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The use of SSR-markers in rice breeding for resistance to blast and submergence tolerance

E. Dubina[1,2,*], P. Kostylev[3], S. Garkusha[1,2], M. Ruban[1], S. Lesnyak[1], Y. Makukha[1], S. Korzh[1], D. Nartymov[1] and O. Gorun[1]

[1]Federal Scientific Rice Centre, Laboratory of Informational, Digital and Biotechnologies, Belozerny 3, RU350921 Krasnodar, Russia
[2]Kuban State Agrarian University, Faculty of Agronomy, Department of Biotechnology, Biochemistry and Biophysics, Kalinina 13, RU350004 Krasnodar, Russia
[3]Agricultural research center 'Donskoy', Laboratory of Rice Breeding and Seed Production, Nauchny Gorodok 3, RU347740 Zernograd, Russia
[*]Correspondence: lenakrug1@rambler.ru

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Abstract. The identification of effective specialized DNA markers providing the clear control of target locus inheritance by the trait of submergence tolerance has been conducted. Among the studied set of microsatellite markers, two the most informative SSR-markers - RM 7481, PrC3 showed high efficiency in detecting intraspecific polymorphism of rice varieties and lines used in the work. With the use of these markers the clear genotype marking the obtained hybrid rice plants by this trait has been conducted and it is has been verified by phenotype evaluation as a result of laboratory trials. The plant samples carrying the target gene in heterozygous and homozygous state has been selected. About 400 backcrossed self-pollinated rice lines with introgressed and pyramided resistance genes *Pi-1, Pi-2, Pi-33, Pi-ta, Pi-b* to *Pyricularia oryzae Cav*. were obtained within the frameworks of program to develop genetic rice sources resistant to blast. The conducted testing for resistance to blast and the assessment by economically valuable traits have allowed to select the prospective rice samples. The plant samples of F2 and BC1F1 generations with combination of resistance to blast genes (*Pi*) and submergence tolerance gene (*Sub1A*) in homozygous and heterozygous state that is confirmed be the results of analysis of their DNA have been obtained. The obtained hybrid plants are being tested in breeding nurseries for a complex of economically valuable traits. The best plants will be selected and send to State Variety Testing system. Their involving in rice industry will reduce the use of plant protection chemicals against diseases and weeds, thereby increasing the ecology status of the rice industry.

Key words: rice, blast resistance genes, submergence tolerance genes, PCR analysis, DNA markers, rice varieties.

INTRODUCTION

Rice is the world's third most cultivated cereal crop. The Krasnodar region is the largest rice-growing area in Russia, having more than 80% of the country's sown area (Dubina, 2019). The main limiting biotic stress factors that prevent high rice yields and

reduce plant productivity are diseases, weeds, and pests. Unfavourable climatic conditions (high air temperatures, lack of water (drought), etc.) also restrain the growth of rice yields and threaten the food security of the region (Kumar & Bhagwat, 2012).

The introduction of genes (functions) of resistance to biotic and abiotic stressors into rice varieties with high productivity that are adapted to local environmental conditions, marker control, and the pyramiding of several target genes in one genotype, are considered relevant and promising directions in breeding (Singh et al., 2009; Negrao et al., 2011).

Genetic resistance is a strategy that allows for the obtaining of high yields of, to some extent, more environmentally friendly rice that meets the requirements of the modern consumer without loss and risk (Zelenskiy, 2016).

The use of SSRs makes it possible to accelerate the breeding process, and therefore, today, molecular breeding technologies are one of the prioritized and dynamically developing scientific directions for breeding programs (Dubina, 2019; Dubina et al., 2015; Mackill & Ni, 2001).

At the International Rice Research Institute (IRRI), using DNA marker selection and phytopathological testing, a number of monogenic lines, 'IRBLs', were developed on a genetic basis for local breeding varieties, carrying resistance genes *to Pyricularia oryzae Cav.*: *Pi-ta, Pi-b, Pi-z, Pi-k, Pi-5, Pi-i, Pi-1, Pi-9,* and *Pi-zt* (Dubina, 2019). Such lines are worthwhile both for breeding blast resistant rice varieties and for monitoring the racial composition of the blast pathogen. A number of ABL lines with the resistance genes *Pi-ta, Pi-b, Pi-z,* and *Pi-40* were developed at the National Institute of Crop Science (NICS) in South Korea using DNA marker selection (Mackill & Ni, 2001; Choi et al., 2006).

However, these varieties in domestic breeding programs for this trait can only be used as donors, because their growing seasons are over 155–170 days (Zelenskiy, 2016). For rice cultivation in the Russian Federation, the growing season for such varieties should be no more than 125 days (Dubina et al., 2015). Therefore, the development of a domestic pool of varieties with genes for resistance to these traits is extremely necessary.

An important stage in the history of rice breeding for resistance to water stress (long-term flooding) was the identification of the *Submergence 1A* or *Sub1A* locus, which controls this trait (Catling, 1992; Xu et al., 1996; Ito et al., 1999; Xu et al., 2006). It regulates the response to ethylene and gibberellin, which limits carbohydrate intake and the state of shoot rest in conditions of flooding, and promotes tolerance to prolonged and deep submergence (Xu et al., 2000). The Sub1 region is within the markers CR25K and SSR1A and covers more than 182 thousand base pairs. This interval encodes three genes for ethylene response factors, designated *Sub1A, Sub1B*, and *Sub1C,* but only *Sub1A* increases submergence tolerance in plants (Mackill & Ni, 2001). Researchers from the University of California at Riverside (USA) found that *Sub1A* allowed rice plants to survive by growing new shoots after a dry period (Kende et al., 1998; Steffens et al., 2006). Work on the development of varieties for long-term flooding has been carried out for several decades at IRRI (Hattori et al., 2009). Recently, a number of different genes for resistance to long-term flooding have been studied, including *Sub1A*. This work is significant for short rice varieties such as Swarna (Collard et al., 2013). One variety has been released in the Philippines as PSB Rc68 (Septiningsih & Kretzschmar, 2015).

