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Genetic diversity among salt-tolerant rice (*Oryza sativa* L.) landraces cultivated in the coastal districts of Bangladesh

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ABSTRACT

The aim of this study was to determine genetic diversity and relationships among the salt-tolerant rice landraces cultivated in the coastal districts of Bangladesh. DNA extracted from seedlings of nine salt-tolerant local rice landraces and three established salt-tolerant rice varieties was subjected to random amplified polymorphic DNA (RAPD) analysis using a number of random decamer primers. Polymorphic bands generated with five primers were scored and used for determining polymorphic information contents (PIC) and in deriving a dendrogram using the Jaccard similarity coefficient-based unweighted pair-group method with arithmetic means (UPGMA). The five primers generated 84 reproducible bands of the size range 0.24-1.90 kbp and 73% of the bands were polymorphic. The UPGMA dendrogram showed five major clusters at genetic similarity of 0.55. The PIC values ranged from 0.71 to 0.90 with an average of 0.85. The pair-wise similarity index values ranged 28.8-97.1% among the twelve genotypes. The three established rice verities formed a single cluster and the local landraces formed four clusters. Our result revealed high level of genetic diversity among the local rice landraces. The RAPD markers identified could be useful in developing high-yield salt-tolerant rice strains with improved grain quality.

Key words: RAPD markers, genetic diversity, *Oryza sativa* L., salt-tolerant rice landraces

Introduction

Rice (*Oryza sativa* L.) serves as an important part of the diet of more than three billion people around the world and the principle source of calories and nutrients in the developing countries (Skaria et al., 2011). Rice is genetically diverse for having many landraces and progenitor species. Nearly 10,000 landraces of rice may have thrived in Bangladesh, of them about 4,000 traditional landraces have been registered and stored by the Rice Gene Bank of the Bangladesh Rice Research Institute (BRRI), Dhaka, Bangladesh. Beside this medium-term repository, an identical set of Bangladeshi rice landraces have been held in a trust at the International Rice Research Institute (IRRI), Manila, Philippines, for long-term storage (Bashar & Sarker, 1997; Jackson, 1999). Genetic diversity of crop species is a valuable asset for agricultural development but in practice, less than 15% of the potential diversity is utilized (Hossain et al., 2012). Thus many alleles of many important traits of economically important crop species are neglected and the alleles may eventually vanish from the gene pool.

Many countries use their best croplands for intensive rice cultivation. The operations have affected some of the croplands adversely. About 35% of rice-growing cropland is currently facing varying degrees of soil salinity due to the development of underground salt dome, exacerbated by irrigation, water-logging, aquifer depletion, deforestation and salt mining (Akbar, 1986). Soil salinity has become a major

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concern in rice production in the coastal regions of Bangladesh. Southern portion of three western coastal districts of Bangladesh, namely Satkhira, Khulna and Bagerhat had become highly prone to soil salinity due to the chronic reduced flow of fresh water through the Modhumati river and its distributaries. Over 70% of the land of the Satkhira region is having soil salinity level 4–16 dS/m (Karim & Iqbal, 2001). The coastal districts of the Meghna river delta, namely Lakshmipur, Noakhali and Feni also face salinity, but to a lesser degree. Salinity in Noakhali district, for example, is moderate (4–8 dS/m), but it extends to larger areas compared to Lakshmipur and Feni (Karim & Iqbal, 2001). In Bangladesh, the coastal soil salinity gradually increases from November through March following the Monsoon cycle. The increased rainfall from March through October brings down salinity to a tolerable level. The severe salinity in the dry season limits the farmers of the western coastal districts to grow only a single rice crop per year. A high salt-tolerant cultivar of rice could be highly useful to the subsistence rice farmers of this region.

