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DETECTION OF SALT TOLERANT RICE GENOTYPES AT THE SEEDLING STAGE USING MOLECULAR MARKERS: A GENOTYPIC ANALYSIS

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ABSTRACT

Selection of salinity tolerant genotypes of rice based on the phenotypic variation is not consistent and thereby, will hinder the advancement in breeding. However, the recent introduction of the use of molecular markers, microsatellites or simple sequence repeats (SSRs) to detect the salt tolerant rice genotypes is reliable. The objective of the present study was to screen the four indigenous rice genotypes collected at Coastal Saline Research Centre, Ramanathapuram, Tamil Nadu, India, for the identification of salt tolerant genotypes at the seedling and reproductive stage under saline and non-saline conditions using the microsatellite markers. Two selected SSR markers were used to determine salinity tolerance in rice genotypes. SSR based marker identified three genotypes as tolerant at least tolerant to one of the two SOS markers but one variety was susceptible for both the markers. The detected salt tolerant genotypes can be a probable germplam resource for prospective breeding program.

KEYWORDS: Na^+ / K^+ ratio, SOS genes, Salt stress, SSRs, RM315 markers.

INTRODUCTION

Salinity, which is considered as a severe limitation to increase the production of rice worldwide, is one of the leading soil problems in rice growing countries followed by drought.[1] It has also been well recognized that salt tolerance varies with rice.[2] Salinity affects the growth and development of the rice plant owing to the abiotic stress on the plants directly.[3] Salt stress, which has been shown to cause osmotic stress, ionic toxicity, oxidative stress and nutritional imbalance in plants, affects the germination and seedling growth of the

plant. It also decreases the leaf expansion which leads to the diminished the photosynthetic area and dry matter production.[4]

In India, rice (Oryza sativa), one of the most important staple food, cultivated in about 42.2 million hectares of the total arable land area of 148 million hectares yielded an average of 2.12 metric tons per hectare.[5] Abiotic stress, a key hazard to crop production worldwide, negatively affects the mean yield of major crops by greater than 50%.[6] It has been estimated that the total area which is under salinity is 953 million hectares and it accounts for only 8% of the total land surface. Consequently, coastal areas are predominantly susceptible to salinity owing to the saline water intrusion and also the increase in sea level due to the global warming.[7]

Although rice is comparatively tolerant to salinity at the germination stage, its panicle initiation and pollination stage are sensitive to salinity and as a result, it is directly associated with crop yield.[8 – 10] Moreover, screening of rice genotypes at seedling stage is relatively easier than reproductive stage and also rapid but it is very complex at the reproductive stage. Other than the reproductive stage of the rice plant, environmental and less sense heritability of salt tolerance also affect the salt tolerance of the plant.[1]

It has been reported that the detection of major gene locus for salt tolerance next to a microsatellite marker can be used by breeders to select more efficiently and to better understand salt tolerance, at vegetative and reproductive growth stages. SSR or microsatellite markers, which are found to be principle model for constructing genetic maps[11], supporting selection and studying genetic diversity in genotype, play an important role in detecting the gene for salt tolerance and thereby, it can be useful for plant breeders to develop new cultivars. In the light of the above findings, the aim of the present study was to screen the four indigenous rice genotypes collected at Coastal Saline Research Centre, Ramanathapuram (Fig.1), using the microsatellite markers to identify salt tolerant genotypes at the seedling and reproductive stage under saline and non-saline conditions.

Fig.1: Collection of indigenous rice varieties from Ramanathapuram Districts, Tamil Nadu, India.

MATERIALS AND METHODS

Collection of Indigenous Rice Varieties

Rice Samples were collected from Ramanathapuram district in Tamil Nadu, India, which is located in the southern part in the latitude between 9°.05′ and 9°.50′ in North and longitude between 78°.10′and 79°.27′ in East, having a hot tropical climate with temperature between 22.3°- 37.8°C and very high relative humidity (80 - 90%) in coastal tracts. The rainfall distribution is less than 870 mm per annum in Ramanathapuram district. Paddy is being cultivated in rain fed conditions. Observations for the past 20 years clearly indicated that the North East Monsoon in this district commences by the last week of October and exists till December. Due to high intensity of rainfall during this season water get stagnated and becomes a common problem for the cultivation of irrigated dry crops. This restricts the farmers to take up only paddy as a mono crop under the water stagnant conditions. After the harvest of the rice crop, the entire cultivable area is kept vacant due to non suitability of underground water which is saline in nature and also with more chlorine content due to sea water intrusion. The sea water intrusion has already extended up to 50 km from the coastal zone and expected to extend further in future. Based on these experiences we can presume that the crop cultivation in this district should depend only with saline water and this condition is found all over the district. The pH of soil ranges from 8.1 to 8.6 and the EC is more than 3.0 in most of the area. In our present study, four indigenous rice genotypes were collected with the help of the scientists working in Coastal Saline Research Centre, Ramanathapuram. Sample leaves from four indigenous paddies which is named as RMD 1 to

4, were collected at the early stage of rice crop cultivated in the coastal belt (14 – 16 day old). For each sampling, the three topmost fully expanded leaf positions were sampled from four hills per genotype. While a part of the samples were preserved in refrigerated condition another part was oven-dried (70°C to constant weight), ground and preserved as powder for further analysis.

