphis sp.		55°42′15″ N, 37°31′32″ E	May, 2019	*Malus domestica*
Dysaphis affinis		55°41′22″ N, 37°51′34″ E	May, 2019	*Pyrus calleryana*
Dysaphis devecta		55°42′19″ N, 37°31′26″ E	May, 2019	*Malus domestica*
Dysaphis plantaginea		55°41′18″ N, 37°51′24″ E	May, 2019	*Malus domestica*
Eucallipterus tiliae		55°41′45″ N, 37°33′52″ E	May, 2019	*Tilia* sp.
Hyalopterus pruni		55°42′46″ N, 37°37′16″ E	May, 2019	*Prunus cerasus*
Hyperomyzus lactucae		55°41′38″ N, 37°51′55″ E	May, 2019	*Ribes nigrum*
Impatientinum asiaticum	(1)	55°36′35″ N, 37°40′43″ E	September, 2018	*Impatiens parviflora*
	(2)	55°40′20″ N, 37°41′02″ E		
	(3)	55°39′51″ N, 37°40′05″ E		
	(4)	55°42′48″ N, 37°35′11″ E		
	(5)	55°50′30″ N, 37°38′04″ E		
Macrosiphum rosae		55°43′00″ N, 37°36′54″ E	May, 2019	*Rosa* sp.
Myzus cerasi	(1)	55°41′40″ N, 37°34′00″ E	May, 2019	*Prunus cerasus*
	(2)	55°42′45″ N, 37°37′17″ E		
Rhopalosiphum lonicerae		55°41′25″ N, 37°51′36″ E	May, 2019	*Lonicera tatarica*
Rhopalosiphum padi	(1)	55°42′03″ N, 37°34′35″ E	September, 2018	*Triticum* sp.
	(2)	55°41′31″ N, 37°51′25″ E	May, 2019	*Prunus padis*
	(3)	55°41′36″ N, 37°34′12″ E		
Schizaphis graminum	(1)	55°42′03″ N, 37°34′35″ E	September, 2018	*Triticum* sp.
	(2)	55°41′47″ N, 37°33′53″ E	May, 2019	

DNA isolation kit for agarose gels Cleanup Mini (Evrogen, Moscow) under the manufacturer's instructions. The purified PCR products were sequenced by Evrogen (Moscow). The newly acquired *COI* gene sequences were registered in the GenBank database under accession numbers MT302332–MT302357; *gltA Rickettsia*, under MT302358–MT302364; *ftsZ Wolbachia*, under MT302365–MT302368; and 16S *Spiroplasma*, under MT302369.

Sequence chromatograms were analyzed using the DNASTAR Lasergene 6 software (Clewley, 1995; Burland, 2000). The newly obtained sequences were compared to the previously available ones using the Barcode of Life (BOLD, http://www.barcodinglife.com/) and GenBank databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Dendrograms were plotted using the neighbor-joining method, Kimura evolutionary model, and 1000 bootstrap replications in the MEGA 6.06 software (Tamura et al., 2013). DNA sequences in the dendrograms were provided with the GenBank and PubMLST (in the case of *Wolbachia*) registration numbers on the right from the isolate name. The divergence between nucleotide sequences was calculated based on *p*-distances (per-site differences) using the MEGA 6.06 software (Tamura et al., 2013).

Results

Diversity of aphid species

To identify aphid species, the DNA barcoding method was used. The nucleotide sequences of *mitochondrial COI gene* were used to identify at least 21 aphid species in 32 samples, with 19 of them identified to a species level, and two, to a genus level, namely *Dysaphis* sp. from the apple tree *Malus domestica* and *Anoecia* sp. from the white dogwood *Cornus alba* and the common dogwood *Cornus sanguinea*. In latter cases, the sequence similarity was the highest with *Dysaphis apiifolia* (97 %) and *Anoecia fulviabdominalis* (96 %), respectively. The aphids collected represent four families as follows: Anoeciidae, Callaphididae, Chaitophoridae, and Aphididae (Fig. 1). Evolutionary divergence values amount to 6–16 % between the aphid genera, 0.8–6.6 % between the *Dysaphis* species and 6.3 % between *Aphis* species. Two mtDNA haplotypes were recorded in three species, namely *Aphis pomi*, *Chaitophorus tremulae*, and *A. pisum* (see Fig. 1). The differences between *COI* haplotypes were 0.2 % in *A. pomi*, 0.6 % in *C. tremulae*, and 0.16 % in *A. pisum*.