Despite the fact that there are no flooding problems associated with the prolonged flooding of rice fields in Russia, this gene can be used as a factor for controlling the harmful weeds of rice fields - the *Echinochloa* species (Dubina et al., 2017; Dubina et al.,

2018). This strategy is not currently used in Russia due to several problems which are linked, firstly, to the deficiency of varieties carrying this gene, and secondly, to the high mortality of rice seedlings that do not have this gene on account of a lack of oxygen caused by raising the water level to combat weeds (Kostylev et al., 2015; Azarin et al., 2016; Azarin et al., 2017). Consequently, a rational approach to allowing rice defense without herbicides is to develop and grow varieties that are capable of anaerobic germination and are resistant to prolonged flooding (Steffens et al., 2006; Negrao et al., 2011).

Varieties with *Sub1A* developed by foreign colleagues can only be taken as donors in domestic breeding schemes for this trait due to their long growing seasons (Xu et al., 1996; Xu et al., 2000; Steffens et al., 2006; Xu et al., 2006; Hattori et al., 2009; Collard et al., 2013). This study was conducted with the following objectives: to identify of informative DNA-markers for blast resistance genes (*Pi-1, Pi-2, pi-33, Pi-ta, Pi-b*) and flooding tolerance (*Sub1A*); to introgress these genes in rice lines for the southern region of the Russian Federation; to identify promising lines with improved tolerance to blast and submergence. This research is of great importance for the development of fundamental and theoretical foundations, along with practical approaches to the use of SSR markers in rice breeding (Fukao et al., 2011; Kostylev et al., 2015; Mickelbart et al., 2015; Dubina et al., 2017; Dubina et al., 2018).

MATERIALS AND METHODS

Plant materials

Varieties of Asian origin, Khan Dan, Swarna, TDK-1, IR-64, CR 1009, Inbara-3, and BR-11, became donors for the introduction of *Sub1A,* a gene that confers tolerance to long-term flooding. The domestic rice varieties Lenaris, KP- 25, KP-163, and KP-24-15 were used as maternal forms. In the breeding program to increase blast immunity, the above-mentioned domestic varieties and breeding samples became the maternal forms, and the varieties and lines of foreign breeding became donors of target genes: BL-1 (*Pi-b*), C104-Lac (*Pi-1*), C101-A-51 (*Pi-2*), C101-Lac (*Pi-1, Pi-33*).

Breeding scheme

In the used breeding schemes, plants of donor and recipient forms, along with hybrid plants of BC (backcrossing) generations, were planted in growing vessels in artificial climate chambers (or in growing plots, depending on the season of the year) in triplicate with an interval of 3–10 days to synchronize flowering. Hybridization of rice plants was carried out by pneumocastration and pollination using the 'TVELL' method (Los, 1987).

Fig. 1 shows our proposed accelerated breeding scheme for developing a modern set of new genotypes resistant to biotic and abiotic stressors.

At the first stage of the breeding program, we crossed the domestic rice varieties and the rice varieties with resistance genes to *Pyricularia oryzae* (*Pi-ta, Pi-33, Pi-1, Pi- b,* and *Pi-2*) and also with the donor variety Khan Dan (paternal form), which possesses a gene for tolerance to long-term flooding, *Sub1A*, in its genotype. For each combination F1 generation was obtained. Then the first backcrossing event was carried out. We have planned to carry out two backcrossing events, since it is known that the replacement of the genome of the recurrent parent (RP) with backcrossing in BC2 is 87.5% (Jena et al., 2003; Dubina et al., 2018).

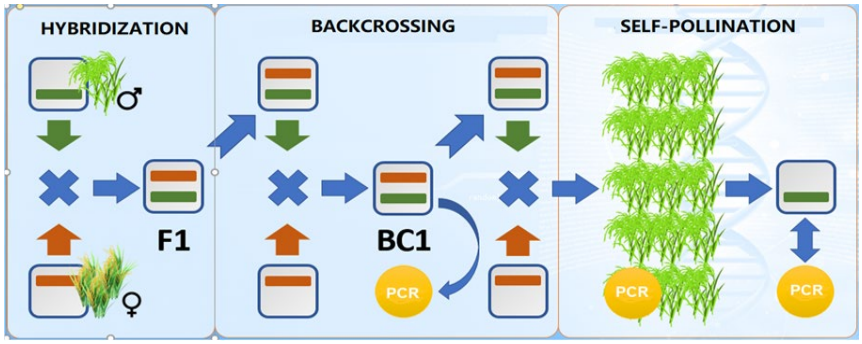


Figure 1. Scheme of introducing target genes into Russian rice varieties using molecular marking.

Note: F1 – the first generation hybrids; BC1 – the first backcrossing with the recurrent parent form; PCR – polymerase chain reaction.

Marker monitoring of the dominant allele of this transferred gene by PCR analysis was carried out at all stages of the breeding scheme, starting with BC1 (the first backcrossing event). Based on the results of DNA analysis, only plants that had dominant alleles of target resistance genes in their genotypes were selected for further study in the breeding nurseries for economically valuable traits.

Genome DNA extraction from the plant tissue

For molecular genetic studies, DNA samples of the analyzed rice plants were isolated from the freshly cut part of the leaf blade at the stage of 4–5 leaves by the CTAB method with some modifications (Murray, 1980).

PCR analysis for identification of *Pi*-genes

PCR was performed with 40–50 ng of DNA in a final volume of 25 μL. The following composition of the reaction mixture was used: 0.05 mM deoxyribonucleoside phosphates (dNTPs), 0.3 mM each primer, 25 mM KCL, 60 mM Tris-HCL (pH 8.5), 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 1.5 mM $MgCl_2$, 1 unit of Taq-DNA polymerase with standard buffer (SibEnzyme).

DNA amplification was carried out under the following conditions: Initial DNA denaturation at 94 °C - 4 min. Thirty cycles: 1 min - denaturation at 94 °C; 1 min - primer annealing at 55 °C; 1 min elongation at 72 °C. Finally: 5 min at 72 °C. The primers used in the studies (Table 1) were synthesized by Syntol (Moscow).

DNA amplification was performed on Tertsik amplifiers (DNA technology, Moscow) and Bio Rad (made in Germany).

The amplification reaction products were separated by electrophoresis in 8% polyacrylamide gel (PAGE).

Visualization of the result of electrophoretic separation of PCR products was performed using ethidium bromide.

