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Diversity and haplotypes of rice genotypes for seedling stage salinity tolerance analyzed through morpho-physiological and SSR markers

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ABSTRACT

Rice is sensitive to salinity at seedling and reproductive stages. A wide genetic variation was reported in rice for salinity tolerance, a pre-requisite for any plant breeding programme. In the present study, we have characterized a set of 192 rice genotypes for their seedling stage salinity tolerance by combining morpho-physiological and molecular markers. The experiment was conducted under control (EC ~ 1.2 dS m^{-1}) and saline stress conditions (EC ~ 12 dS m^{-1}) using hydroponics. There was a significant difference among the genotypes for the ten characters studied. Under saline conditions, the chlorophyll concentration, root length, shoot length, and K^+ concentration decreased as compared to control condition and the opposite was true for Na^+ concentration. Na^+ concentration showed strong negative correlation with chlorophyll concentration, K^+ concentration, root length and shoot length. Thirteen SSR markers associated with *Saltol* region on chromosome 1 were screened across the 192 rice genotypes, of which ten gave scoreable results. Polymorphism Information Content (PIC) and genetic diversity indices showed that the markers RM 493 and RM 10793 were highly useful for distinguishing genotypes. Hierarchical cluster analysis revealed eleven clusters for phenotypic and genotypic data, but very low correspondence was observed between two dendrograms. The haplotypes of genotypes for *Saltol* associated markers, when compared with FL 478, revealed that the genotypes CST 7-1 and Arvattelu could be good candidates for novel genomic regions governing salinity tolerance in rice.

1. Introduction

Soil salinity is widespread problem in both irrigated and non-irrigated crop production systems. Circa, more than 800 million hectares of world's land area are salt-affected (including both saline and sodic soils), which amounts to more than 6% of the world's total land (FAO, 2014). In India, salt affected soils currently constitute 6.73 million ha in different agro-ecological regions. This is expected to increase to 16.2 million ha by 2050 (CSSRI Vision 2050). Rice (*Oryza* spp.) is an important cereal crop and is mainly used for human consumption. Rice provides 50–80% of daily calorie intake among the poor class of the society. It's a staple food and cash crop for over three billion people in the world (Ma et al., 2007). Salinity is the second most important abiotic stress, after drought, limiting rice production in 30% of the rice-growing area worldwide (Tanji, 1990; Wu and Garg, 2003). Rice is sensitive to salinity, at seedling and reproductive stages. Seedlings of rice die at salt level of 10 dS m^{-1} (Munns et al., 2006). Salinity is a complex quantitative trait with low heritability (Shannon, 1985; Yeo and Flowers, 1986) and phenotypic responses of plants to salinity are greatly influenced by environment (Gregorio and Senadhira, 1993; Gregorio, 1997; Krishnamurthy et al., 2015a, 2015b; Tack et al., 2015). The situation is further exacerbated by the side effects of using landraces (Pokkali and Nona Bokra) for transferring salt tolerant genes into traditional local varieties.

In view of aforesaid drawbacks of traditional breeding procedures, there is an increased exploitation of molecular breeding methods (Gregorio et al., 2002; Yamaguchi and Blumwald, 2005; Ismail et al., 2007; Thomson et al., 2010). The collaborative efforts of International Rice Research Institute (IRRI) with researchers of India, Bangladesh and Philippines have lead to success in this direction. A major QTL associated with Na^+-K^+ ratio and seedling stage salinity tolerance, named *Saltol*, was located on chromosome 1 (Gregorio, 1997; Bonilla et al., 2002). This QTL was indentified through AFLP genotyping using a subset of tolerant and sensitive Recombinant Inbred Lines (RILs) developed from a cross between two *indica* rice varieties IR29 (salinity sensitive) and Pokkali (salinity tolerant). This QTL explained 43% of the variation for seedling shoot Na^+-K^+ ratio when tested on a set of 54 RILs (Bonilla et al., 2002). Later, this region was saturated with RFLP and SSR markers. Since then this region was the most exploited QTL for seedling stage salinity tolerance. Many attempts were made across the

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globe to introgress *Saltol* QTL into the locally popular varieties (Huyen et al., 2012, 2013; Linh et al., 2012; Usatov et al., 2015; Singh et al., 2016). The popular donor among these studies was FL478, a salt tolerant RIL from Pokkali × IR 29 cross. This line was found to contain a < 1 Mb DNA fragment from Pokkali at 10.6–11.5 Mb on chromosome 1, flanked by IR 29 alleles (Kim et al., 2009). But, our personal experience had shown that this region when introgressed into other backgrounds (popular high yielding varieties) did not show the level of tolerance which was found in FL 478. Thus, this *Saltol* region may be affected by genetic background into which it was transferred or apart from *Saltol*, there may be other key regions which are also contributing to seedling stage salinity tolerance (Alam et al., 2011). This experiment was framed to test the second part of above hypothesis.

