International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(3): 40-46

Received: 20-03-2020
Accepted: 23-04-2020

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Molecular characterization of rice (*Oryza sativa* L.) Genotypes in response to salinity stress

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DOI: https://doi.org/10.22271/chemi.2020.v8.i3a.9461

Abstract
Rice *(Oryza sativa* L.) is the primary food source for over a third of the world's population. The rice production is limited by various biotic and abiotic stress factors. Of the abiotic stresses, high soil salinity, largely contributed by NaCl, is one of the major factors limiting the distribution and productivity of large crops, including rice. Looking to this problem, using molecular approaches total of 10 SSR primers of rice microsatellite were used to screen 24 genotypes of rice (*Oryza sativa* L.). Eight of these primers were successful in producing polymorphic as well as monomorphic alleles in 24 rice genotypes. The selected molecular markers (RM 145, RM160, RM7075 and RM 253) showed polymorphism in 24 rice cultivars.

Keywords: DNA, Marker, PCR, SSR

Introduction
In agricultural systems, much of the deduction that differentiates yield output from harvestable yield [1] in plants is attributed to the abiotic stresses-salinity, low temperature and drought. Rice (*Oryza sativa* L.) is the main food supply for over a third of the world's population. Rice production is limited by various biotic and abiotic stresses. One of the essential factors restricting the distribution and productivity of major crop plants, including rice, is the high soil salinity of the abiotic stresses, caused largely by NaCl. The increasing number of salt-affected hectares of productive arable land, together with the growing demand for food to feed the ever-rising world population, puts considerable emphasis on mediating salinity stress in plants. In many parts of India and elsewhere, significant yield declines have been observed in the rice fields influenced by salt [2, 3]. Salinity continues to be a significant restriction on the growth and extension of rice farming in areas where rice supply has not been sustained by a rising population with increasing demand. Cultivating resistant varieties on saline fields can also reduce salinity through the biological reclamation process. Today, salinity is the second most prevalent soil issue in rice-growing countries next to drought and considered a severe restriction for the rice production worldwide [4]. The production of salt-tolerant rice cultivars using traditional and new molecular techniques would help to solve the world food security problem. Stress, may lead to development, growth, and productivity changes, and extreme stress can threaten survival. Over recent years, substantial attention has been paid to elucidating the molecular basis of plant salt tolerance, and various genes linked to salt tolerance have been found in rice and other crops. Screening of the germplasm at the seedling stage is readily appropriate as it is based on a basic selection criterion; it makes it impossible to screen easily at the vegetative and reproductive level [5]. Controlled screening has the benefit of reduced environmental effects and the hydroponic system is free from the difficulties associated with soil stress factors. Due to the wide environmental impact and poor narrow sense heritability of salt tolerance, traditional plant selection approaches for salt tolerance are challenging. This impedes the production of an accurate, quick, and effective screening technique. However, DNA markers appear to be the best candidates for efficient evaluation and selection of plant material. Recent advancements and technological improvements in DNA marker technology make it possible to reduce breeding time and accuracy where major environmental factors contribute to poor selection performance.

Microsatellite markers have been shown to be suitable for creating genetic maps, aiding in the discovery and analysis of genetic variation in germplasm [6, 7]. SSR markers play an important role in identifying genes for salt tolerance that can enable plant breeders to grow new cultivars. Therefore, in view of the above aspects, the present study was planned to identify the salt susceptible and tolerant genotype of rice by means of SSR markers.

Material and methods

Experimental material

Seeds of 24 rice genotypes used in the study, consisting of 14 genotypes, were obtained from the Main Rice Research Station, Anand Agricultural University, Nawagam, six genotypes from the Central Soil Salinity Research Institute, Karnal and four genotypes from Kerala Agricultural University, Kerala. Seedlings (Table 1) have been grown in a nutrient solution and have undergone numerous salt tension therapies.