Table 1. Nucleotide sequences of primers for identifying blast resistance gene

Marker name	Gene	Nucleotide sequence of primers (5'→3')	
Rm 224	*Pi-1*	F	ATCGATCGATCTTCACGAGG
		R	TGCTATAAAAGGCATTCGGG
Rm 144		F	TGCCCTGGCGCAAATTTGATCC
		R	GCTAGAGGAGATCAGATGGTAGTGCATG
Rm 72	*Pi-33*	F	CCGGCGATAAAACAATGAG
		R	GCATCGGTCCTAACTAAGGG
Rm 310		F	CCAAAACATTTAAAATATCATG
		R	GCTTGTTGGTCATTACCATTC
Rm 527	*Pi-2*	F	GGCTCGATCTAGAAAATCCG
		R	TTGCACAGGTTGCGATAGAG
SSR140		F	AAGGTGTGAAACAAGCTAGCAA
		R	TTCTAGGGGAGGGGTGTGAA
Pi-ta	*Pi-ta*	F1	GCCGTGGCTTCTATCTTTACATG
		R1	ATCCAAGTGTTAGGGCCAACATTC
		F2	TTGACACTCTCAAAGGACTGGGAT
		R2	TCAAGTCAGGTTGAAGATGCATCGA

PCR analysis for identification of *Sub1A* gene

To identify *Sub1A* gene in hybrid rice plants we used microsatellite molecular markers with specific primers (Table 2) associated with the locus responsible for resistance to prolonged immersion of rice under water.

The amplification was carried out in the 'Tertsik' DNA amplifier under the following conditions: at the first stage - denaturation for 5 min at 94 °C, then at the second stage - 5 cycles according to the following protocol: denaturation - 35 s at 94 °C; annealing of primers - 45 s at 60 °C; elongation - 30 s at 72 °C. The final stage included elongation at 72 °C for 5 min.

The amplification reaction products were separated by electrophoresis in 2% agarose gel. The electrophoresis was carried out at a voltage of 120–130 V for an hour. To visualize the electrophoresis result, an agarose gel plate was placed in the GelDocXR (BioRad) and then was photographed in ultraviolet light.

Table 2. Nucleotide sequences of primers for identification of *Sub1A*

Marker name	Nucleotide sequence of primers (5'–3')	
Sub1A203	F	GATGTGTGGAGGAGAAGTGA
	R	GGTAGATGCCGAGAAGTGTA
Sub1A_6	F	GATGTGTGGAGGAGAAGTGA
	R	GGTAGATGCCGAGAAGTGTA
Sub1A_7	F	GATGTGTGGAGGAGAAGTGA
	R	GGTAGATGCCGAGAAGTGTA
RM7481	F	CGACCCAATATCTTTCTGCC
	R	CATTGGTCGTGCTCAACAAG
RM285	F	CTGTGGGCCCAATATGTCAC
	R	GGCGGTGACATGGAGAAAG
RM219	F	CGTCGGATGATGTAAAGCCT
	R	CATATCGGCATTCGCCTG
RM 464A	F	AACGGGCACATTCTGTCTTC
	R	TGGAAGACCTGATCGTTTCC
RM 285	F	CTGTGGGCCCAATATGTCAC
	R	GGCGGTGACATGGAGAAAG
Sub1A	F	CAGGAATAAGTAGGCACATCA
	R	GGACCAAGAACAAAGTCAAA
AEX	F	AGGCGGAGCTACGAGTACCA
	R	GCAGAGCGGCTGCGA
PrC1	F	TTGC GAGCTAGCTGTCTGAA
	R	TAGTCCACGCGCTAATGTGA
PrC3	F	CAATAAGACTCGGGCTGTGC
	R	TAGTCCACGCGCTAATGTGA
GnS2	F	CTTCTTGCTCAACGACAACG
	R	TCGATGGGGTCTTGATCTCT

The field trials

To assess for agricultural significant traits, the analyzed breeding rice plants with *Pi-* genes were sown on a rice irrigation system after predecessor of perennial grasses at the FSI ESOS "Krasnaya" branch of 'Federal Scientific Rice Centre' (FSBSI), Krasnoarmeysky district. All agrotechnical works and phenological observations were conducted in pursuance of FSBSI's generally accepted methodology (Practical guidance…, 1980). The timing of the vegetation phases before flowering and full ripeness were taken into account. Lodging and shedding were estimated in the phase of full ripeness. Varietal weeding was conducted in order to remove atypical plants in the experimental area. For biometric analysis, 25 typical plants of promising samples were selected, which were distinguished by agricultural significant traits and their resistance to *Pyricularia oryzae* (Dospekhov, 1979; Podkin, 1981).

The phytopathology testing

Evaluation of rice donor lines BL-1 (*Pi-b*), C104-Lac (*Pi-1*), C101-A-51 (*Pi-2*), C101-Lac (*Pi-1, Pi-33*), and IR 83260-2-10-5-2-1-B (*Pi-40*), and breeding samples for resistance to the local population of *Pyricularia oryzae Cav.*, was carried out on a vegetation plot of FSBSI according to methodological guidelines Kovalenko, 1988). As susceptible controls, we used rice varieties Volgogradsky and Pobeda 65; as a resistant control, we used the rice variety Avangard.

Rice plants were infected with *P. oryzae* fungi culture in the phase of 4–5 leaves by spraying them with a suspension of its conidia by spraying from the sprinkler at a normal flow rate of a suspension of 0.5 mL per plant For better adhesion, 'Tween' was added to the suspension at the rate of 1 drop per liter of water (Fig. 2, Kolomiets, 1990).

Figure 2. Artificial infection of donor lines and breeding material with the phytopathogenic fungus *Pyricularia oryzae Cav.*

Note: a – the spraying of analyzed rice plants by suspension of *P. oryzae*; b – the plants, treated by the suspension of *P. oryzae*; c – the growing of the *P. oryzae* conidia on the slide in Petri dishes for the evaluation of the *P. oryzae* spores vitality by microscoping while rice plants infecting period.

The estimation of plants damaged by *Pyricularia oryzae* was conducted on the 14th day after inoculation, in accordance with the express method for assessing rice varietal resistance to blast. The assessment was carried out by taking into account two indicators: type of reaction (in points) and degree of damage (in percentages), using the ten-point scale of the International Rice Research Institute (Kovalenko, 1988; Kolomiets, 1990): (1) resistant, 0–1 points - the absence of damage, small brown spots, covering less than 25% of the total leaf surface; (2) medium resistant, 2–5 points - typical elliptical blast spots, 1–2 cm long, covering 26–50% of the total leaf surface; (3) non-resistant,

6–10 points - typical elliptical blast spots, 1–2 cm long, covering 51% and more of the total leaf surface.