The apple aphid *A. pomi*, bird cherry-oat aphid *Rhopalosiphum padi*, black cherry aphid *Myzus cerasi*, and Asian balsam aphid *Impatientinum asiaticum* were found in two or more collection sites (see Table 1). Two aphid species were found in the same plant species in the following cases: *Hyalopterus pruni* and *M. cerasi* in cherry trees, *A. pomi* and *Dysaphis devecta* in apple trees, *Anuraphis subterranea* and *Dysaphis affinis* in pear trees, with the last aphid species coexisting on the leaves of the same tree (see Table 1). One aphid species was observed in different plant species in the following two cases: *R. padi* in bird cherry trees and wheat and *A. pomi* in apple trees and cotoneaster.

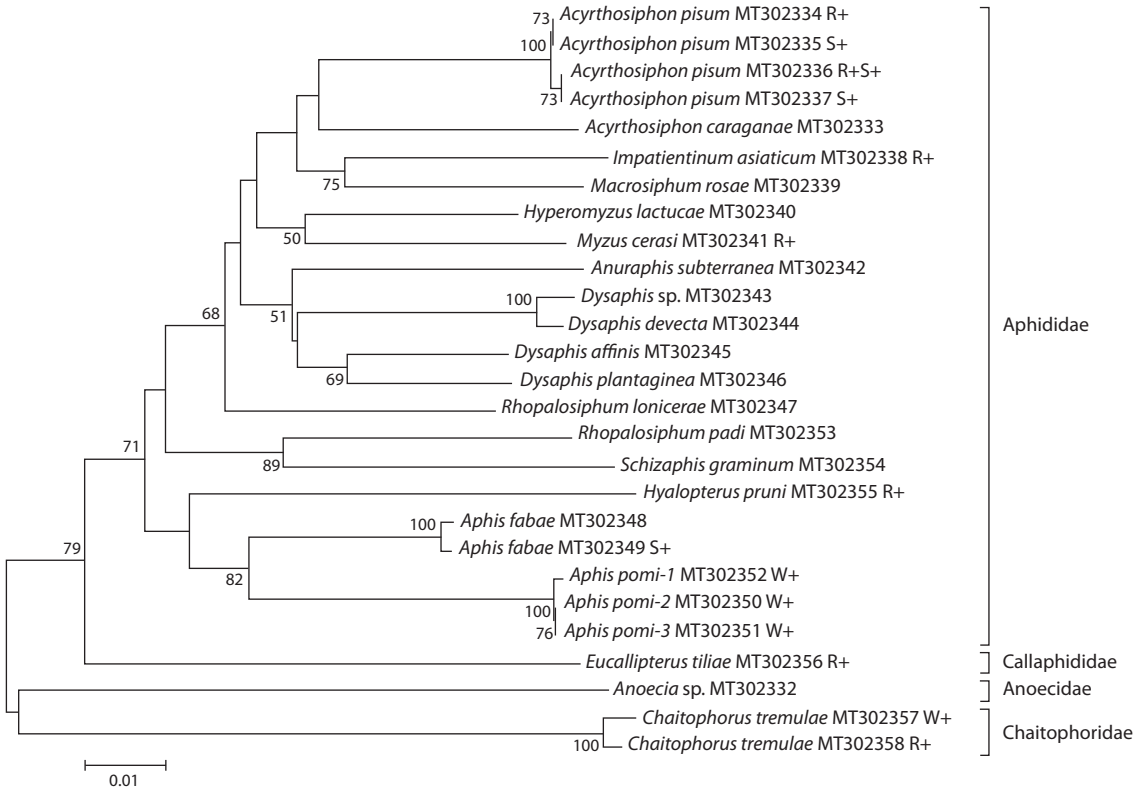

Fig. 1. Phylogenetic reconstruction of the studied aphids species based on the analysis of the 630-bp nucleotide sequences of the mitochondrial *COI* gene.

