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Introgression of the rice blast resistance genes *Pi1, Pi2* and *Pi33* into Russian rice varieties by marker-assisted selection

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Abstract

Marker-assisted selection (MAS) was adopted for introgression of broad-spectrum blast resistance genes *Pi1, Pi2* and *Pi33* into elite Russian rice varieties. It was shown that microsatellite markers RM 224, RM 527 and RM 310 may be effectively used to transfer *Pi1*, *Pi2* and *Pi33* genes into selected Russian genotypes of rice. Based on the highly productive variety Kuboyar, we obtained the lines, 200-5668 (*Pi 1+2+33* IL14 × Kuboyar), 207-5671 (*Pi 1+2+33* IL28 × Kuboyar) and 208-5674 (*Pi 1+2+33* IL28 × Kuboyar) carrying three pyramided genes *Pi1, Pi2* and *Pi33* in homozygous state. The lines 200-5668, 207-5671 and 208-5674 are used as an improved resistance donor source to obtain hybrids and pyramiding additional *Pi* genes in order to provide stable long-term resistance for rice blast disease.

Key words: *Magnaporthe grisea*, DNA-markers, blast resistance, marker-assisted selection, rice

Introduction

Development of novel technologies that would shorten the period of selection process and allow increasing reliability of breeding material analysis is presently important for breeding crops (Jiang et al. 2015). One of these approaches is the technology of molecular markers (Usatov et al. 2014). DNA marker analysis provides several significant advantages in comparison with analysis of morphological and physiological traits. For example, presence or absence of a DNA marker does not depend on environmental conditions, growth stage of type of a tissue studied (Ali et al. 2014). DNA markers are not affected by selection pressure. Moreover, they are distributed throughout the genome and usually characterized by high level of polymorphism (Singh et al. 2015). Almost any gene, locus or even individual alleles may be marked, suggesting the existence of a number of such specific markers (Platten et al. 2014). For example, DNA markers are successfully used in breeding of rice with respect to its resistance to blast, a disease induced by imperfect fungi *Magnaporthe grisea* (Herbert) Borr. (anamorph *Piricularia oryzae* Cav.) (Fjellstrom et al. 2004; Khanna et al. 2015). The rice blast is widespread in all parts of the world, in which rice is cultivated. It manifests the occurrence of lesions of different shape and color that may be located on leaves, leaf sheaths, stem nodes, panicles and seeds. Injured leaves die, stems break, panicles undergo premature drying or produce poor seeds that eventually lead to about 80% yield losses and decrease in quality of grain (Deng et al. 2006).

Presently the *Pi1, Pi2* and *Pi33* genes that are involved in the resistance of rice against the pathogens are identified, and their allelic variants, which provide resistance to different strains of *Magnaporthe grisea* are marked (Liu et al. 2002; Berruyer et al. 2003; Singh et al. 2015). Therefore, combination of several targeted resistance genes on the basis of elite varieties of rice is believed to be a promising strategy for selection of the pathogen resistant varieties (Hittalmani et al. 2000; Jiang et al. 2015). The present study was aimed at the development of the breeding material of rice with the rice blast resistance genes *Pi1, Pi2* and *Pi33* combined in one genotype by marker-assisted selection.

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Materials and methods

The highly productive elite varieties Buyarin and Kuboyar, bred in Russia were used as the recipients. Lines, C101-A-51 (*Pi2*) and C101-LAC (*Pi1* and *Pi33*) were used as donors. Originally, the rice variety Kuboyar was developed by individual selection from the hybrid population Kuban3 × Boyarin. Kuboyar was more preferred as the recurrent parent based on the agro-morphological traits such as filled grains per panicle and grain yield (Table 1). However, the cross breeding Kuboyar with the lines C101-A-51 and C101-LAC was characterized by low fertility. Therefore, we used an additional recipient variety Boyarin in the selection scheme. First generation hybrids (Boyarin × C101-A-51 and Boyarin × C104-LAC) were used to obtain F_2 population. The F_2 generation was characterized by higher heterogeneity that evidenced of considerable genetic differences between the parental lines. Out of 500 and 700 plants of the F_2 generation were selected 22 and 30 rice plants based on traits of earliness, short stature, non-shattering and spikelets fertility based on for each combination of crossing, respectively. The selected plants were analyzed by PCR for the presence of introduced alleles. A schematic representation of the procedure used for pyramiding the blast resistance genes is shown in Fig. 1.

