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Validation of SSR markers associated with Submergence Tolerance in Rice (Oryza sativa L.)

[1]Kirill V. Azarin, [1]Alexander V. Usatov, [2]Andrey V. Alabushev,
[2]Pavel I. Kostylev, [1]Maxim S. Makarenko and [1]Aleksey A. Kovalevich

[1]*Southern Federal University, Rostov-on-Don, Russia*
[2]*All-Russian Research Institute of Grain Crops after I.G. Kalinenko, Zernograd, Russia*

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Corresponding Author:
Kirill V. Azarin
Southern Federal University,
Rostov-on-Don, Russia
Email: azkir@rambler.ru

Abstract: The present work is devoted to the validation of SSR markers associated with submergence tolerance (*Sub1A*) QTLs in rice. It was shown that only microsatellite markers RM 7481 differentiates donor lines and recipient variety. Based upon the results of DNA analysis with the marker of the RM 7481 of the F_2-plants, the 14 homozygotes by the locus *Sub1A*, 40 samples carried *Sub1A* in the heterozygous state, 22 plants inherited only alleles from the recipient variety Novator were identified. Survival analysis of parental and hybrid plants (21 days after germination) showed that the most submergence tolerance forms were donor lines of *Sub1A* loci and F_2-plants carrying *Sub1A* locus in homozygous and heterozygous state according to the data of molecular genetic analysis. Thus, it was shown that RM 7481 microsatellite marker is effective for breeding of submergence tolerance rice using Russian elite cultivars.

Keywords: *Sub1A*, Submergence Tolerance, DNA-Markers, Rice

Introduction

Development of the submergence tolerant versions of rice cultivars due to the fact that extensive growing areas in South and Southeast Asia are prone flash floods during the monsoon season (Septiningsih *et al*., 2009; Linh *et al*., 2013). Another aspect of the use this trait is the transition from seedling to direct sowing seeds. In Russia complete flooding is used to weed control because they can not overcome deep layer of water. Therefore, it is necessary the submergence-tolerant rice cultivars. A considerable stage in the history of breeding for submergence tolerance in rice it was mapped on chromosome 9 the major Submergence 1 (*SUB1*) QTL (Xu *et al*., 2006).

As was shown *SUB1* QTL carries three related Ethylene Response Factor (ERF)-like genes (*Sub1A*, *B* and *C*), however, only *Sub1A* was strongly induced in the tolerant varieties in reaction to flooding (Xu *et al*., 2006; Fukao *et al*., 2009). In Russia like in several countries cultivated rice belongs to the subspecies japonica, but *Sub1A* is absent from all japonica cultivars. Therefore introgression the *Sub1A* QTL into the elite rice cultivars of Russia by marker-assisted selection is a real an urgent objective.

Nevertheless, association microsatellite locus alleles with specific trait in a certain population do not always correlate with that trait in population other origin (Neeraja *et al*., 2007; Singh *et al*., 2010; Usatov *et al*., 2015; 2016). In this connection, the validation of SSR markers associated with submergence tolerance in rice for to transfer the *Sub1A* locus into genotype of the high-yielding Russian variety of rice was conducted.

Materials and Methods

Plant Materials

The highly productive Russian variety Novator was used as the recipient. Varieties Inpara-3, BR-11, TDK-1, CR-1009 was used as a donor *Sub1A* region. For validation of SSR markers associated with submergence tolerance in rice the hybrids by crossing varieties Novator with *Sub1A* gene donors were obtained. The F_1-plants were characterized by a high sterility (90-95%), indicating a significant genetic difference between the parental forms. Out of the second generation were selected 20 rice plants based on traits of earliness, short stature, plant height, grain content to panicles, non-shattering and spikelets fertility based on for each combination of crossing, respectively. The selected plants were analyzed for the presence of introgression locus.