Table 1: List of the rice genotypes for phenotypic and molecular screening

1.	IR-64
2.	POKKALI
3.	VYTILLA-1
4.	VYTILLA-2
5.	VYTILLA-3
6.	CSR-30
7.	CSR-13
8.	CSR-36
9.	CSR-10
10.	CSR-27
11.	CSR-23
12.	JAYA
13.	NARMADA
14.	GR-12
15.	GAR-13
16.	GR-104
17.	GAR-1
18.	GR-6
19.	GR-7
20.	GR-9
21.	DANDI
22.	GR-11
23.	GURJARI
24.	SLR-51214

Pretreatment of rice genotypes

The seeds of 24 rice genotypes were raised in hydroponic solution and subjected to different treatments of salt stress. This cultivars were further utilized for molecular screening. Rice seeds from different sources (Table 1) were surface sterilized by following treatments 1.2% sodium hypochloride, 20 min and 0.1 % mercuric chloride, 10 min. subsequently; seeds were rinsed thoroughly with distilled water and placed on cotton wet pad for seven days with three replicates in each treatment. Uniform size seedlings were subjected to 150mM of NaCl (for five days) in nutrient solution in culture room, respectively. After three days seedling were transferred to 250mM of NaCl (for 72 hours) nutrient solution and later to 300mM of NaCl for 48 hrs (Fig 1).

Fig 1: Seedlings of rice genotypes grown on salinity stress (300mM, NaCl) for 48hrs

Isolation of genomic DNA

Genomic DNA was extracted using the CTAB (Cetyltrimethylethyl Ammonium Bromide) method by Li *et al.*, 2001 with some modifications. The freshly collected leaf sample (0.5gm) of each line was crushed in liquid nitrogen and powdered samples were then taken in 10 ml centrifugal tubes. Added to the tube 3 ml of freshly prepared pre-warmed (70°C) extraction buffer and 10µl proteinase K. The tube was vigorously shaken by hand to form slurry and incubated at 65°C in water bath for 60 minutes with intermittent shaking and swirling for 5 minutes. Once, the supernatant was carefully decanted using a wide pipette to avoid shearing in a new 1.5ml Eppendorf tubes and step II was repeated. The supernatant was transferred to a new tube and nucleic acid precipitation was done with equal volumes of ice cold isopropanol. To get maximum precipitation, the samples were maintained at –20°C for a minimum period of 12 hrs. The precipitated samples were then centrifuged at 10,000 rpm for 20 min. The pellets were retained and twice washed with 70% ethanol, air dried and suspended in 200 µl of TE buffer (pH 8.0) in 1.5ml Eppendorf tube, 2µl/100µl of DNase free RNase A were added to the samples and kept for 1 hour at 37°C. The activity RNA degradation was terminated at 65°C for 10 minutes.

Quantity and quality of DNA

The good quality DNA samples with a ratio of 1.7 – 1.8 at O.D 260/280 in spectrophotometric measurements were retained for PCR amplification. The stocks were diluted to a final concentration of 30 ng/µl of DNA and used for further application. The quantification of DNA was carried out using Nanodrop spectrophotometer machine. The quality of DNA was checked on 0.8% (w/v) agarose gel prepared in 1.0X TBE (Tris 45mM, Boric Acid 45mM and EDTA 1mM) containing 2.0 µl of Ethidium bromide (EtBr; 1mg/1ml). The already extracted genomic DNA from the stock (5µl) was mixed with 1µl of 6X agarose gel loading dye. The 5µl sample was loaded in each well using micropipette. The gel was provided a potential difference of 5-6 V/cm for an hour. The bands were visualized under UV light using a Tran's illuminator. The samples which were resolved into discrete high molecular weight bands near the well with no shearing were considered to be of good quality.

Molecular validation

A total 10 SSR primers reported to be linked with tolerant trait in rice were screened to produce distinct polymorphic banding patterns in tolerant and susceptible lines in the present studies. Each PCR reaction was carried out with 25µl reactions containing 0.5µl of each primer, 0.5µl of deoxyribonucleotides, 2.5 µl 10X Assay buffer, 1.0µl of DNA and 0.5 unit of Taq DNA polymerase. The temperature profile used for PCR amplification includes 94◦C for 5 min, followed by 32 cycles of 94◦C for 1 min, 55◦C for 1 min, 72◦C for 1 min, and finally by 5min at 72◦C for the final extension. Annealing temperature was adjusted based on the specific requirement of each primer combination.