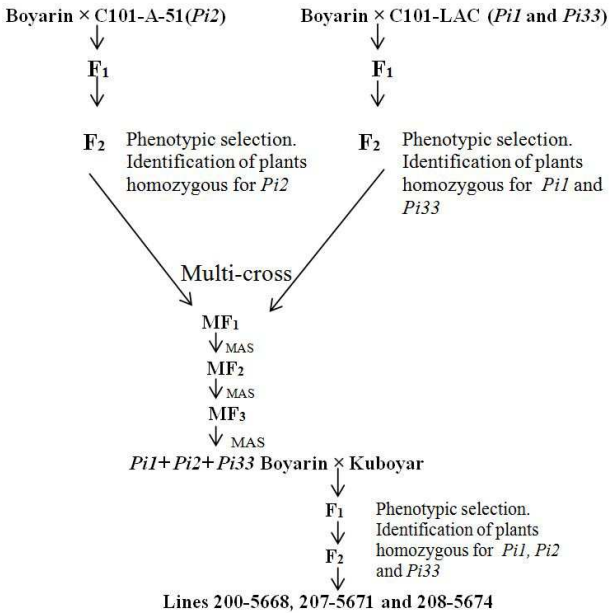

Fig. 1. The molecular pyramiding scheme for the development of blast-resistant lines with introgression of *Pi1, Pi2* and *Pi33* resistance genes

Pi1, Pi2, Pi33 genes were identified during the selection process by microsatellite markers. To identify the gene *Pi-2* we used the RM 527 and RM 140 markers. To identify the gene *Pi1* we used RM 144 and RM 224 markers, and for the *Pi33* gene RM 72 and RM 310 markers were used (http://gramene.com).

Table 1. Reaction to blast under artificial conditions

Genotypes	Genes	Blast isolates	
		Rostov isolate	Krasnodar isolate
Boyarin	-	4	5
Kuboyar	-	5	5
C101-A-51	*Pi2*	1	3
C101-LAC	*Pi1+Pi33*	1	1
200-5668 (*Pi 1+2+ 33*IL14xKuboyar)	*Pi1+Pi2+Pi33*	1	1
207-5671(*Pi 1+2+ 33* IL28×Kuboyar)	*Pi1+Pi2+Pi33*	1	1
208-5674(*Pi 1+2+ 33* IL28×Kuboyar)	*Pi1+Pi2+Pi33*	1	1

Score 0 to 2 = Resistant, 3 = Moderately resistant and 4 to 5 = Susceptible

To perform the molecular genetic analysis, genomic DNA was isolated from leaf tissue as described in (Boom et al. 1990), with our modification (Markin et al. 2015). Polymerase chain reaction was carried out in 25 µl reaction mixture of the following composition: 67 mM Tris-HCl buffer, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$, 0.1 mM mercaptoethanol, 0.25 mM of each dNTP (dATP, dCTP, dTTP and dGTP), 20 pM primers, 2.5 units of Taq-polymerase and 15 ng isolated DNA. Amplification was performed in the Thermocycler PalmCycler (Corbett Research, Australia). Thermal regime of the reaction was chosen individually for each pair of primers on the basis of their sequences. For majority of reactions the optimal thermal regime was as follows: 1) denaturation at 94°C for 5 min, 2) 35 cycles at the following thermal and time regime: primer annealing at 55-60°C for 30s, 30s elongation at 72°C, denaturation at 94°C, 30s, 3) 8 min final elongation at 72°C. Amplification products were analyzed by electrophoresis in 2% agarose gel supplemented with ethidium bromide in Tris-Borate buffer. The obtained gels were photographed with the gel-documenting system (GelDoc 2000, BioRad,

United States). Gene Ruler 1 Kb DNA Ladder (Fermentas, Lithuania) was used as a molecular weight marker.