The farmers of the eastern coastal districts namely Noakhali and Feni can grow two or three rice crops a year. Rice landraces in these districts form 60% of the top 20 rice varieties grown there in the dry season (Bose et al., 2001) and these varieties are exposed to salinity particularly during the early vegetative and the later reproductive growth stages (Mass & Hoffman, 1977; Shannon et al., 1998). Conserving these landraces and characterizing their genetic endowments would provide with valuable resources for breeding salt-tolerant rice cultivars (Rabbani et al., 2008). The landraces have been shown to be excellent sources of novel alleles (Evenson & Gollin, 1997; Tanksley & McCouch, 1997; Jackson, 1999; Hoisington et al., 1999; Guevarra et al., 2001). Since the entire Ganges-Brhamaputra delta of Bangladesh is threatened with increasing salinity (Alam, 1996), there is a necessity to widen the genetic base for donors of salt tolerance traits for adaptability of the rice cultivars to the changing ecological conditions in the coastal areas of Bangladesh.

Conventional approach for screening rice genotypes can be capital-, technology- and labor-intensive. Analysis of DNA polymorphism offers several advantages over measuring morphological or physiological traits in identifying genetic variations among salt-tolerant rice landraces. Molecular markers are reliable tools in evaluating genetic variations and elucidating relationships within and among species (Rahman et al., 2007; Tehrim et al., 2012). There are several molecular methods for analysis of genetic variations. Of them, the polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) method is the simplest yet rapid and effective (Williams et al., 1990). The method requires no prior knowledge of the genome sequence and only a small amount of genomic DNA is sufficient for the analysis (Yu & Nguyen, 1994; Karp et al., 1997). RAPD has been used extensively in genotype identification and gene mapping (Rabbani et al., 2008; Pervaiz et al., 2009). Here we used the RAPD method to analyze genetic variation among 12 salt-tolerant rice cultivars and identified high level of genetic polymorphisms. The markers thus identified can be used in developing high-yield varieties of salt-tolerant rice cultivars with improved grain quality.

Materials and Methods

Seeds and seedlings

Seeds of three established rice verities namely Pokkali, BRRI dhan47 and BRRI dhan53 and nine local landraces namely Dular, Panbira, Siral, Gota, Minikit, Ashrafuli, Swarna Pajam, Jira dhan and Ghunshi (Table 1) were collected from the Genetic Resource Centre (GRC) of BRRI. The seeds were incubated at 50°C for three days to break dormancy. Then the seeds were rinsed with distilled water and placed on a wet filter paper in a Petri dish and the Petri dish was placed in a dark incubator set at 28°C. Ten seeds of identical genetic background were placed in each Petri dish. Aseptic techniques were followed throughout the process. The germinated seeds were potted in rows and columns and the pots were housed in a glass greenhouse.

Genomic DNA extraction

DNA was extracted from leaves of the seedlings as described previously (Zheng et al., 1995). Briefly, a 2.0 cm slice of leaf was homogenized in a microfuge tube using a polished glass or polypropylene pestle. The resulting paste was mixed with 0.4 ml of extraction buffer (50 mM Tris-HCl pH 8.0, 25 mM EDTA, 300 mM NaCl and 1% SDS) and the tube was incubated at 60°C for 10 minutes with occasional agitations. The tube was cooled down and the lysate was extracted with 0.5 ml of a mixture of chloroform and isoamyl alcohol (24:1). The tube was centrifuged at 10,000x*g* for 8 minutes and the upper aqueous layer of the supernatant was transferred to a new tube. Equal volume cold isopropanol was added to the tube and mixed. DNA was precipitated by

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centrifugation at 8,000x*g* and the pellet was washed twice with 70% ethanol. The washed pellet was air-dried and then suspended in 0.1 ml of PCR-grade water. DNA concentration was measured spectrophotometrically and the presence of long (10-20 kbp) DNA molecules in the samples was ensured by resolving 1.0 µg of the samples in 0.8% agarose gel. The DNA samples were stored at -80^{o}C until used.