Analysis of sodium and potassium content from Paddy leaf samples

Ground and preserved powder was analyzed by flame photometry (Digital Flame Analyzer, Cole Parmer, Illinois, USA) for potassium and sodium content after 48 h of extraction with 1N HNO_3, following the procedure described by Yoshida et al. (1976).[12] Briefly the leaves powders are first crushed by hand to make them into small crumbs in a plastic container for homogenization of the sample. Then the leaves are crushed by means of a micro-electric grinder to obtain a very fine grind. Clean the blade of the grinder with a brush after each grinding to avoid mixing the samples. After grinding of each sample, store in a pill box and label it on the cap, this allows the stoker plant powder until analysis and mineralization.

1g of each samples in crucibles are weighed by the precision analytical balance. Heat the contents of the crucibles at a temperature of 100°C for 10 minutes until powder colour changes from black to grayish white. Samples allowed to cool then added to each crucible 20mL of 1N nitric acid HNO_3. The samples were heated on a hotplate till boiling. Cooled and filtered through ash less filter paper in a 100mL volumetric flask which is then made upto the mark with distilled water. The determination of sodium and potassium in the flame spectrophotometer using stock solutions samples corresponding to each.

Isolation of Paddy Leaf DNA

Fresh leaf tissues of 14 – day old seedlings were chosen for DNA isolation using the modified CTAB method. Leaf samples were grinded with extraction buffer and SDS was followed by incubating the leaf sap at 65°C for 10 min. 100µl NaCl and 100 µL CTAB were added sequentially and mixed well; and incubated again at 65°C for 10 minutes and the suspensions were transferred to a new plate. 900µL chloroform: isoamyl (24:1) was added and mixed well using a shaker. Then the sample was centrifuged at 5700 rpm for 10 minutes. After that the supernatant were transferred into new eppendorf tubes. Then added 600µL ice-cold isopropanol and shaken slowly and then centrifuged at 5700 rpm for 15 minutes. The supernatant was decanted and air dried for the minimum period of one hour. Pellets were washed with 70% ethanol (200µL), spanned for 15 minutes at 5700 rpm and then air-dried

for 1/2-1 hours. Then the ethanol was removed and air-dried. The pellets were resuspended in 30µl 1 X TE buffer for further use.

PCR amplification of the isolated DNA

Amplification reactions were carried out in a thermal cycler with 25µL of PCR reaction mixture containing 2.5µL of 10 X Taq-buffer, 1.0µL of 50mM MgCl2 solution, 0.25µL of 2.5mM dNTPs mixture, and 1.0µL of each of the forward and reverse rice microsatellite primer (RM8094) & the primer for SOS genes at a concentration of 10pmole/µL, 0.1µL (1unit) of 5 unit/µL Taq-DNA polymerase, 4µL of purified genomic DNA (100ng), and 15.15µL of PCR grade water. The primer used in this study was RM315; rice – microsatellite (RM) marker, linked with a drought tolerant QTL, located on the rice 1st chromosome was obtained from Integrated DNA Technologies, USA.

The other PCR reagents were obtained from Fermentus Life Science, USA. The thermal cycling conditions for the first cycle were 97°C for 5 min and 55°C for 2 min. For the next 35 cycles, the temperature regime was 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The final extension was at 72°C for 10 min. The amplified products were resolved in native polyacrylamide gel (6%) following the protocol given by Sambrook and Russel (2001)[13] and documented in a gel documentation system (Perkin Elmer, Geliance 200 imaging system). The length of the amplified DNA bands (microsatellite alleles) were determined with reference to the 100 bp DNA ladder (Fermentus Life Sci. USA) included in the gel as a size marker. The molecular weight (nucleotide base pairs) of the most intensely amplified bands for each microsatellite marker was analyzed using the Gel Doc software.

RESULTS AND DISCUSSION

Analysis of Paddy leaf samples for sodium and potassium content was carried out by flame photometry and the results are tabulated in Table no: 1

Table 1: Na+/K+ ratio in the leaves of Indigenous Rice varieties.