Table 2: List of primer (SSR) were used for screening of salt susceptible and tolerant rice genotypes

Sr. No	Primer Name	Primer Sequence 5' to 3'	Reference
1.	RM7075 (F)	TATGGACTGGAGCAAACCTC	(8)
	RM7075(R)	GGCACAGCACCAATGTCTC	
2.	RM336 (F)	CTTACAGAGAAACGGCATCG	(8)
	RM336(R)	GCTGGTTTGTTTCAGGTTCG	
3.	RM223(F)	GAGTGAGCTTGGGCTGAAAC	(9)
	RM223(R)	GAAGGCAAGTCTTGGCACTG	
4.	RM253(F)	TCCTTCAAGAGTGCAAAACC	(8)
	RM253 (R)	GCATTGTCATGTCGAAGCC	
5.	RM128 (F)	AGCTTGGGTGATTTCTTGGAAGCG	(10)
	RM128 (R)	ACGACGAGGAGTCGCCGTGCAG	
6.	RM160(F)	AGCTAGCAGCTATAGCTTAGCTGGAGATCG	(10)
	RM160(R)	TCTCATCGCCATGCGAGGCCTC	
7.	RM280(F)	ACACGATCCACTTTGCGC	(10)
	RM280(R)	TGTGTCTTGAGCAGCCAGG	
8.	RM145 (F)	CCGGTAGGCGCCCTGCAGTTTC	(10)
	RM145(R)	CAAGGACCCCATCCTCGGCGTC	
9.	RM8094 (F)	AAGTTTGTACACATCGTATACA	(11)
	RM8094(R)	CGCGACCAGTACTACTACTA	
10.	RM10745 (F)	TGACGAATTGACACACCGAGTACG	(11)
	RM10745(R)	ACTTCACCGTCGGCAACATGG	

Seven microliters of PCR product was mixed with 2 µl of 6X tracking dye and loaded onto the well. The gel was run at 80 V current (constant) to separate the amplified bands. The standard DNA marker (50 bp) was also run along with the samples. The separated bands were identified under UV trans illuminator and photographed using Alpha Ease FC 4.0.0 Gel documentation system (Alpha Innotech Corporation, USA) and analyzed.

Result and Discussion

It is readily acceptable to test genotypes at the seedling stage, because it is based on a basic selection criterion [4]. Screening under stable conditions results from decreased environmental impacts, and the hydroponic system is free from problems associated with the stress factors associated with soil. The modern plant selection strategies for salt tolerance are not simple due to the broad environmental impact and the poor sense of salt tolerance heritability. This impedes the production of accurate, quick and effective screening techniques. DNA markers tend to be the best candidates for an accurate assessment and collection of plant material. Recent advances and technical advances in DNA marker technology allow reduction of breeding time and accuracy where pronounced environmental effects hamper poor selection efficiency [7, 12]. It has been shown that SSR or microsatellite markers are suitable for creating genetic maps,

assisting selection and researching genetic variation in germplasm. SSR markers [13] play a significant role in the discovery of salt resistance genes that could be useful for plant breeders in developing new cultivars. The goal of this study was to screen genotypes of rice at the seedling stage using microsatellite markers to identify salt tolerant genotypes.

DNA spectrophotometric analysis revealed an average concentration of DNA at 1775ng/µl. It was achieved mainly to determine the DNA sample concentration to be used for further SSR analysis. The A260 / A280 DNA absorbance ratio ranged from 1.80 to 2.07 with an average value of 1.94. As the ratio of A260 / A280 to pure DNA was 1.8–2.2 [14]. The findings of the present investigation were identical and the content of DNA was fairly strong for further research.

Microsatellite (simple sequence repeat) analysis

Total of 10 primers of SSR were used to select among 24 rice genotypes (Oryza sativa L) for stress on salinity. Eight of these primers managed to generate both polymorphic and monomorphic alleles using 24 rice genotypes (Table 2, Fig 1 to 6).