Primers and PCR amplification

Random decamer primers were obtained from Operon Technologies (Alameda, CA, USA). A number of primers were tested before selecting five primers (Table 2) that reproducibly generated a number of polymorphic DNA bands with the rice DNA samples. PCR amplification was performed as described previously (Williams et al., 1990) with slight modifications. Briefly, the reaction mixture (25 µl) contained 1x *Taq* DNA polymerase buffer (Life Technologies, Grand Island NY, USA), 0.25 mM dNTP mixture, 0.4 µM of a oligonucleotide primer, 1.5 mM $MgCl_2$, 1.0 ng of template DNA and 1.0 unit of AmpliTaq DNA polymerase (Life Technologies). Amplification was carried out in a Multigene thermocycler (Labnet International, Woodbridge, NJ, USA). The thermocycler was programed as the following: 94°C for 5 minutes (one cycle); 94°C for 30 sec, 35°C for 60 sec and 72°C for 120 sec (40 cycles); 72°C for 5 min (one cycle) and 4°C hold. The amplification products along with appropriate DNA size markers were eletrophoretically resolved in 1.0% agarose gel at a constant voltage of 7.5 volts/cm of gel length for 45 minutes. The gel was stained with ethidium bromide and documented using a Panasonic Lumix DMC-FS20 digital camera (Panasonic Malayasia, Kuala Lumpur, Malaysia).

Table 1. *Identities and some phenotypic descriptions of the 12 rice verities tested.*

Identity (Ecotypes)	Common names	BRRI accession numbers	Comments
V-1	Pokkali	5309	Moderately salt-tolerant
V-2	BRRI dhan47	5327	BRRI-released as moderately salt-tolerant
V-3	BRRI dhan53	5251	BRRI-released as moderately salt-tolerant
V-4	Dular	5318	Local landrace
V-5	Panbira	5330	Local landrace
V-6	Siral	5301	Local landrace
V-7	Gota	5329	Local landrace
V-8	Minikit	5338	Local landrace
V-9	Ashrafuli	5250	Local landrace
V-10	Swarna Pajam	5325	Local landrace
V-11	Jira dhan	5326	Local landrace
V-12	Ghunshi	5328	Local landrace

Table 2. *The used five primers, their DNA sequences, the size ranges of the amplified DNA bands, the number of polymorphic and monomorphic bands and polymorphic information contents obtained for each of the primers used in analyzing genomic DNA of 12 rice cultivars.*

Primer name	DNA sequences	GC contents (%)	Size range of the bands (in bp)	TA	PA	%	MA	%	PIC
OPC-05	5'GATGACCGCC3'	70	550-1300	14	11	78.67	3	21.43	0.859
OPA-01	5'CAGGCCCTTC3'	70	230-1400	23	16	69.56	7	30.44	0.816
OPF-14	5'TGCTGCAGGT3'	60	330-1900	14	10	71.42	4	28.58	0.902
OPL-03	5'CCAGCAGCTT3'	60	250-1500	12	9	75.00	3	24.00	0.718
OPB-07	5'GGTGACGCAG3'	70	300-1100	21	15	71.42	6	28.58	0.879
Total				84	61		23		
Average		66	332-1440	16.8	12.2	73.21	4.6	26.60	0.847

MA= Monomorphic allele, **PA**= Polymorphic allele, **PIC**= Polymorphic information content, **TA**= Total alleles

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RAPD data analysis

Amplification profile of all twelve rice genotypes were compared relative to the GeneRular 1.0 kb DNA size markers (Fermentas, New Delhi, India) using Alpha-Ease FC 4.0 software (Alpha Innotech, San Leandro, CA, USA). Polymorphism Information Content (PIC) value for each primer was calculated using the formula: $PIC=1-\sum Pij^2$, where Pij is the frequency of the j^{th} allele for primer i and the summation extends over n^{th} alleles (Anderson et al., 1993). All genotypes were scored for the presence and absence of the RAPD bands for all twelve rice varieties and the data was transformed to binary data for the presence (1) or absence (0) of a DNA band. A data matrix was made for estimation of genetic distance between the varieties with NTSYS-pc version 2.2 software program (Rohif, 2000). Estimation of genetic similarity was calculated between all pairs of the rice variants applying the Jaccard similarity coefficient. In addition, NTSYS-pc version 2.2 was used to develop UPGMA (unweighted pair group method with arithmetic averages) dendrogram to investigate the similarity-based interrelationship among the rice cultivars.