Several important agronomic traits of the parental lines and the pyramided lines were tested. These were days to flowering (DF), days to ripening (DR), plant height (PH), panicle length (PL), filled grains per panicle (FGP), spikelet fertility (%) (SF), thousand grain weight (TGW) and grain yield (GY). In Russia, the main rice crop is concentrated in two locations namely, Rostov and Krasnodar regions. In this connection, the gene pyramids along with their parental lines were evaluated for blast resistance using two *M. grisea* isolates collected from Rostov and Krasnodar regions, respectively. Preliminary disease resistance assessment to the local population of *Magnaporthe grisea* was carried out according to the standard evaluation system for rice (IRRI 2002). The collected data were subjected to analysis of variance (ANOVA). All data were represented by an average of the four biological replicates.

Results and discussion

We performed hybridization of the standard-variety Boyarin, subspecies *japonica*, sensitive to rice blast with the lines C101-A-51 and C101-LAC, subspecies *indica*, which were used as donors of the *Pi1, Pi2* and *Pi33* rice blast resistance genes, in order to develop resistant lines of Russian rice varieties.

The molecular analysis of rice samples by 2 microsatellite markers of the *Pi1* locus showed that only RM 224 provided reliable well-reproducible spectra and informativeness for identification of the *Pi1* gene. The results of DNA analysis with the marker of the *Pi-* gene of the F_2-plants from the cross breeding

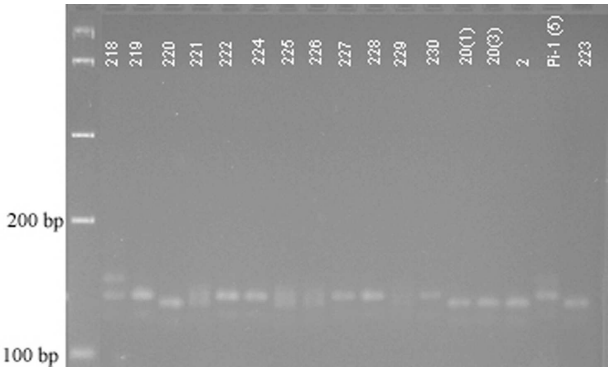

Fig. 2. The electrophoregram of amplification products of genomic DNA of rice with the primer RM 224. Molecular weight marker – 1 Kb

combination Boyarin × C104-LAC are shown in Fig. 1. It was shown that plants with the numbers 220, 20(1), 20(2), 2 and 223 carried only the allele inherited from the maternal line (variety Boyarin). Samples No. 219, 222, 224, 227, 228 and 230 are homozygous for the donor allele of the *Pi1* locus.

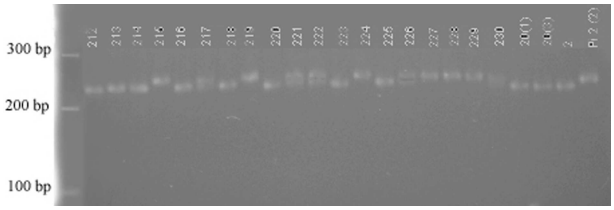

Fig. 3. The electrophoregram of amplification products of genomic DNA of rice with the primer RM 527. Molecular weight marker – 1 Kb

The SSR-markers RM 527 and RM 310 were shown to be most informative for analysis of allelic states of the loci *Pi2* and *Pi33*. Data of electrophoretic analysis of PCR product of the RM 527 marker, which is linked to the *Pi2* gene, are shown in Fig. 2. Donor resistance allele of the parental line C101-A-51, which is marked in Fig. 2 as *Pi2(2)*, was found homozygous in six samples. Plants Nos. 217, 221, 222 and 226 carried both donor and maternal alleles, which were heterozygous for the *Pi2* locus. Other samples carried only the allele inherited from the variety Boyarin and thus, were rejected. An example of PCR analysis of rice samples carrying the microsatellite marker RM 310 is shown in Fig. 3. Donor allele of the *Pi33* gene was identified in plants No. 21, 227, 25, 28 and 31, which were homozygous for this locus. Samples 32 and 33 carried only the allele inherited from the variety Boyarin.