The intensity of disease development (IDD,%) was calculated by the formula (1):

$$IDD = \sum \frac{a \cdot b}{9n} \quad (1)$$

where IDD – the intensity of disease development, %; $\sum(a \cdot b)$ – the sum of the products of the number of infected plants multiplied by the corresponding damage score; n – the number of recorded plants, pcs (pieces).

Depending on the damage score, all varieties were conventionally divided into 4 groups: (1) resistant; (2) intermediate; (3) susceptible; (4) highly susceptible.

The laboratory express method for submergence tolerance

Evaluation of donors and domestic rice varieties for tolerance to prolonged flooding was carried out in laboratory conditions. The seeds of hybrid plants were grown in test tubes (Skazhennik et al., 2009; Linh et al., 2013). Further, when the seeds germinated, the tubes were placed in an artificial climate chamber with a light regime of day for 12 h and night for 12 h at a temperature of 30 °C. After the sprouts reached a length of 2–3 cm (Fig. 3, a), the plants in the tubes were filled with water (Fig. 3, b) and on the 15th day the plants were assessed for tolerance to flooding (Fig. 3, c). The evaluation of the plants for submergence tolerance was conducted visually (Vergara et al., 1976; Catling, 1992; Manangkil et al., 2008).

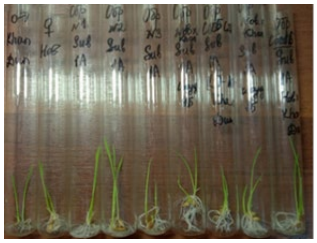
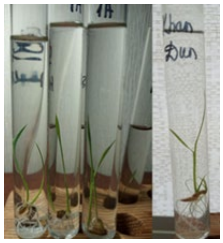
a)  b)  c) 

Figure 3. Laboratory-based express method testing the submergence tolerance of rice plants.
Note: a – growing hybrid rice plants seeds with Sub1A – gene in the tubes; b – the flooding sprouts with the water; c – the evaluation of hybrid plants for submergence tolerance.

Biometric rates (the plant height, the panicle length, the grain mass from one panicle, the mass of 1,000 grains) of experimental plants were counted by the software Microsoft Office Excel 2010 and STATISTICA 10.0 for Windows.

RESULTS AND DISCUSSION

Study of Sub1A genes/loci that control the defense reactions of rice plants to prolonged immersion under water - a factor in weed control

Recently, the usage of nature-saving technologies while growing agricultural crops and developing prospective germplasm resistant to stressors has been increased (Dubina et al., 2015, 2017, 2018).

Earlier, we mentioned that the *Sub1A* gene contributes resistance to prolonged flooding and that it could be used as a factor for combating weeds in rice cenosis.

Thirteen microsatellite markers were taken from the NCBI database (www.ncbi.nih.gov) for visualization of the donor and recessive alleles of the gene *Sub1A*. Their sequences are shown in the Materials and Methods section. For each specific pair of primers, protocols for the optimal composition of the reaction mixture and the amplification reaction program were developed, as a result of which, when performing SSR analysis under these conditions, the amplification products were clearly visualized (Figs 4–6).

Each molecular marker was tested on contrasting varieties: those both resistant (Khan Dan, TDK-1, CR 1009, Swarna, IR-64, BR-11, Inbara-3) and susceptible (KP-23, Contact, Boyarin, KP-25, KP-163, Flagman, Lenaris) to submergence tolerance.

It has been seen from the Fig. 4 that there is no polymorphism between analyzed plants by Sub1A203 locus. Therefore, this locus can't be used for the identification of the Sub1A-gene donor alleles.

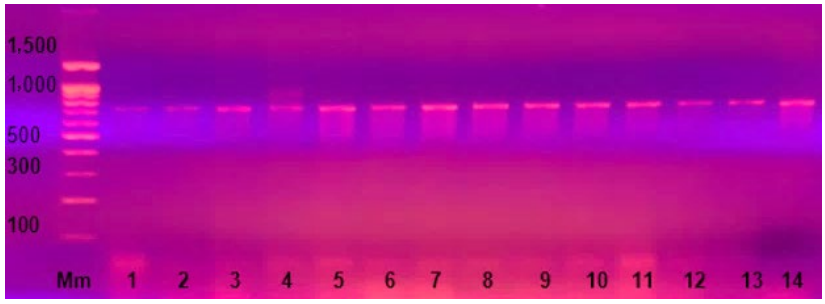


Figure 4. Visualization of amplification products at the Sub1A203 locus.

Note: Mm – molecular weight marker pBR322/BsuR I (supplier: Helicon, Russa), base pairs (bp); 1 – Khan Dan; 2 – TDK-1; 3 – CR 1009; 4 – Swarna; 5 – IR-64; 6 – BR-11; 7 – Inbara-3; 8 – KP-23; 9 – Contact; 10 – Boyarin; 11 – KP-25; 12 – KP-163; 13 – Flagman; 14 – Lenaris. The molecular mass is always measured in base pairs (bp).

Fig. 5 shows the allele variants of tolerant (1–7) and susceptible (8–14) rice varieties by the trait 'submergence tolerance'. The tolerant allele has PCR-product with size of 105 bp (base pairs), the susceptible one has 95 bp allele by this locus.

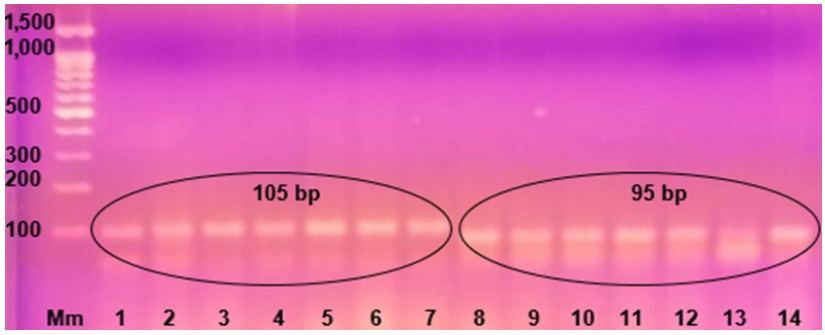


Figure 5. Visualization of amplification products at the RM 7481 locus.