Variety	Na ug/g	K ug/g	Na+/K+ ratio
RMD-1	1.60	8.00	0.2
RMD-2	2.80	2.00	1.4
RMD-3	3.10	1.58	1.9
RMD-4	7.80	1.90	4.1

The variety (Table.1) RMD 1 showed very lower; Na+/K+ ratio which may be an indication that this variety shows saline tolerance when compared with all other varieties. This is in line with the findings of Mishra et al., 1997.[14] This Low Na+/K+ ratio of ion uptake is positively correlated with a high level of salt tolerance and can be taken into consideration as a desired characteristic while screening rice lines.

All the four varieties showed the genomic DNA was isolated as shown in the Fig.2 and amplification for RM 315 marker which is an indication that all the four verities have general tendency for salt tolerance as shown in the figure 3. But in case of the SOS genes (SOS1 and SOS2) the variety RMD1 showed the presence for both. Surprisingly the same variety (which was later identified as *Kalarpala*i by seed enthusiast later) has very low Na+/K+ ratio. This corroborated the property of RMD1 for saline resistance.

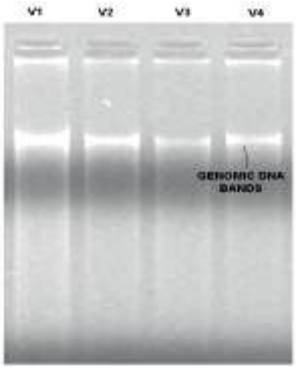

Fig. 2: Genomic DNA from Rice Leaves [Variety 1 (V1), Variety 2 (V2), Variety 3 (V3), Variety 4 (V4)].

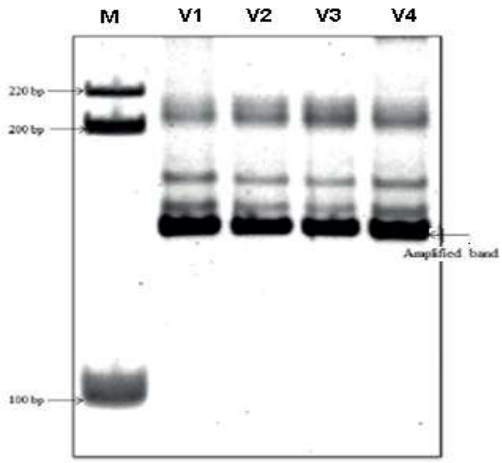

Fig.3: DNA bands amplified from leaves of Rice genotypes V1, V2, V3 & V4 using microsatellite RM 315 markers.