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Molecular Marker Analysis

For molecular genetic analysis 7 SSR microsatellite markers flanking the locus *Sub1A* were selected (http://www.gramene.org). Descriptions of the primers are shown in Table 1. Polymorphism between donors and recipient was surveyed using these markers. 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 (NH4)2SO4, 2.5 mM MgSO4, 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 (Usatov *et al*., 2014). Amplification was performed in the thermo cycler Palm Cycler (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 3 min, (2) 30 cycles at the following thermal and time regime: Primer annealing at 55-59°C for 35 and 30 sec elongation at 72°C, denaturation at 94°C, 35 sec, (3) 7 min final elongation at 72°C (Usatov *et al*., 2014). 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.

Evaluation of Submergence Tolerance

In a laboratory experiment, rice seeds were soaked in water for 12 h and then were sown in the soil (Azarin *et al*., 2016). Deep flooding was simulated in an aquarium with a 50 cm layer of water (Fig. 1). Control plants were grown in physiological normal conditions. After 21 days the survival rate was measured.

Fig. 1. The evaluation of submergence tolerance

Table 1. SSR markers linked with submergence tolerance locus *Sub1A*

SSR locus	Forward primer	Reverse primer	Annealing temp (°C)	Product size (bp)
RM 219	cgtcggatgatgtaaagcct	catatcggcattcgcctg	55	202
RM 316	ctagttgggcatacgatggc	acgcttatatgttacgtcaac	55	210
RM 444	gctccacctgcttaagcatc	tgaagaccatgttctgcagg	55	139
RM 464	aacgggcacattctgtcttc	tggaagacctgatcgtttcc	55	262
RM 7481	cgacccaatatctttctgcc	attggtcgtgctcaacaag	59	95
RM 8303	aggggagaggacacacacac	ggatcctcctgcaaaatcaa	59	129
RM 23877	tgccacatgttgagagtgatgc	tacgcaagccatgacaattcg	59	327

Statistical Analysis

The collected data were subjected to Student's t-test. All data were represented by an average of the three biological replicates and the standard deviations.

Results

Parental SSR Polymorphism Screening

In this study, 7 microsatellite markers associated with the *Sub1A* loci were checked with three donor lines and recipient variety in order to find out polymorphic markers to further use for screening the *Sub1A* of the crossing populations. The molecular analysis of parent's varieties by 7 SSR markers of the *Sub1A* loci showed that only RM 7481 differentiates donor lines and recipient variety and it is informative for identification of the *Sub1A* region in hybrid population.

In the case of the presence *Sub1A* locus in the genome of the test plants the length of amplified fragments according to electrophoregram was about 95 base pairs (Fig. 2). The fragments with the same molecular weight on electrophoregram (~ 95 bp) were identified for donor lines BR-11, Inpara-3, CR-1009 and TDK-1, while the amplification product of the RM 7481 marker for variety Novator was a distinctive molecular weight (~ 70 bp).

Molecular Marker Analysis

The electrophoretic analysis of PCR products with marker RM 7481 are shown in Fig. 3. The donor allele of parental line CR-1009 was founded in the homozygous state in the samples 2, 3, 5, 9, 13 and 17. The plants no: 2, 4, 6-8, 10, 11, 16, 18 and 19 were heterozygous by Sub1A locus, i.e., carried both alleles of donor and recurrent parent. Allele inherited from the variety Novator was revealed in other samples. Thus, upon the results of PCR analysis with marker RM 7481 were identified 14 F_2-plants homozygotes by the locus *Sub1A*, 40 samples carried *Sub1A* in the heterozygous state, 22 plants inherited only the allele from the variety Novator.