Individuals infected with *Wolbachia*, *Rickettsia* or *Spiroplasma* are designated W+, R+, S+, respectively.

Table 2. The occurrence of the symbionts in aphids

Aphid species (sample)	*N* of individuals studied	Symbiont genus and the number of infective		
		Spiroplasma	*Rickettsia*	*Wolbachia*
Acyrthosiphon caraganae	20	0	0	0
Acyrthosiphon pisum	28	24	17	0
Anoecia sp. (1)	17	0	0	0
Anoecia sp. (2)	12	0	0	0
Anuraphis subterranea	11	0	0	0
Aphis fabae	40	0	0	0
Aphis pomi (1)	11	0	0	11
Aphis pomi (2)	36	0	0	36
Aphis pomi (3)	26	0	0	26
Chaitophorus tremulae	9	0	3	2
Corylobium avellanae	10	0	0	0
Dysaphis sp.	9	0	0	0
Dysaphis affinis	9	0	0	0
Dysaphis devecta	10	0	0	0
Dysaphis plantaginea	30	0	0	0
Eucallipterus tiliae	16	0	5	0
Hyalopterus pruni	10	0	10	0
Hyperomyzus lactucae	18	0	0	0
Impatientinum asiaticum (1)	18	0	11	0
Impatientinum asiaticum (2)	18	0	8	0
Impatientinum asiaticum (3)	18	0	14	0
Impatientinum asiaticum (4)	18	0	8	0
Impatientinum asiaticum (5)	18	0	7	0
Macrosiphum rosae	27	0	0	0
Myzus cerasi (1)	8	0	1	0
Myzus cerasi (2)	23	0	0	0
Rhopalosiphum lonicerae	12	0	0	0
Rhopalosiphum padi (1)	30	0	0	0
Rhopalosiphum padi (2)	12	0	0	0
Rhopalosiphum padi (3)	6	0	0	0
Schizaphis graminum (1)	18	0	0	0
Schizaphis graminum (2)	30	0	0	0
Total (%)	578 (100 %)	24 (4 %)	84 (14 %)	75 (13 %)

Symbiotic bacterial infection

The presence of *Wolbachia*, *Spiroplasma*, and *Rickettsia* was analysed in 578 specimens of 21 aphid species and the infections were detected in *A. pisum* (*Spiroplasma* and *Rickettsia*), *I. asiaticum*, *M. cerasi*, *H. pruni*, *Eucallipterus tiliae* (*Rickettsia*), *A. pomi* (*Wolbachia*), and *Chaitophorus tremulae* (*Rickettsia* and *Wolbachia*) (Table 2). The *C. tremulae* specimens infected with *Rickettsia* and *Wolbachia* had different mtDNA haplotypes (see Fig. 1).

Rickettsia were found in 84 aphid specimens representing six species; *Wolbachia*, in 75 specimens of two species; and *Spiroplasma*, in 24 specimens of one species (see Table 2). Individual aphid specimens are typically infected by only one type of symbiotic bacteria out of three. However, *Rickettsia*/*Spiroplasma* coinfection was recorded in *A. pisum* in 13 of 28 individual specimens. In adult *A. pisum* specimens, 100 % were infected with one or two types of endosymbionts. The presence of *Rickettsia* in *I. asiaticum*, *M. cerasi*, *H. pruni*, *E. tiliae*, and *C. tremulae* and *Wolbachia* in *A. pomi* and *C. tremulae* has been described for the first time.