Results

We tested over a dozen of random decamer RAPD primers. Five of these primers generated a variety of polymorphic bands with the DNA samples extracted from the 12 rice variants. Rest of the primers that generated only monomorphic bands, were excluded from further analysis. A typical band pattern generated by one of the five RAPD primers is depicted in Figure 1. The five RAPD primers generated a total of 84 reproducible bands that can be readily scored. Each of the rice variants generated an average of 7 bands and each of the RAPD primer generated an average of 16.8 bands. Out of the 84 bands scored, 61 bands (or 73.21%) were reproducibly polymorphic and 23 bands (or 26.6%) were monomorphic. Of the five primers (Table 2), the primer OPC-05 showed highest polymorphism (78.56%) and produced 14 bands of which 11 were polymorphic and the primer OPA-01 showed least polymorphism (69.56%) and produced 23 bands of which 16 were polymorphic.

The similarity matrix used in determining the level of relatedness of the cultivars yielded pairwise genetic similarity indices as shown in Table 3. The pairwise genetic similarity observed, (from highest to lowest similarity) are as the following: Pokkali and BRRI dhan47 (97.1%), BRRI dhan47 and BRRI dhan53 (91.4%), Dular and Panbira (90.5%), Pokkali and BRRI dhan-53 (88.6%), Swarna Pajam and Jira dhan (84.4%), Swarna Pajam and Gunshi (75.9%), Jira dhan and Ghunshi (74.2%), Gota and Siral (66.7%), Ashrafuli and Swarna Pajam (59.0%), Ashrafuli and Jira dhan (54.8%), Panbira and Gota (43.6%), BRRI dhan53 and Minikit (38.80%), BRRI dhan-47 and Minikit (38.0%), Jira and Siral (36.0%), BRRI dhan47 and Panbira (35.7%), Minikit and Panbira (30.5%), BRRI dhan47 and Dular (33.3%), and Panbira and Ashrafuli (28.8%).

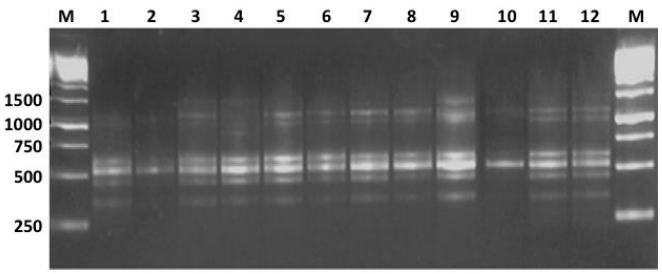

Figure 1. *DNA banding pattern of the 12 rice variants (V-1 through V-12) generated with the primer OPL-03. The sizes of the makers (in bp) are indicated on the left. M- DNA size markers.*

Genetic relatedness between the 9 local landraces as well as the Pokkali, BRRI dhan47 and BRRI dhan53 based on their shared allele was calculated using Jaccard's similarity coefficient method followed by clustering into a dendrogram (Figure 2). The 12 rice cultivars fell into five clusters (CLU), CLU-1 through CLU-5 in the similarity coefficient of 0.55. As expected, the established salt-tolerant strains Pokkali, BRRI dhan47 and BRRI dhan53 formed a single cluster (CLU-1). The local landraces formed clusters of two or three variants but not necessarily by the localities where the landraces are generally cultivated.

Discussion

Genetic diversity analysis of the traditional salt-tolerant rice landraces is essential in germplasm characterization and conservation and in identifying salt-tolerant donor parents. Phenotypical studies such as morphological and physiological analyses have long been the means of studying the taxonomy and variability among plant species. RAPD markers are among the most widely used DNA markers for many purposes including genome mapping, genotype identification and tagging important genes (Tragoonrung et al., 1996; Rabbani et al., 2008; Pervaiz et al., 2009).