Note: Mm – molecular weight marker pBR322/BsuR I (supplier: Helicon, Russia); 1 – Khan Dan; 2 – TDK-1; 3 – CR 1009; 4 – Swarna; 5 – IR-64; 6 – BR-11; 7 – Inbara-3; 8 – KP-23; 9 – Contact; 10 – Boyarin; 11 – KP-25; 12 – KP-163; 13 – Flagman; 14 – Lenaris.

Fig. 6 also demonstrates the allele variants of tolerant (1–7) and susceptible (8–14) rice varieties by the trait 'submergence tolerance'. The tolerant allele has PCR-product with size of 703 bp, the susceptible one has 203 bp amplicon by this locus.

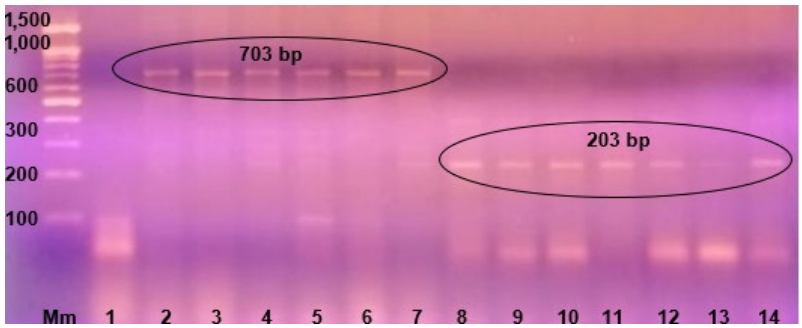


Figure 6. Visualization of amplification products at the PrC3 locus.

Note: Mm – molecular weight marker 100 bp + 1.5 Kb (supplier: comp. Syntol, Russia); 1 – Khan Dan; 2 – TDK-1; 3 – CR 1009; 4 – Swarna; 5 – IR-64; 6 – BR-11; 7 – Inbara-3; 8 – KP-23; 9 – Contact; 10 – Boyarin; 11 – KP-25; 12 – KP-163; 13 – Flagman; 14 – Lenaris.

The figures show that from the studied set of microsatellite markers, high efficiency in identifying the intraspecific polymorphism of the rice varieties and lines used in the work was shown by the two most informative SSR markers - RM 7481 and PrC3. The results were introduced into the breeding program for the development of rice varieties tolerant to prolonged flooding.

To compare molecular genetic studies, phenotypic analysis of parental forms was conducted, as described in the Materials and Methods section. As a result of the experiment, it was noted that the plants of the cultivated domestic rice varieties (maternal form) were elongated. This can be explained the fact that they belong to the *japonica* species, and it is known from literary sources (Vergara et al., 1976; Catling, 1992) that this species is characterized by the presence of genes (SK1/Sk2) which cause increased growth. This mechanism is based on the activation of ethylene accumulation, which reduces the amount of abscisic acid and increases the level of gibberellic acid, which cause increased growth of plants (Steffens et al., 2006; Hattori et al., 2009). For field conditions, this leads to the risk of the lodging of plants when water is discharged, and subsequent plant death.

In rice plants of the donor variety Khan Dan with the *Sub1A* gene, stunted growth was observed - i.e., they were in a state of rest. After 15 days in the full flooding regime, the water from the tubes was drained, and within 2–3 days the plants came out of the stressed state and began vegetation.

Recently, a BC1F2 population was obtained, which was verified by PCR analysis for the existence of the *Sub1A* gene in the genotype (Figs 7, 8).

From 184 plants of the hybrid combination Novator x Khan Dun, 143 plants carrying the target gene were selected, which were sown at the lysimetric site of the FSBSI for assessment by economically valuable traits. The selected plants with the *Sub1A* gene were involved in the backcross with recurrent parental forms. The work on saturating crosses was started with the aim of producing rice lines with a complex of valuable traits of the recurrent parental form, an effective *Sub1A* gene for submergence tolerance, and a vegetation period of up to 125 days.

Fig. 7 shows that hybrid plants No. 64/3, 64/5, 66/2, 66/3, 66/4, 66/5 have a specific allele of the Sub1A gene. Samples No. 64/3, 64/5 are homozygotes for this locus, and No. 66/2, 66/3, 66/4, 66/5 have maternal and paternal alleles. Plants that did not have the Sub1A gene in the genotype as a result of analysis of their DNA were rejected.

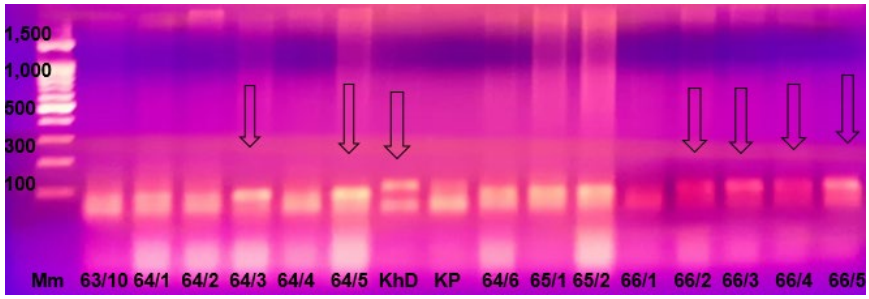


Figure 7. Results of PCR-analysis by RM7481locus.

Note: Mm – molecular weight marker 100 bp + 1.5 Kb (supplier - Syntol, Russia); 63/10–66/5 – hybrid plants; KhD – donor variety Khan Dan; KP – KP-163 line (maternal form); the arrows show the dominant allele.

The electrophoresis gel in Fig. 7 shows that only the hybrid sample 37/8 had a dominant allele of the *Sub1A* gene in its genotype. The rest of the samples had a recessive allele and were thus rejected.

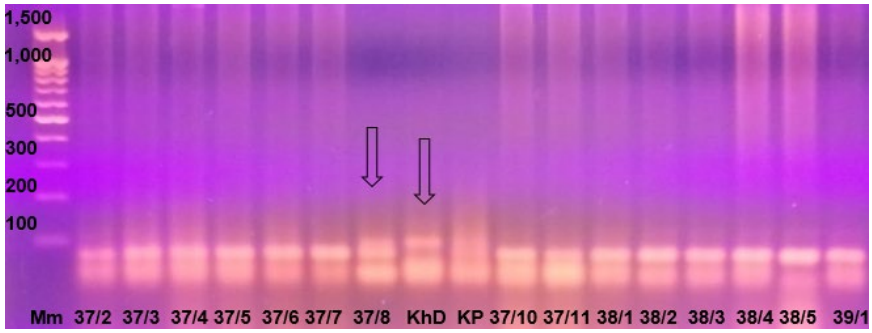


Figure 8. Results of PCR analysis by RM7481 locus.