The RM336 marker was used to discriminate genotypes against salinity tolerance. Pokkali is treated as a positive international salinity tolerance check and IR-64 as a salt-sensitive genotype negative check. Genotypes with identical banding patterns to Pokkali were considered tolerant and different Pokkali patterns were considered susceptible. Using microsatellite markers RM336 the genotypes Vytilla-1, Vytilla-2, Vytilla-3, CSR-30, CSR-36, CSR-27, Dandi and SLR-51214 were found to be tolerant and showed a single 196 bp amplicone. On the other hand, in GR-11, IR-64, Narmada, GR-104, GR-12, GAR-1, GAR-13, Jaya, CSR-10, GR-6, GR-7, GR-9, CSR-13 and CSR-23, this 196bp amplicon was missing. Using the RM336 salinity-tolerant rice genotype marker, [8] reported an amplicon of 196bp in size. They concluded that salinity tolerant rice genotypes could be discriminated against salt susceptible. The number of alleles per marker varies from a single monomorphic allele to two RM160 alleles. Salt-tolerant land race Pokkali showed unique double bands with RM160, while other genotypes showed only a single band with this primer. The unique double band (205bp and 160bp) in Pokkali, CSR-30, Dandi, CSR-27, CSR-13, CSR-23, Jaya, SLR-51214, Vytilla-1, Vytilla-2, Vytilla-3 and CSR-10 may be due to duplicate loci for this marker. Whereas susceptible rice genotypes include GR-11, GR-12, GR-104, IR-64, GAR-13, GAR-1 with a product size of 160bp (Fig 2). The phenomenon was reproducible and may have meaning in the distinct existence of these genotypes. Lack of salinity stress marker that separated alleles for salt-tolerant genotypes and salt-sensitive genotypes, partially due to the nature of the salt tolerance phenotype and the various pathways of the individual rice genotype. This marker on chromosome 9 in the region of the major salt tolerance QTL was identified earlier in Pokkali. Different research conducted [10] using the RIL population derived from Nona Bokra / Pokkali/IR46 and recorded QTL on chromosome 9, suggesting the existence of a salt tolerance gene using RM 160. In fact, chromosome 9 has a small number of chromosomal regions for salt resistance characteristics, in regard to the minimum amount of alloys required for salinity stress in the rice genome. Thus, using RM 160 which is located on the short arm of chromosome 9, it may be possible to distinguish susceptible and tolerant genotypes for salinity stress in rice. The RM128 marker was used to evaluate responsive and resistant rice genotypes where Pokkali and IR-64 respectively were taken for salinity as positive and negative checks. Two RM128 amplicons displayed distinct salt-tolerant genotypes, while others displayed only one amplicon with that tag. The genotypes with identical banding patterns were sensitive relative to CSR-23 and had a discrepancy in pattern to CSR-23 when RM128 was used as a marker. For RM128, 405bp were made, both Dandi and CSR -23, but susceptibles showed 180bp genotype size amplicons including control (Fig 3). Pokkali was detected as salt-tolerant genotype when RM7075 was used as a marker and this banding pattern was contrasted with other rice genotypes. In compared with land-race salt tolerant Pokkali, R M7075 marker RM7075 for chromosome 8 were also found (absence of 350-bp sensitive amplicone) to genotypes Dandi, Vytilla-2, Jaya, SLR-51214, GR-9, GSR-30, CSR-27, CSR-36, CSR-13 and CSR-23 whereas 350-bp amplicon were also identified in GR-11, GR-104, GR-12, GAR-1, IR-64, GAR-13,Gurjari, GR-6, GR-7 and Narmarada. Similarly, [8] phenotypic rice genotypes for salinity resistance using RM7075 could be further evaluated. The RM253 marker has been reported for chromosome 7 and used to estimate salinity tolerance genotypes. The bands obtained from all genotype were compared to the Pokkali. The genotypes with the same banding pattern as Pokkali were adaptive and were found to be reactive with different patterns as Pokkali. Tolerant was detected with RM253, Vytilla-1, CSR-27, GAR-1, GAR-13, Gurjari, GR-12, GR-9, GR-104, Jaya, Narmada, and SLR-51214. In comparison, the RM253 marker displays a different amplicon style and was found to be sensitive for GR-11, IR-64, GR-6 and GR-7. In comparison to Pokkali genotype, the marker RM253 developed an amplicon of 180bp size that was capable of identifying 18 tolerant and four susceptible genotype. Use RM 253, rice genotypes for salinity tolerance have been evaluated by similar scientists and their findings in tolerant and sensitive discriminating genotypes are identical to the present findings. Pokkali showed double bands with the RM280 marker, while other genotypes only presented the single band with the primer. Due to the duplicate loci of this marker, the special double band (100-160bp) in Pokkali, Dandi, CSR-13, CSR-23, CSR-301, CSR-36, Jaya, SLR-51214, Vytilla-1, Vytilla-2, Vytilla-3, GAR-13, may be attributable to GR-11, GR-12, GR-104, GAR-1, Narmada, GR-9, gurjari, GR-6and GR-7 which displayed an amplicon of 160bp (Fig 4) for this marker. The Pokkali land resistance race showed two amplicons of RM 280, sized 145bp and 160bp. The model was reproducible and may have a meaning in the distinct existence of the genotypes. This marker is situated on chromosome 4, where there is no marker alleles, which can vary between salt tolerant genotypes of those which are sensitive to salt like marker RM280, which can be useful for further salinity screening and improvements of rice for the distinction [10] between different salinity genotypes. The RM145 marker displayed (Fig 5) an unequivocal scoring band of 24 separate genotypes of specific responses to tolerance to saline. Pokkali had a special double band with RM145, while other genotypes displayed the single band only. A double rice genotypes, including GR-11, GR-51214, GAR-1, and Narmada, have shown 196-bp product number, could be due to duplicate locos of this marker, the uniquely dubbed (196-80bp) Pokkali CSR-30, Dandi, CSR-27, CSR-23, Jaya, Vytilla-1-1-2, Vytilla-2 and CSR-23. The GR-7, GR-6, Gurjari, GR-9 and GR-12 observed an absence of amplified bands. A major quantitative trait locus (QTL) for salt tolerance "Saltol" was mapped to chromosome 1, which is