Bacteria of the genus *Rickettsia*. Aphid *Rickettsia* were clustered with the *R. bellii* group (Fig. 2). *Rickettsia* in *E. tiliae* and *C. tremulae* had species-specific *gltA* alleles. *Rickettsia* in *A. pisum* had two alleles, $gltA^1$ and $gltA^2$ (GenBank entries

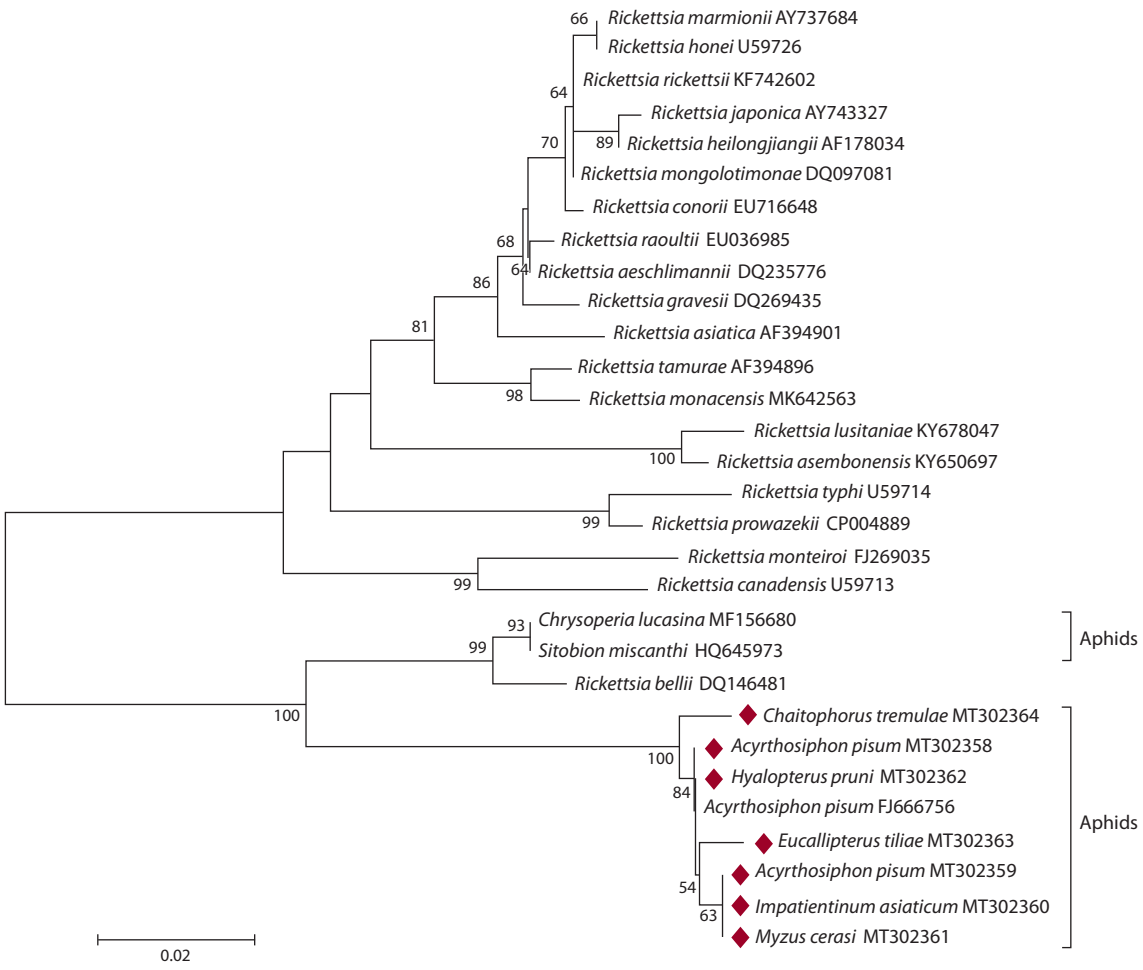

Fig. 2. Phylogenetic reconstruction of *Rickettsia* diversity according to the analysis of 343 bp of the *gltA* gene.
Bacteria from aphids are indicated by host species. The bacterial isolates obtained in this study are highlighted with diamonds.