SSR-analysis of rice samples, which were selected from the F_2 generation on the basis of morphological traits, allowed us to identify forms, which carried combinations of resistance alleles of the *Pi1* and *Pi33* genes in homozygous state. These plants were crossed with rice samples, the DNA analysis of which revealed homozygosity of the *Pi2* gene. Early ripening forms characterized by priority morphotype were further selected from the progeny. The selected samples were analyzed for the presence of *Pi1*, *Pi2* and *Pi33* genes by marker-assisted technology. Eventually, in the F_4 generation, we identified forms carrying three pyramided *Pi1*, *Pi2* and *Pi33* genes in homozygous conditions. These lines were used to introgress the resistance genes into the elite highly productive variety Kuboyar.

Table 2. The characteristics of newly developed lines of rice by *Pi* loci

Lines	*Pi-1*	*Pi-2*	*Pi-33*
196-5666	+ | -	+ | +	+|-
197-5666	+ | +	- | -	-|-
198-5666	+ | -	+ | -	-|-
199-5668	+ | +	+ | +	-|-
200-5668	+ | +	+ | +	+|+
201-5668	+ | +	+ | +	+|-
202-5668	+ | +	+ | +	-|-
203-5668	+ | -	+ | -	-|-
204-5671	+ | +	+ | -	-|-
205-5671	+ | +	- | -	-|-
206-5671	+ | +	- | -	-|-
207-5671	+ | +	+ | +	+|+
208-5674	+ | +	+ | +	+|+
209-5674	+ | -	+ | +	+|+

+ = Donor's resistance allele, - = Recipient's susceptible allele

The F_1 obtained from the cross *Pi1*+ *Pi2*+ *Pi33* Boyarin × Kuboyar were used in a series of backcrosses, which provided introduction of donor resistance alleles into the genotype of the recurrent parental form (the variety Kuboyar). Presence of the transferred alleles should be assessed by the method of molecular markers as described above. In the present work we selected perspective lines with priority for agro-morphological traits, from the F_3 population. Russia is the northernmost region of the world where rice is grown. Due to the deficiency of biologically active temperatures during the year one of the major agronomic traits is the number of days to ripening. The selected plants were analyzed by PCR. PCR analysis of linked markers allowed us to identify samples that carried different *Pi* genes (Table 2). Samples 200-5668, 207-5671 and 208-5674 were homozygous by *Pi1*, *Pi2* and *Pi33* loci. Moreover, we identified three lines that carried all three *Pi* loci, though some of them were in heterozygous condition.

Evaluation of agronomical traits of the pyramided lines showed that selected lines were similar with the recipient parent for all the agro-morphological traits (Table 3). However, developed lines had lower spikelet fertility by 3-5 %, but with a longer panicle length of 1-3 cm over Kuboyar. The plant height of 208-5674 (*Pi 1+2+33*, IL. 28 × Kuboyar) was shorter than that of Kuboyar by 8 cm. This is a desirable trait for seed production and enhances lodging resistance. The evaluation of the pyramided lines and parental lines for blast resistance shown in Table 1. Two isolates were used to screen the gene pyramided lines under artificial inoculation conditions. The lines 200-5668(*Pi 1+2+33* IL14 × Kuboyar), 207-5671 (*Pi 1+2+33* IL28 × Kuboyar) and 208-5674 (*Pi 1+2+33* IL28 × Kuboyar) carrying the blast resistance genes *Pi1*, *Pi2* and *Pi33* in homozygous condition were found to be resistant against both the isolates tested whereas parental recipient verity was susceptible.

Presently, newly developed lines 200-5668 (*Pi 1+2+33* IL14 × Kuboyar), 207-5671(*Pi 1+2+33* IL28 × Kuboyar) and 208-5674 (*Pi 1+2+33* IL28 × Kuboyar), carrying *Pi1*, *Pi2* and *Pi33* genes, are being used as the improved donor source of blast-resistance in order to obtain hybrids resistant to the broad-spectrum of the disease races, and to pyramiding the additional resistance *Pi* genes.