Note: Mm – molecular weight marker 100 bp + 1.5 Kb (supplier: Syntol, Russia); 37/2–39/1 hybrid plants; KhD – donor variety Khan Dan; KP – KP-23 line (maternal form); the arrows show the dominant allele.

In the hybrids KP-163 × KhanDan and KP-23 × KhanDan, segregation took place in a ratio of 15:5 and 14: 6, respectively, or approximately 3: 1, i.e. close to Mendelian.

The deviations in segregation of the two combinations can be explained by the influence of selection and gene linkage. The best plants were selected to obtain F3-progenies.

The proposed accelerated scheme for the development of a modern set of rice lines carrying the target genes will make it possible to develop varieties that correspond to the environmental conditions of the South of Russia, with increased productivity, milled rice quality, resistance to *P. oryzae* and drought, and submergence tolerance, to combat weeds in the future. The proposed scheme will contribute to the production of environmentally

friendly products, saving money for rice producing enterprises, since it will allow for a significant reduction in, if not a complete abandonment of the use of pesticides, which will increase the ecological status of the rice production and economy of the region. Ultimately, it will have a beneficial effect on the health and longevity of the nation.

Development of a modern set of rice lines with genes *Pi-ta, Pi-1, Pi-b, Pi-2, Pi-40*, and *Pi-33*, which controls rice protective reactions against the pathogen *Pyricularia oryzae Cav.*

Breeding rice varieties with increased resistance to blast disease (the most dangerous disease in the world), and introducing these into production, is becoming increasingly necessary.

In the program aimed at pyramiding blast resistance genes in one genotype based on the rice varieties Flagman and Snezhinka, and large grain lines with a short growing period, KP-163 and KP-24-15, in artificial climate chambers and on the growing plot of the FSBSI, about 400 backcross self-pollinated lines of the BC2F3 population with pyramided blast resistance genes ((*Pi-33, Pi-1, Pi-b, Pi-2*); (*Pi-1, Pi-33, Pi-2*); (*Pi-33, Pi-b*); (*Pi-1, Pi-2*)) were propagated.

Assessment of the donor rice lines BL-1 (*Pi-b*), C104-Lac (*Pi-1*), C101-A-51 (*Pi-2*), and C101-Lac (*Pi-1, Pi-33*), and breeding samples for the local population of *Pyricularia oryzae Cav*. was performed at a vegetation site of the FSBSI according to methodological guidelines (Los, 1987). As the susceptible control, we used the rice varieties Volgogradsky and Pobeda 65, and as the resistant control, we used the rice variety Avangard.

The results of assessing the breeding samples for blast resistance and some indicators for economically valuable traits are presented in Table 3.

Table 3. Some characteristics of rice breeding samples with genes *Pi-1, Pi-2, Pi-33, Pi-ta,* and *Pi-b,* grown on the growing plot of FSBSI in 2021

Line name/ origin	Vegetation period, days	Plant height, cm	Mass of 1,000 grains, g	IDD*, %
Flagman (St)	115–117	90–95	26.7–28.4	54.6–70.5
Avangard (St)	115–117	105–110	-	0
1/ Flagman × Bl-1/ Flagman	105–110	75–80	25.4–26.8	20.0
2/ Flagman × IR-36/ Flagman	110–115	80–85	27.8–29.3	12.6
3 /Flagman × C101-A-51	105–110	85–90	27.3–28.4	4.4
4 /Flagman x Bl-1/ Flagman	105–115	75–80	24.4–25.2	23.3
5 /Flagman × Bl-1/ Flagman	105–115	75–80	25.3–27.1	17.6
6 /Flagman × A-51/ Flagman	100–105	85–90	27.5–29.1	7.8
7 /Flagman x Bl-1/ Flagman)	105–110	80–85	25.3–27.3	18.7
8 [Fl × IR-36/ Fl] × [Fl× Bl-1/ F]	112–117	85–90	27.7–29.3	16.4
9/Flagman× A-51/ Flagman	100–105	85–90	28.4–30.1	6.8
10/(Flagman x Bl-1/ Flagman	105–110	80–85	25.6–26.6	17.6
11/Flagman x Bl-1/ Flagman	110–115	80–85	25.5–26.4	25.6
12 /Flagman × A-51/ Flagman	105-110	85–90	29.2–30.4	12.3
13 /Flagman × C101 Lac/ Flagman	110–115	80–85	27.3–28.1	17.4
14/Flagman × C101 Lac/ Flagman	110–115	80–85	28.4–29.2	13.3
15/Flagman× C101 Lac/ Flagman	110–115	80–85	26.9-28.1	10.2
16/Flagman × C101 Lac/ Flagman	110–115	85–90	27.6–28.9	18.5

Table 3 (continued)

17/Flagman × C101 Lac/ Flagman	110–115	85–90	27.7–29.3	10.8
19 [(Flagman x C101Lac\ Flagman)] × [(Flagman × A-51 \ Flagman)]	115–117	80-85	29.3–30.2	6.8
20 Flagman x Bl-1/ Flagman	110–115	80–85	25.4–27.3	25.6
21 [(Flagman x C101Lac\ Flagman)] × [(Fl. × A-51 \ Fl.]	115–117	80–85	27.8–29.2	5.6
22 [(Flagman x C101Lac\ Flagman)] × [(Flagman × A-51 \ Flagman)]	115–117	80–85	28.8–30.1	6.4
23 Flagman × IR-36/ Flagman	118–120	75–80	29.8–30.1	15.6
24 [(Flagman x IR-36\ Flagman)] × [(Flagman ×C101) \ Flagman]	115–117	80–85	28.6–30.1	12.3
25 [(Flagman x IR-36\ Flagman)] × [(Flagman ×C101) \ Flagman]	115–117	80–85	28.7–30.3	11.7
26 [(Flagman x IR-36\ Flagman)] × [(Flagman ×C101) \ Flagman]	115–117	85–90	23.2–25.4	11.5
27 [(Flagman x IR-36\ Flagman)] × [(Flagman×C101) \ Flagman]	115–117	85–90	23.5–25.5	10.8
28 Flagman x Bl-1\ Flagman	110–115	85–90	26.6–29.3	27.5
29 Flagman × A-51/ Flagman	115–120	85–90	28.3–30.3	12.1
30 Flagman x IR-36\ Flagman	115–117	77–80	30.1–30.4	15.6
31 Flagman x C101Lac/ Flagman	110–115	85–90	27.3–28.3	17.8
C101Lac-A-51 (*Pi-2*)	-	-	-	0.0
Bl-1 (*Pi-b*)	-	-	-	8.4
C101Lac	-	-	-	2.0
IR-36 (*Pi-ta*)	-	-	-	1.2
7-5-1 *(Pi-40)*	-	-	-	0.0

∗IDD – intensity of disease development; St – standard rice varieties; Avangard (St) – resistant rice variety.