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Table 3. *Genetic similarity coefficient matrix obtained from the RAPD analysis.*

Ecotypes	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
V1	1.000											
V2	0.971	1.000										
V3	0.886	0.914	1.000									
V4	0.340	0.333	0.365	1.000								
V5	0.364	0.357	0.364	0.905	1.000							
V6	0.400	0.392	0.373	0.389	0.436	1.000						
V7	0.340	0.333	0.315	0.462	0.455	0.667	1.000					
V8	0.388	0.380	0.388	0.327	0.305	0.358	0.352	1.000				
V9	0.426	0.417	0.396	0.309	0.288	0.340	0.309	0.769	1.000			
V10	0.386	0.378	0.356	0.347	0.321	0.354	0.375	0.500	0.590	1.000		
V11	0.391	0.413	0.391	0.380	0.352	0.360	0.353	0.500	0.548	0.844	1.000	
V12	0.474	0.462	0.436	0.356	0.327	0.429	0.386	0.450	0.541	0.759	0.742	1.000

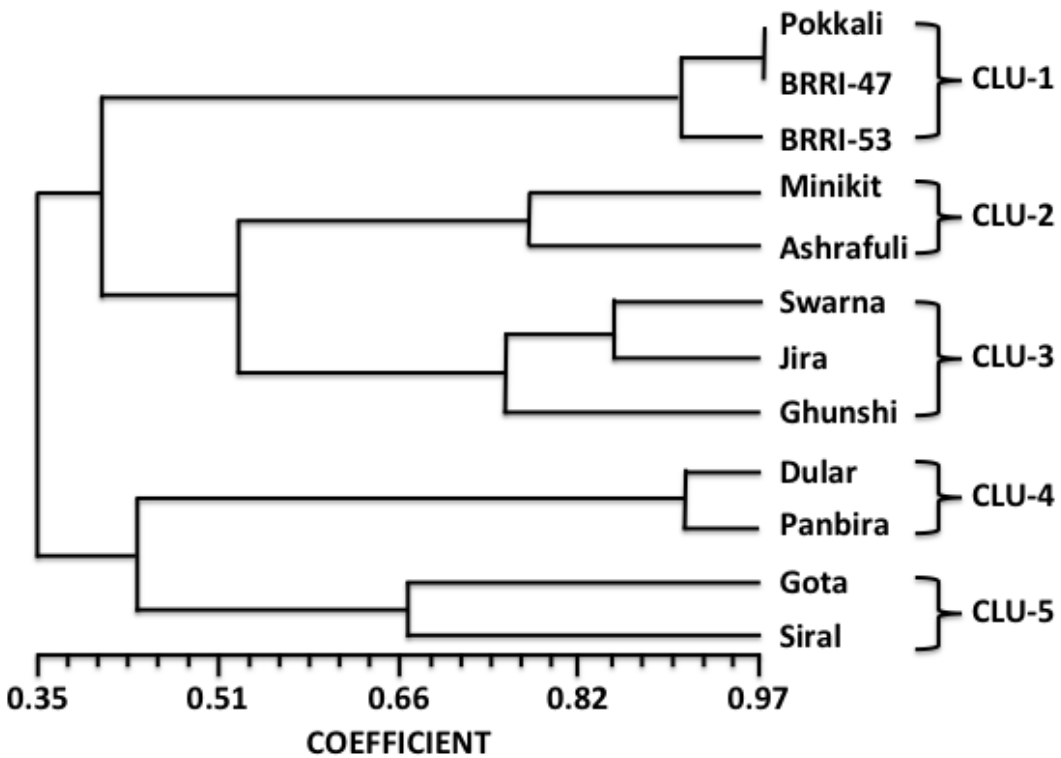

Figure 2. *An UPGMA cluster dendrogram showing the genetic relationships of the 12 rice cultivars tested using five RAPD primers. CLU- cluster.*

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Unlike the morphological and biochemical markers, molecular markers are not stressed by environmental factors and growth conditions (Ovesna et al., 2002). RAPD markers have been extensively used to investigate genotypic variations among different cultivars (Rabbani et al., 2008; Pervaiz et al., 2009; Kanawapee et al., 2011; Tehrim et al., 2012). Here we genotyped and partially characterized 12 rice varieties including three institutionally established salt-tolerant cultivars and nine local landraces grown in the coastal districts of Bangladesh. Farmers' preference for the landraces, despite of the availability of modern high-yield varieties suggest that the landraces offer greater adaptability to the fluctuating coastal environment (Bose & Hossain, 2003). Notably, the costal rice fields of Bangladesh are susceptible to unpredictable rainfall, water logging and seawater intrusion. The landraces are most likely more adaptable to ion toxicities and mineral deficiencies of the coastal soil.