It was previously shown that there are a number of genes (*Pi1, Pi2, Pi33, Pita, Pib, Pi40, Pi9, Piz-t, Piz, Pigm, Pi50* etc.) that provide resistance to a wide spectrum of *Magnaporthe grisea* races (Suh et al. 2009;

Table 3. Agronomic characteristics of the rice genotypes

Genotypes	DF	DR	PH	PL	FGP	SF (%)	TGW	GY
Boyarin	86	116	86	14	115	95	30	7,42
Kuboyar	93	123	96	16	140	94	30	10,33
C101-A-51	124	154	75	21	103	55	25	5,11
C101-LAC	125	155	72	20	98	62	25	4,95
200-5668(*Pi 1+2+33* IL14×Kuboyar)	94	124	92	17	131	91	29	8,81
207-5671(*Pi 1+2+33* IL28×Kuboyar)	94	124	90	19	135	88	29	8,92
208-5674(*Pi 1+2+33* IL28×Kuboyar)	91	121	88	18	124	90	28	8,21
$LSD_{(0.05)}$	6	6	4,5	1,2	8	7	1,1	0,52

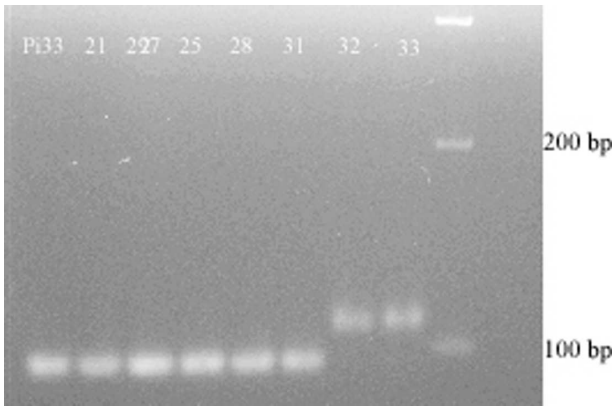

Fig. 4. The electrophoregram of amplification products of genomic DNA of rice with the primer RM 310. Molecular weight marker – 1 Kb

Deng et al. 2006; Hayashi et al. 2004; Liu et al. 2002; Zhu et al. 2012). Rice blast is also a serious problem in Russia. Among several blast resistance genes known, *Pi1, Pi2, Pi33* have been reported to be the most effective genes providing broad-spectrum resistance across various rice sowing regions in Russia. The same high performance was revealed for *Pita* and *Pib* blast disease resistance genes (Mukhina et al. 2011). Introduction of the resistance genes into the elite varieties adapted to certain soil and climatic conditions, as well as pyramiding of several resistance genes in one genotype are considered to be the most promising ways of development of varieties resistant to this pathogen (Jiang et al. 2015). Marker assisted selection (MAS) is presently widely used as a promising tool for breeding programs (Ali et al. 2014; Fjellstrom et al. 2014). Application of MAS provides clear understanding of the targeted gene drift from parental lines (Jiang et al. 2015; Usatov et al. 2014). Therefore, it may be effectively used to effect the selection of plants in order to make the development of new forms which is economical in terms of cost and time. Nevertheless, not only molecular genotyping data, but also classical complex analysis of morpho-physiological traits of cultivated plants both in laboratory model experiments and in natural conditions are required to solve the problems of genetic marking of agronomic characters of crops (Jiang et al. 2015).

Individual plant selection by morphological traits and in combination with MAS allowed us to simplify the selection scheme and obtain pyramided lines, carrying *Pi1, Pi2* and *Pi33* genes in homozygous condition. These lines were characterized for a set of complex traits that corresponded to the agroclimatic conditions for rice breeding in Russia. This material was used as the improved source of resistance to rice blast in order to obtain heterotic hybrids resistant to a wide spectrum of the pathogen races, as well as to introduce additional *Pi* genes so as to develop stable long-term resistance to rice blast.

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