Figs 9 and 10 show some results of the PCR analysis for the identification of the above-mentioned blast resistance genes in the hybrid material.

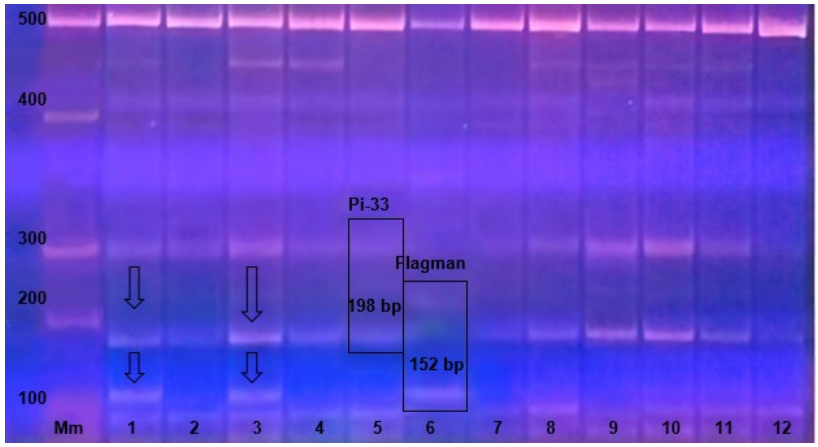


Figure 9. Electropherogram of genomic DNA amplification products of loci RM310 and RM72.

Note: 1–4, 7–12 – analyzed hybrid plants; 5 – donor line C101-Lac of the Pi-33 gene, which determines that the resistance gene is 198 bp; in varieties with a recessive allele, this is 152 bp; Flagman – recurrent parental form.

Fig. 9 shows that plants 2, 4, and 7–12 were homozygous for the dominant allele; plants 1 and 3 were heterozygotes.

Fig. 10 shows that plants 2, 7, and 9 were homozygous for the *Pi-b* gene.

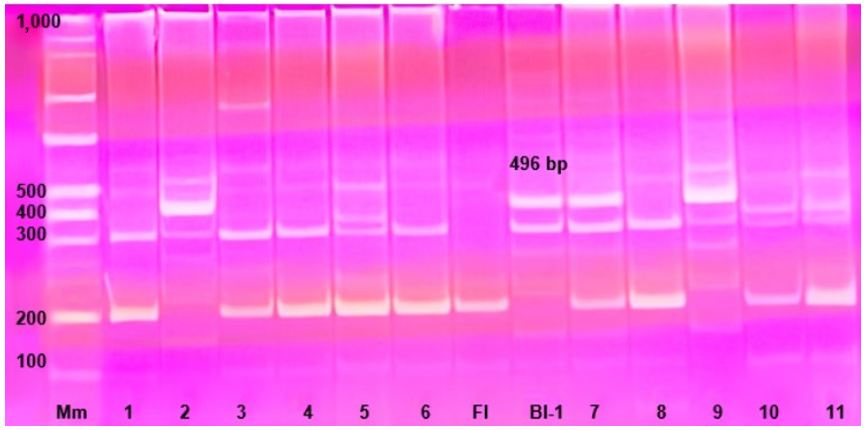


Figure 10. Electropherogram of genomic DNA amplification products of the *Pi-b* locus.
Note: 1–11 – analyzed hybrid plants; Fl – rice variety Flagman (maternal form); Bl-1 – line donor Bl-1 of the *Pi-b* gene, which determines that the resistance gene is 496 bp.

Thus, the results of PCR analysis (Fig. 10) show that samples 2 and 9 had four blast resistance genes in their genotypes (*Pi-1, Pi-2, Pi-33, Pi-b*); samples 4, 8 and 11 had three resistance genes (*Pi-1, Pi-2, Pi-33*); sample 7 had the genes *Pi-33* and *Pi-b*; sample 6 had genes *Pi-1* and *Pi-2*; samples 10 and 12 had the *Pi-33* gene.

The plant forms which had donor resistance alleles in their genotypes were selected and then sown in the field for evaluation by economically beneficial traits. Some of the results of this evaluation are presented in Table 3.

The data from the Table 3 demonstrate that the obtained plants have vegetation period of 110–120 days that corresponds growing conditions in the South of Russia. They have plant height of between 75 cm (undersized plants) and 90 cm (medium-sized), don't lie down and have the mass of 1,000 grains of between 25 g (medium-grained) and 30 g (coarse-grained). The resistance to blast rate is between 4.4 and 20% (for resistant plants) and between 25–27% (for susceptible plants).

Currently, samples with target genes in their genotypes are being studied in the field for agricultural valuable traits, and we also plan to conduct an assessment of blast resistance at test sites of a rice irrigation system after using a predecessor of perennial grasses. The best ones will be selected for further study. This will make it possible to develop varieties appropriate to the environmental conditions of the South of Russia. Increased productivity and blast resistance are two key traits they should possess. Rice resistance to diseases plays an important role in grain production, as it allows rice to be grown without the use of toxic fungicides, which would improve the ecological impact of the rice industry and the economy of the region.

Development of rice lines with complex resistance to blast and submergence tolerance

To diversify the gene pool of rice with complex blast resistance and submergence tolerance, we crossed rice lines with the genes *Pi-2* and *Pi-33* with the Khan Dan variety (*Sub1A*) to obtain breeding material with united genes for submergence tolerance and resistance to disease. The F_2 and BC_1F_2 generations were obtained using climatic chambers.