responsible for low Na+/K+, high K+ absorption and maintenance of Na+/K+ homeostasis in rice shoots. The RM10745 marker is reported to be related to the Saltol locus (Nejadet *et al.*, 2008). Landrace Pokkali contained Saltol QTL for salinity tolerance, and therefore only 205bp amplicon was produced in Pokkali using RM10745. The majority of the cultivars did not consider this form of amplicon. Genotypes, CSR-10, CSR-27, CSR-30, IR-64, GAR-1 Vytilla-1, Vytilla-2, Vytilla-3 and GR-12 produced 180bp amplicone which has not yet been recorded for salinity (Fig 6). Nonetheless, further study and confirmation of the use of this marker in the discrimination against salt-tolerant and susceptible rice genotypes is required.

Eight of these primers succeeded in generating both polymorphic and monomorphic alleles when added to the DNA of the 24 rice cultivars. The marker RM336 produced a 196bp amplicon while the tolerant land race Pokkali showed unique double bands with RM160 and RM128. The marker RM7075 (350bp) identified 14 tolerant and 10 susceptible genotypes especially in comparison to Pokkali, while the marker RM253 (180bp) identified 18 tolerant and four susceptible genotypes compared to Pokkali. RM145 (196 bp-80 bp) displayed distinctive amplicon in 24 different genotypes with differing responses to salinity tolerances. In addition, another RM10745 marker stated to be associated with Saltol locus was also present in Pokkali, which produced a different 205bp amplicon for salinity tolerance.