The five RAPD primers we used yielded a total of 84 bands. Each rice variety yielded an average of seven bands and each primer generated an average of 16.8 bands. Of the 84 bands, 61 (or 73.21%) were reproducibly polymorphic and 23 (or 26.6%) were shared. The mean polymorphic allele (12.2 alleles/locus) found in our study is comparable to the result reported earlier (Tehrim et al., 2012), where 14 RAPD primers were used to study local landraces and several high-yield verities and 7.4 alleles/locus were detected. Skaria et al. (2011) reported an analysis of genetic variability in domesticated rice varieties (*Oryza sativa* L.) of Kerala using RAPD Markers and detected a total 101 allele, out of which 73 alleles were polymorphic and 28 alleles were shared. In another study (Kanwapee et al., 2011) involving Indian quality rice germplasm, Thai improved varieties, Indian local landraces, some rice landraces from China and wild rice (*Oryza rufipogon*) from Northeastern Thailand indicated an average of 10.1 and 8.05 alleles/locus.

Our result (12.2 alleles/locus) is comparable to several other studies (Rabbani et al., 2008; Raghunathachari et al., 2000). Rahman et al. (2007) reported only 53.85% polymorphism among 6 different rice cultivars and inferred a low level of genetic variation and high levels of genetic relatedness among the cultivars. Several other studies also indicated relatively lower levels of polymorphism (65-67%) among local rice landraces (Choudhury et al., 2001; Ashraf et al., 2007; Hashemi et al., 2009). Other reports indicated pairwise similarity found in rice from 0.42-0.85 (Arif et al., 2005), 0.59 to 0.92 (Ren et al., 2003) and 0.50-0.92 (Rabbani et al. 2008). Rekha et al., (2011) observed similarity values ranging from 0.61 to 0.90 for Pakistani and traditional indica rice varieties using 15 RAPD primers. Tehrim et al. (2012) observed similarity values for 35 varieties ranging from 0.53 to 0.94, which is higher than the result obtained in our study. The moderate range of similarity value observed in our study suggests that there is a high level of genetic diversity among the local landraces we tested. Thus, the RAPD marker we identified could serve as a potential tool in the identification and characterization of remote cultivars.

Cluster analysis presents a bottom line about the relationship among the studied cultivars. The twelve genotypes we studied clustered into five distinct groups at the similarity coefficient value of 0.55. The internationally well-known standard for salt-tolerant rice is Pokkali. BRRI had released two moderately salt-tolerant varieties (BRRI dhan47 and BRRI dhan53), which are routinely cultivated in Pirojpur and some southeast coastal areas of Bagerhat, Bangladesh. Pokkali, BBRI dhan47 and BRRI dhan53 formed a distinct group (CLU-1) at the coefficient value of 0.95. This result validated our methods and further implicated that the three verities are related and they are quite distinct from the local landraces. Moderate drought tolerant rice varieties Dular and Panbira fell in the same cluster at the genetic distance of 0.89 and implicated that these two genotypes are genetically similar. Although Gota and Siral are generally maintained and cultivated in different coastal regions of Bangladesh and the variants are morphologically somewhat different, they formed the CLU-5 at the genetic coefficient value of 0.67. Ogunbayo et al. (2005) reported that RAPD markers unravel more differences among rice strains than morphological analyses.

Our study supports and extends previous studies (Ren et al., 2003, Arif et al., 2005, Rabbani et al. 2008, Rekha et al., 2011, Tehrim et al. 2012) that RAPD analysis is a simple, but powerful method for genetic analysis of rice strains. The rice genetic markers that others and we have identified could be used in developing improved and standardized salt-tolerant rice cultivars for routine agricultural use. This will require cloning and further characterization of the markers and transfection and propagation of rice tissue culture and finally, selection of the appropriate strains.

Conclusion

RAPD analysis is a rapid and effective method of investigating genetic variation and relationships among rice

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variants. The rice landraces grown in the coastal districts of Bangladesh include aromatic rice and rice of fine grain quality and the landraces are moderately salt tolerant. RAPD markers of the landraces that are associated with salt tolerance and grain quality could be used in improving rice cultivars with high grain quality and robust salt tolerance.

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