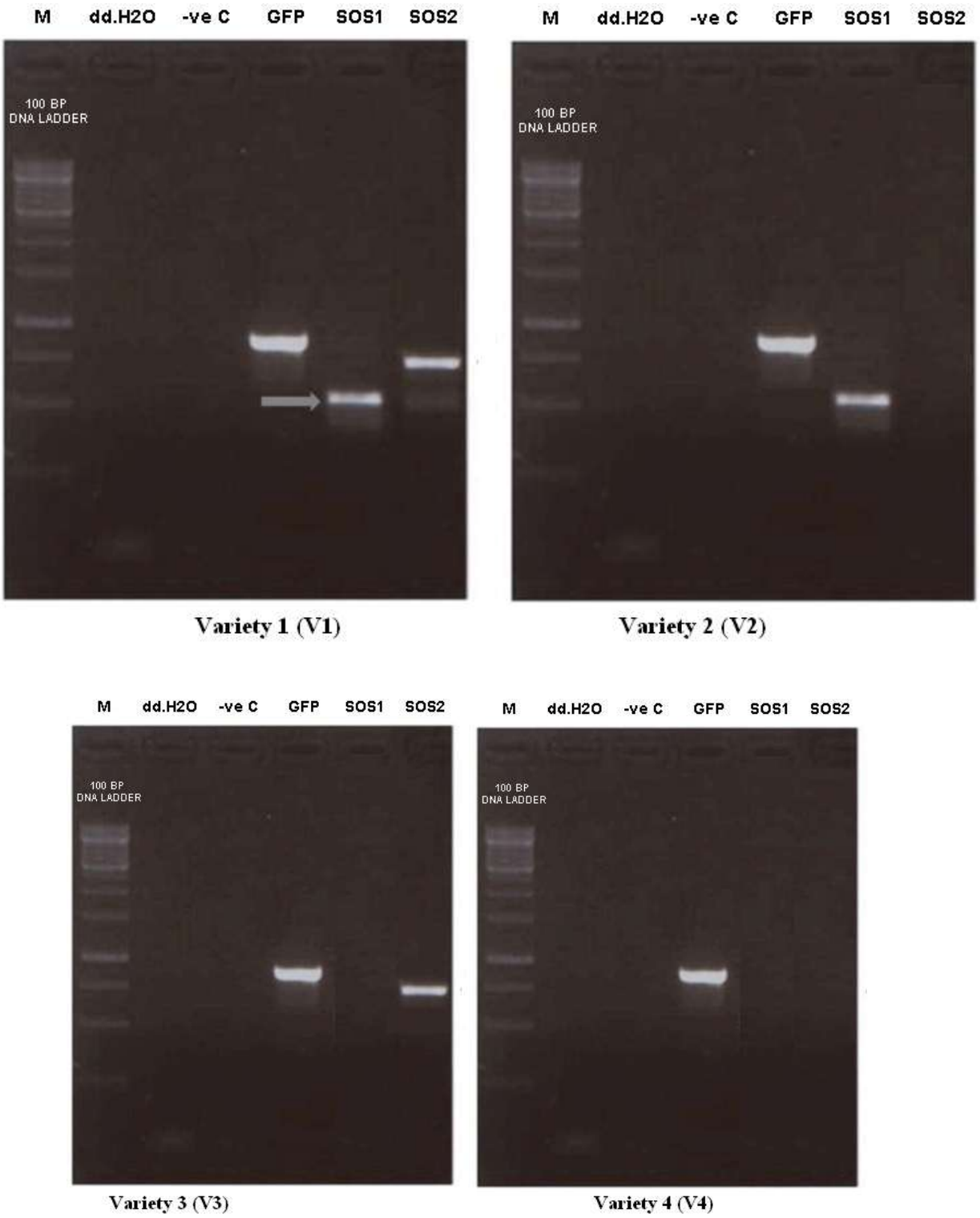

Figure 4: The SOS GENES responsible for salt tolerance in rice plants.

Interestingly though all the four verities showed the presence of RM 315 marker, the RMD2 variety did not responded for SOS2 gene but responded positively for SOS1 gene (Fig.4). The response of RMD3 is reverse in compared to RMD2. RMD3 showed the presence of SOS2 gene. RMD2 and RMD3 verities showed 1.4 and 1.9 Na+/K+ ratio respectively as shown in

the table 1. These values are higher than the value of RMD1. RMD4 did not respond for both SOS1 and SOS2. RMD4 also showed very high Na+/K+ ratio.

The use of ionic composition in rice shoots or rice leaves as an indicator of performance under salinity has been argued controversially in recent years. Yeo & Flowers et al,. 1993, report that leaf sodium concentration in the seedling stage is closely related with survival, but shoot sodium concentration can only account for a small proportion of the variability in survival.[15] Qadar (1988) reports that the potassium status of the rice shoot can be used as an index for salt tolerance. Neither potassium nor sodium concentrations either in the shoot or in the leaves are sufficient as indicators for magnitude of yield loss due to salinity.[16] Garcia et al. (1997) have recently shown that although in rice no significant relationship between shoot sodium and potassium concentrations exists, the sodium potassium ratio has narrow sense heritability.[17] The leaf sodium concentration depends (among other factors) on the retention capacity for sodium in the leaf sheaths.[18] The relation between the two is an indicator for salt tolerance in rice as it captures both desirable high potassium uptake to the plant as a prerequisite for metabolic processes as well as osmotic adjustment and sodium as an indicator for the stress level.

This Low Na+/K+ ratio and the presence of RM315 markers along with SOS genes 1 and 2 positively correlated with a high level of salt tolerance and can be taken into consideration as a desired characteristic while screening rice lines. Molecular marker, which assists in detecting the alleles that are associated with major phenotypic variants, is found to be associated with salt tolerant alleles at seedling stage in rice using phenotypic and marker population. It is also known that salt stress genes were located at loci in chromosomes 1 and 8. A study by Lang et al., (2000) also found that RM223, which is related to salt tolerance gene, is present in chromosome 8.[19]

The results of the current study are in line with a study by Bhowmik SK, 2009 which evaluated 11 rice genotypes using three SSR markers viz., RM7075, RM336 and RM253, found that markers were capable to distinguish the salt tolerant genotypes from the salt sensitive traits by exhibiting the polymorphism among them. The SSR markers including RM7075, RM336 and RM253 used detected 8, 9 and 7 salt tolerant genotypes, respectively.[20]

CONCLUSION

As SSR or microsatellite markers are well known for constructing genetic maps, aiding in selection and studying genetic diversity in germplasms, they play a vital role in detecting salt tolerant gene to assist the plant breeder in developing new cultivars. Accordingly, these markers used in this study showed polymorphism for the rice genotypes. In addition, the detected salt tolerant rice genotypes can be considered as the potential source to improve the salt tolerant genotype.

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