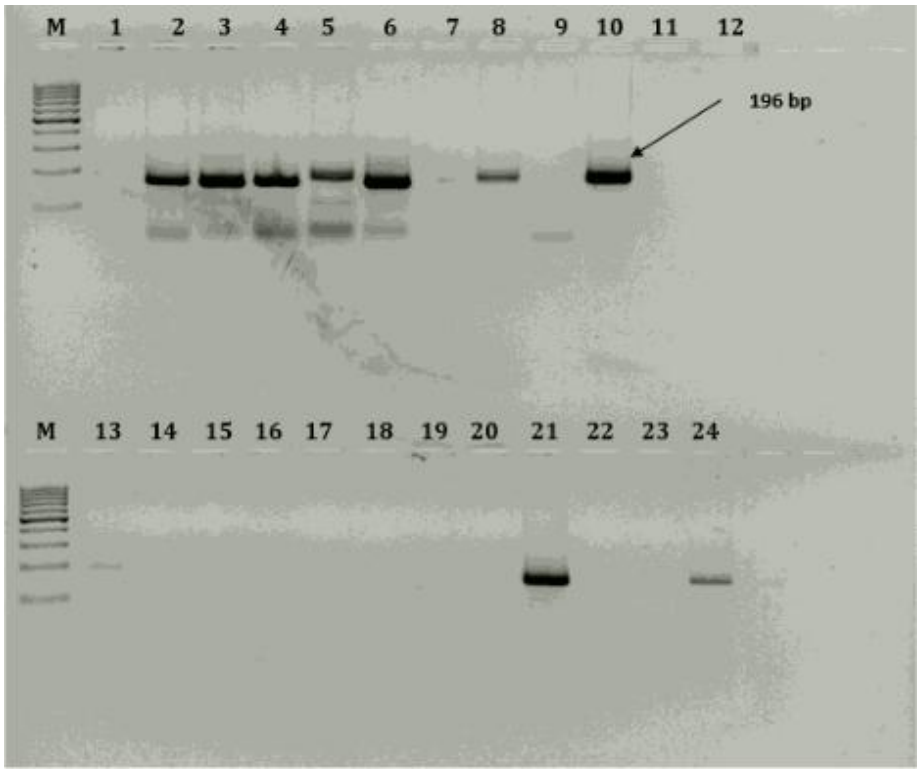

Fig 2: DNA bands amplified from leaves of different rice genotypes using microsatellite marker RM336. M=100bp ladder

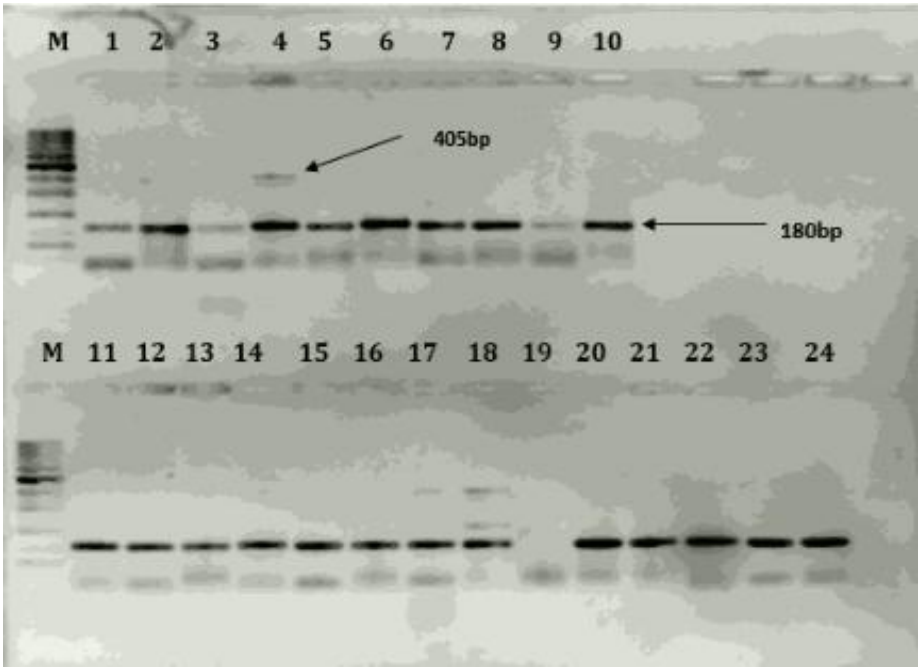

Fig 3: DNA bands amplified from leaves of different rice genotypes using microsatellite marker RM128. M=100bp ladder