MT302358 and MT302359), differing in one nucleotide substitution. The $gltA^1$ allele was observed in one *A. pisum* specimen, and $gltA^2$, in seven. The $gltA^1$ allele was also observed in *Rickettsia* from *H. pruni*, and $gltA^2$, in *Rickettsia* from *I. asiaticum* and *M. cerasi*. The $gltA^1$ allele was identical to the *Rickettsia* DNA from *A. pisum* strain PAR (the USA) registered in GenBank as FJ666756 (see Fig. 2). The $gltA^2$ allele has been discovered for the first time. The newly obtained *Rickettsia* DNA sequences differed significantly from bacterial DNA in *Sitobion miscanthi* aphids (HQ645973) genetically close to *R. bellii* (see Fig. 2). The evolutionary divergence value between the *R. bellii* group and *Rickettsia* group in the present paper amounted to 8.2 %, which greatly exceeded the divergence between, for example, the *R. typhi* and *R. prowazekii* species (2 %).

Bacteria of the genus *Wolbachia*. Within the genus *Wolbachia*, there are 16 phylogenetic supergroups (Glowska et al., 2016). *Wolbachia* from *Aphis pomi* #1 (MT302366) and #3 (MT302367) were clustered with supergroup M bacteria, and *Wolbachia* from *Chaitophorus tremulae* (MT302365) and *A. pomi* #2 (MT302368), with supergroup B (Fig. 3). The DNA differences in the *ftsZ* gene sequence of *Wolbachia* from *A. pomi* #2 (MT302368) and #1 (MT302366) amounted to 14.3 % (67 of 466 bp). At the same time, the *ftsZ* gene allele of *Wolbachia* from *A. pomi* #2 (MT302368) had only three different substitutions (0.6 %, 3 from 469 bp) if compared to the *Wolbachia* DNA from the Aspen leaf aphid *Chaitophorus tremulae* (MT302365).

The sample of *A. pomi* infected with group B *Wolbachia* was collected from cotoneaster, and the samples of *A. pomi* infected with group M *Wolbachia*, from cotoneaster and apple trees (see Table 1). The distance between the collection sites, where *A. pomi* were collected from the same plant species but infected with different *Wolbachia* strains, was over 20 km. At the same time, the distance between the collection sites, where *A. pomi* were collected from different plant species but infected with the same *Wolbachia* strain, did not exceed 4 km (see Table 1).

Bacteria of the genus *Spiroplasma*. *Spiroplasma* observed in *A. pisum* were clustered with the bacteria encountered in *A. pisum* in Japan (AB048263), Britain (JX943566, JX943567), and *A. craccivora* from the USA (KF362032) (Fig. 4). Variation of 16S rRNA genes of *Spiroplasma* in aphids from *A. pisum* collected in geographically separated sites amounted to 0.3–0.6 % (5–8 nucleotide substitutions per 974 bp). All of them are included in the *Spiroplasma ixodetis* clade. The latter also had the symbionts of other insects, such as lady beetles *Anisosticta novemdecimpunctata* (AM087471)