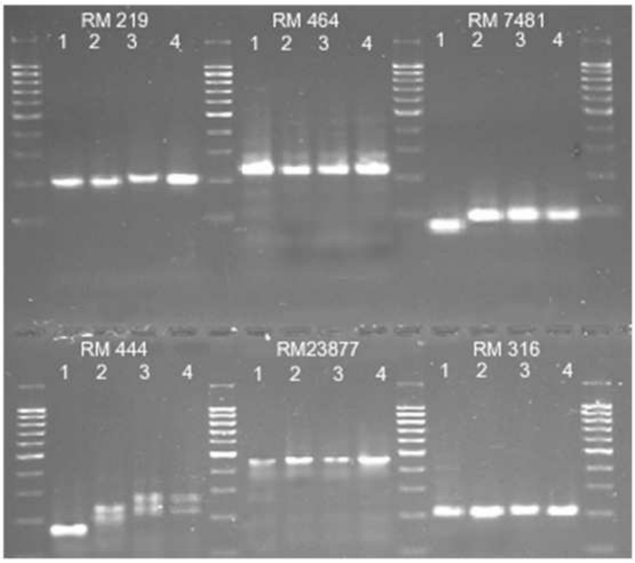

Fig. 2. The electrophoregram of amplification products of genomic DNA of rice. 1- Novator; 2 - BR-11; 3 - CR-1009; 4 - TDK-1. Molecular weight marker-1 Kb

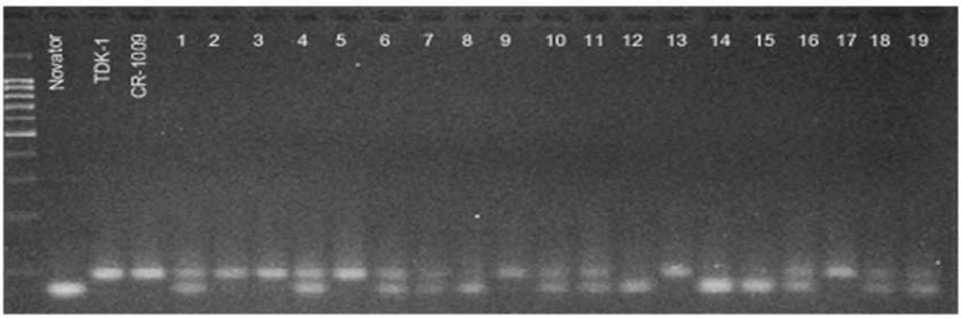

Fig. 3. The electrophoregram of amplification products of genomic DNA of rice with the primer RM 4781. 1-19 - F_2 (CR-1009 × Novator). Molecular weight marker – 1 Kb

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Table 2. The survival rates (%) of rice under control and submergence conditions (21 days after germination)

Samples	Survival rate, (%)	
	Control	Submergence
Novator	98±1.0	42±4.0*
Inpara-3	91±2.0	87±2.5
BR-11	94±2.5	85±1.5*
CR-1009	89±3.5	85±3.0
TDK-1	95±2.1	89±4.7
Inpara-3 × Novator		
Homozygotes (*Sub1A*)	91±2.0	89±1.5
Heterozygotes	93±2.0	85±4.5
Homozygotes (without *Sub1A*)	91±1.5	57±4.0*
BR-11 × Novator		
Homozygotes (*Sub1A*)	92±2.0	85±3.5
Heterozygotes	94±1.0	83±1.1*
Homozygotes (without *Sub1A*)	94±1.5	47±2.7*
CR-1009 × Novator		
Homozygotes (*Sub1A*)	90±2.0	87±2.7
Heterozygotes	90±1.0	87±2.5
Homozygotes (without *Sub1A*)	91±1.5	49±3.0*
TDK-1 × Novator		
Homozygotes (*Sub1A*)	93±2.0	86±3.7
Heterozygotes	95±1.0	87±4.6
Homozygotes (without *Sub1A*)	95±1.5	45±2.2*

Evaluation of Submergence Tolerance

For validation of genic-SSR markers it is very important the consistency of the genotyping data and morpho-physiological response to the stress. Survival analysis of parental and hybrid plants (21 days after germination) showed that submergence significantly decreased survivability of variety Novator and hybrids without *Sub1A* locus. The most submergence tolerance samples at survival rates were donor lines of *Sub1A* loci and hybrids F_2 carrying *Sub1A* locus in homozygous state (deviation from the control < 15%). Hybrid plants (F_2) with locus *Sub1A* in heterozygous state also showed high tolerance to submergence (Table 2).