In order to increase the efficiency of MAS (marker-assisted selection), we developed multi-primer systems for the identification of two target genes at the same time. A protocol of DNA analysis was designed where the specific PCR products of target genes were visualized clearly.

Fig. 11 demonstrates the results of DNA marker systems for the simultaneous identification of two genes: *Pi-2* and *Sub1A*.

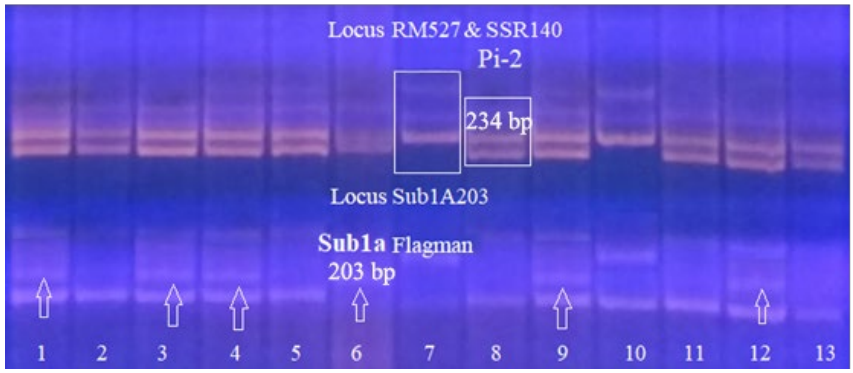


Figure 11. Results of visualization of PCR products of loci RM527 and SSR140 for Pi-2, showing that this resistance gene is 234 bp; and the products of the Sub1A203 locus for Sub1A, which show that this resistance gene is 203 bp in 8% PAAG.

Note: 1–4, 7–13 – analyzed hybrid plants of BC1F2 generation; 6 – rice variety Khan Dan, donor of the Sub1A gene; 7 – Flagman, recurrent parental form.

Fig. 11 shows that samples 3 and 12 had dominant alleles of the *Pi-2* and *Sub1A* genes in a homozygous state in their genotypes; samples 1, 4, 9, and 12 were homozygotes for the *Sub1A* gene and had *Pi-2* in their genotypes in a heterozygous state; sample 10 was recessive homozygous for the two target genes and was thus rejected. Clear identification on the electropherogram made it possible to reliably identify the presence of the dominant alleles of target genes.

The involvement of such varieties in rice production will help avoid the epiphytotic development of disease, preserving rice yields and obtaining environmentally friendly agricultural products.

Thus, through MAS (breeding with the use of molecular markers), an important national economic problem can be solved - namely, rice varieties with increased resistance to blast and high yields can be developed.

The identification of effective specialized DNA markers which provide clear control over the inheritance of the target locus was performed. Of the investigated set of microsatellite markers based on tolerance to long-term flooding, four of the most informative SSR markers, RM 7481, PrC1, Gns2, and PrC3, showed high efficiency in identifying the intraspecific polymorphism of the rice varieties and lines used in the work. The identities of the microsatellite markers RM 7481, PrC1, Gns2, and PrC3, linked to the trait 'tolerance to long-term flooding', were established. The results were introduced into the breeding program for the development of rice varieties tolerant to prolonged flooding (for weed control).

On the basis of the selected informative SSR markers, closely linked with the trait 'tolerance to prolonged flooding', clear genotype marking of the analyzed hybrid rice plants for this trait was carried out, which was confirmed by phenotype assessment during laboratory testing. Plants that had a target gene in a hetero- or homozygous state were selected.

Within the framework of the program for development of rice genetic resources resistant to biotic stressors (blast), about 400 backcross self-pollinated rice lines with introgressed and pyramided genes for resistance to *P. oryzae Cav.* (*Pi-1, Pi-2, Pi-33, Pi-ta, Pi-b,* and *Pi-40*) were obtained. A test for blast resistance and an assessment of the economically valuable traits of experimental plants with introgressive and effective *Pi* genes for the south of Russia were performed. Promising samples were selected.

To increase economic efficiency and to reduce labor costs, multi-primer systems were developed to identify two genes (*Pi and Sub1A*) in a hybrid material simultaneously. The optimal conditions for the amplification reaction were selected, by which the PCR products were clearly visualized.

Samples of F2 and BC1F1 generations with combined genes for blast resistance (*Pi*) and tolerance to long-term flooding (for weed control) (*Sub1A*) in homo- and heterozygous states were obtained, which was confirmed by DNA analysis. The conducted testing of the obtained rice breeding samples for resistance to prolonged flooding under experimental laboratory conditions allowed for the selection of tolerant rice forms in which the breeding processes can be studied according to the complex of economically valuable traits. Their use will reduce the application of plant protection chemicals against diseases and weeds, thereby increasing the ecological status of the rice industry.

CONCLUSIONS

1. As a result of the studies carried out using molecular marking based on PCR in combination with traditional breeding, early maturing rice lines with genes for resistance to flooding Sub 1A, suitable for cultivation in the south of Russia, were isolated.

2. Rice lines have been developed, the genotype of which contains effective blast resistance genes (*Pi-1*, *Pi-2*, *Pi-33*, *Pi-ta*, *Pi-b*). The intro-duction of such varieties into production will allow avoiding the epiphytotic development of the disease, preserving the biological productivity of rice and obtaining environmentally friendly agricultural products.

3. Samples of the F2 and BC1F2 generations were obtained with com-bined blast resistance (Pi) and prolonged flooding tolerance (*Sub1A*) genes as a factor in the control of weeds in homo- and heterozygous state, which is confirmed by the data of their DNA

analysis. The testing of the obtained rice breeding resources for resistance to prolonged flooding under laboratory conditions made it possible to select tolerant rice forms that will be studied in the breeding process for a complex of agronomically valuable traits.

The proposed accelerated scheme for the development of a modern set of rice lines carrying target genes will make it possible to breed varieties that correspond to the agro-climatic conditions of the South of Russia, with increased productivity, cereal quality, resistance to blast, drought, submergence tolerance as a factor in weed control in the future. This will contribute to the production of environmentally friendly products, to save money for rice-producing enterprises, as it will allow, if not completely but to significantly reduce the use of pesticides, that will increase the ecological status of the rice growing industry and improve the economy of the region. Ultimately, this will have a beneficial effect on the health and longevity of the nation.

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