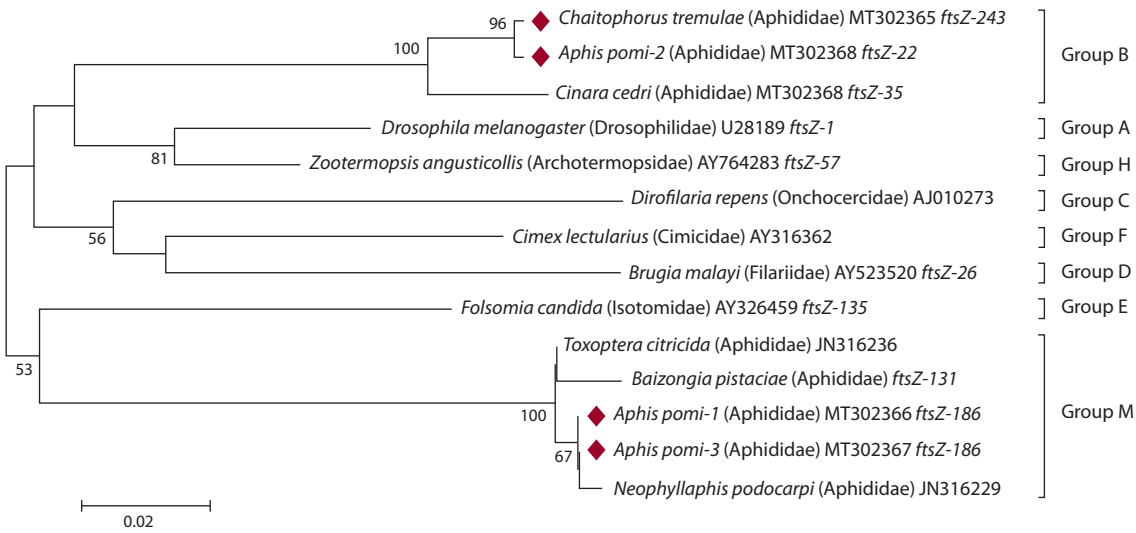

Fig. 3. Phylogenetic reconstruction of *Wolbachia* diversity according to the analysis of 466 bp of the *ftsZ* gene.

Wolbachia supergroups are shown on the right. Bacteria are indicated by host species. The bacterial isolates obtained in this study are highlighted with diamonds.

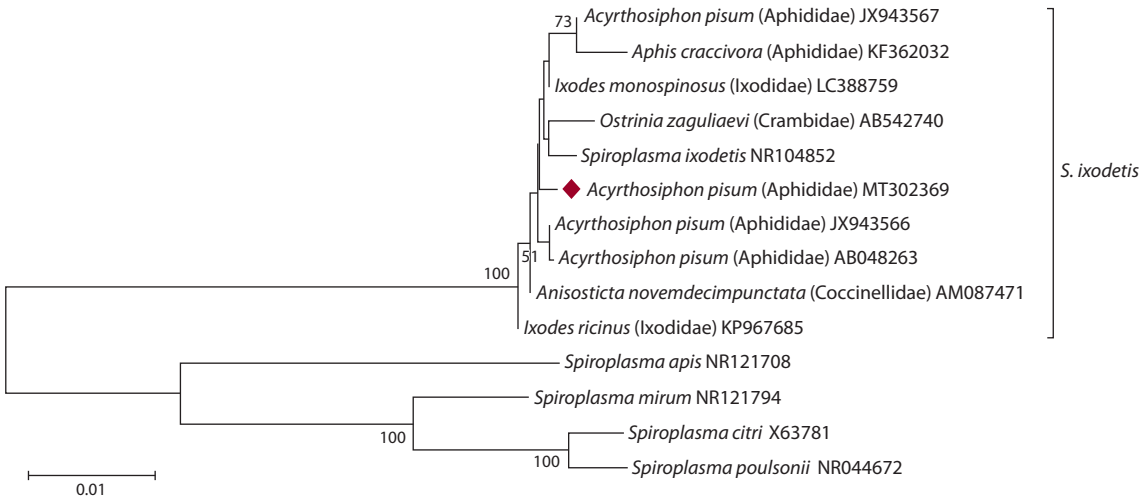

Fig. 4. Phylogenetic reconstruction of *Spiroplasma* diversity according to the analysis of 974 bp of the 16S rRNA gene.