Discussion

The peculiarity of flooding as a stress factor for plants is the combination of high water content and oxygen deficit. The access of oxygen to plants roots is hampered by the extremely low oxygen diffusion in water compared to air (Jung *et al*., 2010; Fukao *et al*., 2011; Mickelbart *et al*., 2015). In this case there are inhibited the seed germination, reduced water absorption and inhibited the process of exudation (Kawano *et al*., 2009; Fukao *et al*., 2011). The absorption of ions through the roots is dramatically decreased. As a result, it is revealed nutrient deficiencies in shoots, which leads to stunted growth and plant death. Development of oxidative stress is the common response to hypoxia and anoxia in plant tissues (Banti *et al*., 2013). Even a short period of flooding in the early stages of plant development significantly reduces crop productivity (Kawano *et al*., 2009; Niroula *et al*., 2012). However, some plant species tolerate oxygen deficiency in the soil. In this regard rice is a unique crop, which is usually grown in flooded soil. However, not all rice varieties according to its genotype can tolerate to complete flooding. The possibility to grow under submergence condition (hypoxia and anoxia) is provided by the locus *Sub1A*, encodes by ethylene-response factor transcription (Xu *et al*., 2006; Mickelbart *et al*., 2015). To save energy and carbohydrates in plants carrying *Sub1A* is inhibited the expression of genes encoding a-amylase and sucrose synthase involved in the metabolism of starch and sucrose (Lasanthi-Kudahettige *et al*., 2007; Banti *et al*., 2013). *Sub1A* allows submerged rice plants to save carbohydrates for use after flooding (Banti *et al*., 2013).

Therefore, the introgression of the *Sub1A* locus into the high-yielding varieties using marker-assisted selection will lead to the development of new submergence-tolerant lines less time consuming (Linh *et al*., 2013; Usatov *et al*., 2015).

In our study, of the seven SSR markers analyzed, only RM 7481 differentiates donor from recipient forms and had a clear codominant inheritance. To validation of the marker RM 7481 the hybrids by crossing Novator (elite rice variety of Russia) with *Sub1A* gene donors (lines BR-11, Inpara-3, CR-1009, TDK-1) were obtained. According to the results of molecular genetic screening with RM 7481, the samples carrying different allelic variants *Sub1A* locus were identified, some of them were homozygous. Investigation of the effect of introduced locus on morpho-physiological characters of rice showed that the most viable samples under submergence were *Sub1A* locus donor lines and hybrid plants (F_2) carrying the *Sub1A* locus in a homozygous state. The second generation hybrids carrying the *Sub1A* locus in a heterozygous state were also highly tolerance to submergence. Comparative study of agronomic traits of the parental and hybrid plants grown under normal field condition demonstrated that homozygotes by *Sub1A* locus have a similar morphotype with the high-yielding cultivar Novator (data not shown). Thus, according to laboratory and field experiments the resulting hybrids have shown a more tolerance to submergence compared to Novator and more adapted to the agro-climatic conditions of rice growing in Russia compared with donor lines.

Conclusion

The present study aimed at the validation of SSR markers associated with submergence tolerance QTLs *Sub1A* in rice to introgression of this QTL into the elite rice variety of Russia. It was shown that only RM 7481 differentiates donor lines and recipient variety and it is informative for identification of the *Sub1A* region in hybrid population. To validation of the marker RM 7481 we obtained hybrid combinations of F_1, then F_2 between the sensitive and tolerant varieties. It was confirmed, that the mode of inheritance of RM 7481 is codominant. Studying the impact of introduced locus on survival of rice plants under submergence showed that the most tolerant plants were the donor lines of *Sub1A* loci and second generation hybrids carrying *Sub1A* in homozygous state according to the data of molecular genetic analysis. Thus, it was shown that RM 7481 microsatellite marker may be effectively utilized for breeding of submergence tolerance rice using Russian elite cultivars.

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Author's Contributions

All the six authors participated in all experiments, data analysis and the entire process of the writing of the manuscript.

Ethics

The authors declare that this manuscript conforms to the ethical standards specified by the American Journal of Agricultural and Biological Sciences.

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