Using the markers associated with *Saltol* QTL and FL478 as tolerant check, many studies were conducted to group the genotypes for salinity tolerance (Islam et al., 2012; Davla et al., 2013; Ali et al., 2014; Babu et al., 2014; Chattopadhyay et al., 2014; Krishnamurthy et al., 2014, 2015c; Dahanayaka et al., 2015; Kordrostami et al., 2016). A simple and precise screening procedure at seedling stage was devised by Gregorio (1997). This has lead to the generation of ample phenotypic data to supplement the genotypic data in the above studies. Coupled with multivariate analysis, haplotype studies have recently become popular, where genotypes are analyzed for different markers and the allelic pattern of these markers is compared with the checks to find novel donors for desirable phenotypes. Such a study was first done by McCartney et al. (2004) for Fusarium head blight resistance QTLs in wheat. Haplotype analysis was tried in rice for seedling stage salinity tolerance as well as using markers associated with *Saltol* (Mohammadi-Nejad et al., 2010; Islam et al., 2012; Babu et al., 2014; Krishnamurthy et al., 2015c; Kordrostami et al., 2016). Most of these studies used landraces and wild relatives to find novel sources of salinity tolerance. The constraints of using these landraces as parents are well known. Hence in the present study, we have used a diverse set of 192 rice genotypes which were not characterized before for seedling stage salinity tolerance. The present study was designed with the following objectives: (1) assessing the genetic diversity of these rice genotypes based on morpho-physiological and molecular data using SSR markers associated with *Saltol* QTL on chromosome 1, (2) identifying the most discriminating and informative markers to aid in the selection of salt tolerant rice genotypes, and (3) identifying salinity tolerant rice genotypes with putative novel genomic regions for salinity tolerance.

2. Material and methods

2.1. Plant materials

We have screened one hundred and ninety-two genotypes obtained from different sources for salinity tolerance at the seedling stage (Supplementary Table 3). Seven varieties and sixty-two advanced breeding lines were evolved at ICAR-Central Soil Salinity Research Institute, Karnal, fifty-one genotypes were landraces, nine genotypes were acquired from IRRI and the remaining were high yielding varieties released from various ICAR institutes and state agricultural universities. The genotypes FL 478 and IR 29 were used as seedling stage salinity tolerant and susceptible checks, respectively.

2.2. Plant culture and salinity treatments

Screening for salt tolerance at seedling stage was performed in hydroponics using Yoshida culture solution (Yoshida et al., 1976), under controlled glasshouse conditions at CSSRI, Karnal, India, with 29–35 °C/21 °C day/night temperature. Relative humidity was 30–40% and photoperiod of 13 h. The seedlings were established on Styrofoam floating grids in 200-l tanks and separate tanks were used for control and salinity treatments. Measured quantity of NaCl was added to the nutrient solution for salinity stress (EC ~ 12 dS m^{-1}) on 14th day after sowing. The solution was replaced every week and the pH was maintained at 4.5–5.5. Control plants were grown at the same time in nutrient solutions without NaCl. The entire experiment was conducted in an augmented block design with checks replicated after every 78 genotypes. Fifteen seedlings were grown per genotype, but only five plants of uniform growth per genotype were considered for data collection after 14 days of saline treatment.

2.3. Measurement of morpho-physiological characters

To evaluate the salinity tolerance of these 192 genotypes, ten traits were recorded, namely, salt injury (sal), root length (cm), shoot length (cm), total chlorophyll concentration (mg g dw^{-1}), Na^+ and K^+ concentrations (mM g dw^{-1}) of root and shoot and Na^+/K^+ ratio of root and shoot. Salinity screening data for per cent survival and total leaf area affected were recorded according to the salt injury score (Sal) of Standard Evaluation System (SES) for rice (IRRI, 2013). The salinity SES scores of the seedlings in culture solution were taken on 28th day after sowing (14 days after salinization). Here, genotypes with visual scores between 1 and 5 were considered as tolerant and 7–9 as susceptible. Growth changes in terms of root length and shoot length in response to salinity stress were measured for each genotype 14 days post salinization. Shoot length was measured from the base of the plant to the tip of the longest leaf while root length was measured from the base of the plant to the tip of the longest root.

Genotypes showed leaf yellowing from the tip 4 days after salinization. To compare differences among genotypes, total chlorophyll concentration was measured using DMSO (dimethyl sulphoxide) according to Hiscox and Israelstam (1979). The concentrations of sodium and potassium in the root and shoot nitric-perchloric acid digests were determined for each genotype grown in saline and control conditions using Flame photometer.

2.4. DNA extraction and microsatellite markers

Leaf tissues (150–200 mg) were ground to a fine powder in liquid nitrogen and used for DNA extraction. Genomic DNA was extracted from the ground tissue following modified CTAB method as described by Zheng et al. (1995). The DNA concentration was quantified by a UV–vis spectrophotometer (NanoDrop 2000c, Thermo Scientific Products, USA) and was adjusted to a final concentration of 30 ng μl^{-1}. For molecular analysis, the sequences of 13 microsatellite primer pairs spanning the *Saltol* region on chromosome 1 were downloaded from Genome Databases, Gramene (http://www.gramene.org/). Primers were synthesized by Sigma-Aldrich Corporation, Bangalore, India.

2.5. PCR amplification and gel electrophoresis

PCR reactions were carried out on a Biometra TGradient Thermocycler (Imperial Life Science (P) Limited, Gurgaon, India). The reaction volume was 10 µl containing 50 ng template genomic DNA, 1.8 µl 10× Taq polymerase assay buffer (with 16 mM $MgCl_2$), 1 µl of dNTPs, 0.5 µl of each primer and 1 U TaqDNA polymerase (Merck Specialities Private Limited, Mumbai, India). The temperature cycles were programmed as initial denaturation at 94 °C for 5 min followed by 35 cycles at 55 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and 7 min at 72 °C for final extension. Aliquots of 10.0 µl amplified PCR products, along with DNA ladder were resolved by electrophoresis on 3% agarose gel in 1× TBE buffer, stained with ethidium bromide. Gels were scanned with a gel documentation system (Alpha-Imager Private Limited, Bangalore, India).

2.6. Data analysis

Morphological data was summarized using MS Excel. Correlations were worked out among various traits recorded under saline stress. For