Insect bacteria are indicated by host species. The bacterial isolate obtained in this study is highlighted with a diamond.

and the moth *Ostrinia zaguliaevi* (AB542740). Nucleotide variation within this clade did not exceed 0.6 %. However, nucleotide variation between the clades of the genus *Spiroplasma* reached 10–16 %.

Discussion

The study has demonstrated that at least 21 aphid species were represented in the 32 samples investigated. Most aphid species studied (18 of 21) belong to the family Aphididae, while the families Anoeciidae, Callaphididae, and Chaitophoridae are each represented by one species. Two species were not confined to their plant hosts, whereas 19 aphid species were encountered on particular plant species.

For the first time, *Rickettsia* infection has been observed in *I. asiaticum*, *M. cerasi*, *H. pruni*, *E. tiliae*, and *C. tremulae*, and *Wolbachia* infection, in *A. pomi* and *C. tremulae*. As opposed to other studies (see Zytynska, Weisser, 2016), aphid species collected in Moscow in the present research were mostly infected by *Rickettsia* rather than *Wolbachia.* The specimens of the Aspen leaf aphid *C. tremulae* infected with *Rickettsia* and *Wolbachia* have different mtDNA haplotypes, although it has yet to be studied in a larger number of samples whether cytoplasmic components are inherited together in this particular aphid species. *Rickettsia* and *Spiroplasma* coinfection was recorded in half of the specimens of the green pea aphid *A. pisum.* Many authors have already noted the presence of *Rickettsia* and *Spiroplasma* among the *A. pisum* facultative symbionts in Europe (Nyabuga et al., 2010; Ferrari et al., 2012; Gauthier et al., 2015), the USA (Russell et al., 2013; Smith et al., 2015), and Japan (Tsuchida et al., 2002). Among 28 *A. pisum* specimens in the sample from Zvenigorod, infection rates were 61 % (*Rickettsia*) and 86 % (*Spiroplasma*),

which is several times as large as the previous figures, 8 and 3 % in 119 isofemale lines from 81 populations in Japan (Tsuchida et al., 2002), 48 and 9 % among 318 specimens in the USA (Russell et al., 2013), 4 and 22 % in 30 lines (Nyabuga et al., 2010), 23 and 27 % in samples collected from eight legume species from England and Germany (Ferrari et al., 2012). It is possible that these high bacterial infection rates only existed for a short period, since both *Spiroplasma* and *Rickettsia* reduce the life span of aphids and inhibit their fertility (Simon et al., 2007, 2011; Mathé-Hubert et al., 2019). According to our observations, prolonged laboratory cultivation of *A. pisum* previously resulted in a loss of *Spiroplasma* symbiotic bacteria.

Aphid *Rickettsia* in our collections form a separate cluster. It was shown earlier that *Rickettsia* from *A. pisum* were attributed to the *R. bellii* group based on a comparison of four bacterial genes (Weinert et al., 2009). Other bacterial genes in different aphid species are to be studied to find out whether they form a separate species within the genus *Rickettsia.* The bacterial group *R. bellii* is the basal group of *Rickettsia* formed before the pathogenic spotted fever group and murine typhus group (Stothard et al., 1994). Four genetically different alleles of the *Rickettsia gltA* gene were discovered in the six aphid species studied, two of them encountered in *A. pisum*. One allele (MT302359) was observed in specimens from three aphid genera, another one (MT302358), in two genera, and the two remaining species had unique allelic variants of symbionts (MT302363-64). Thus, we may assume that different aphid species were infected with *Rickettsia* independently.

Spiroplasma discovered in *A. pisum* based on DNA analysis of the 16S rRNA gene is clustered with the bacteria previously observed in *A. pisum* and *A. craccivora.* It has been shown that *Spiroplasma* in *A. pisum* cause male offspring deaths at early larval stages, i. e. androcide or male-killing (Simon et al., 2011), and are genetically close to the *Spiroplasma* that caused androcide in aphid predators, i. e. lady beetles (Tinsley, Majerus, 2006) and moths (Tabata et al., 2011). All these bacteria are a part of the *Spiroplasma ixodetis* group. *S. ixodetis* is an endosymbiont described in ticks, but it is also common in other arthropods. Phylogenetic studies have shown that *S. ixodetis* strains are subjected to multiple horizontal transfers between ticks and other arthropods including aphids (Binetruy et al., 2019).

The present study has discovered *Wolbachia* infection in the apple aphid *A. pomi* for the first time. *Wolbachia* was found in 100 % of *A. pomi* specimens in our collections, even though this symbiont had not been observed in this aphid species from Greece earlier (Augustinos et al., 2011). The apple aphid *A. pomi* from three collection sites in Moscow (#1, 2, and 3 in Table 1) were infected with two *Wolbachia* strains, one attributed to supergroup B and another, to supergroup M (see Fig. 3), which had been described as prevalent in the aphid species of Spain, Portugal, Greece, Israel, and Iran (Augustinos et al., 2011), China (Wang et al., 2014), and the Azores (Moreira et al., 2019). Since the data shows the group's low genetic variation, one might assume that it emerged quite recently and spread rapidly across aphid populations (Wang et al., 2014).

The results allow us to assume that infection of *A. pomi* with a specific *Wolbachia* strain is not linked to the plant host species, but rather to its habitat. The noteworthy fact of discovering allelic variants of the *Wolbachia ftsZ* gene with only slight differences in various aphid species (*A. pomi* and *C. tremulae*) may imply the possibility of bacterial gene exchange during horizontal transfers of *Wolbachia* among insects, for example, through parasites or plants. This hypothesis is corroborated by the discovery of closely related alleles of *Wolbachia* genes in other unrelated insects (Ilinsky, Kosterin, 2017; Shaikevich et al., 2019). Symbiont DNA diversity is persuasive evidence of maternal inheritance and horizontal transfer being the key distribution mechanisms of facultative bacteria in aphids.

Conclusions

While laboratory research results provide an increasingly better understanding of the role of aphid symbionts, data on symbiont distribution in aphid populations in natural ecosystems are still insufficient. The present paper provides the first report on the genetic diversity of bacterial endosymbionts in previously understudied aphid species. Aphid population screening in Moscow and the Moscow Region made it possible to newly identify *Rickettsia* infection genetically different from infection with *R. bellii*, to which aphid symbionts are typically attributed, in six aphid species (Weinert et al., 2009). Whether these bacteria should be considered a new species is to be decided by studying a larger number of *Rickettsia* alleles. An apple aphid *A. pomi* infection with two *Wolbachia* strains has been discovered for the first time, one being in the supergroup B strain, which is genetically close to *Wolbachia* from Aspen leaf aphids. The second *Wolbachia* strain from *A. pomi* belongs to supergroup M. Regardless of the strain, 100 % of *A. pomi* specimens in the present study were infected with *Wolbachia*, and we thus may assume that there is a selection mechanism of infected specimens in place, which involves the reproductive manipulations discovered in *Spiroplasma* from *A. pisum* (Simon et al., 2011). However, most aphid generations are asexual, and if symbiont infections are maintained in natural aphid populations by reproductive effects, then this reproductive phenotype has to be of great value for the insect host. The obvious advantage of androcide is that it prevents inbreeding in the aphid population (Simon et al., 2011). As for now, it is unclear whether these advantages outweigh the possible side effects of symbiont infection during the asexual phase of the lifecycle. The positive effect, i. e. resistance against natural enemies (parasitoid wasps, pathogenic fungi, and heat stress), seems to be the key driver of an increasing incidence of *Wolbachia*, *Spiroplasma*, and *Rickettsia* facultative endosymbionts in aphid populations.

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ORCID ID

D.A. Romanov orcid.org/0000-0003-3340-9278
E.V. Shaikevich orcid.org/0000-0002-6504-5547

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