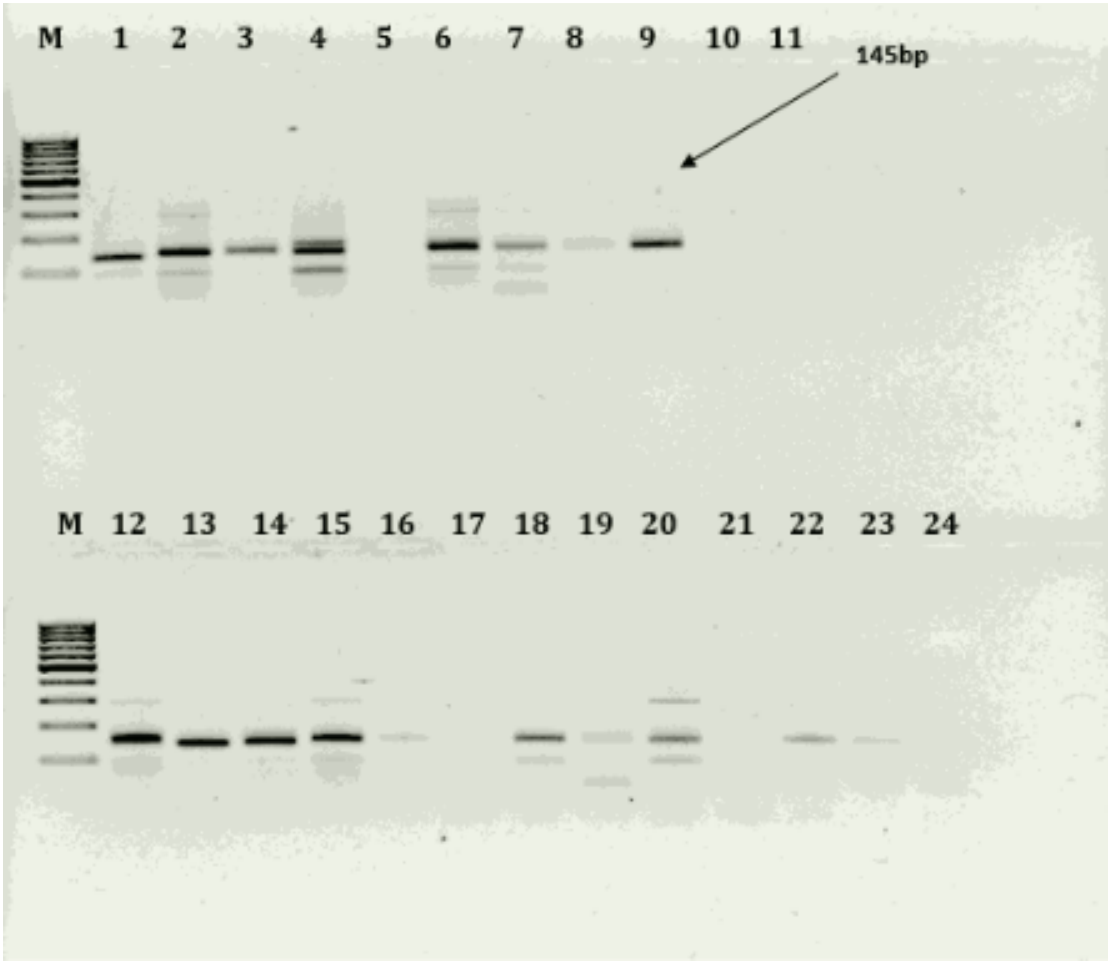

Fig 4: DNA bands amplified from leaves of different rice genotypes using microsatellite marker RM280. M=100bp ladder

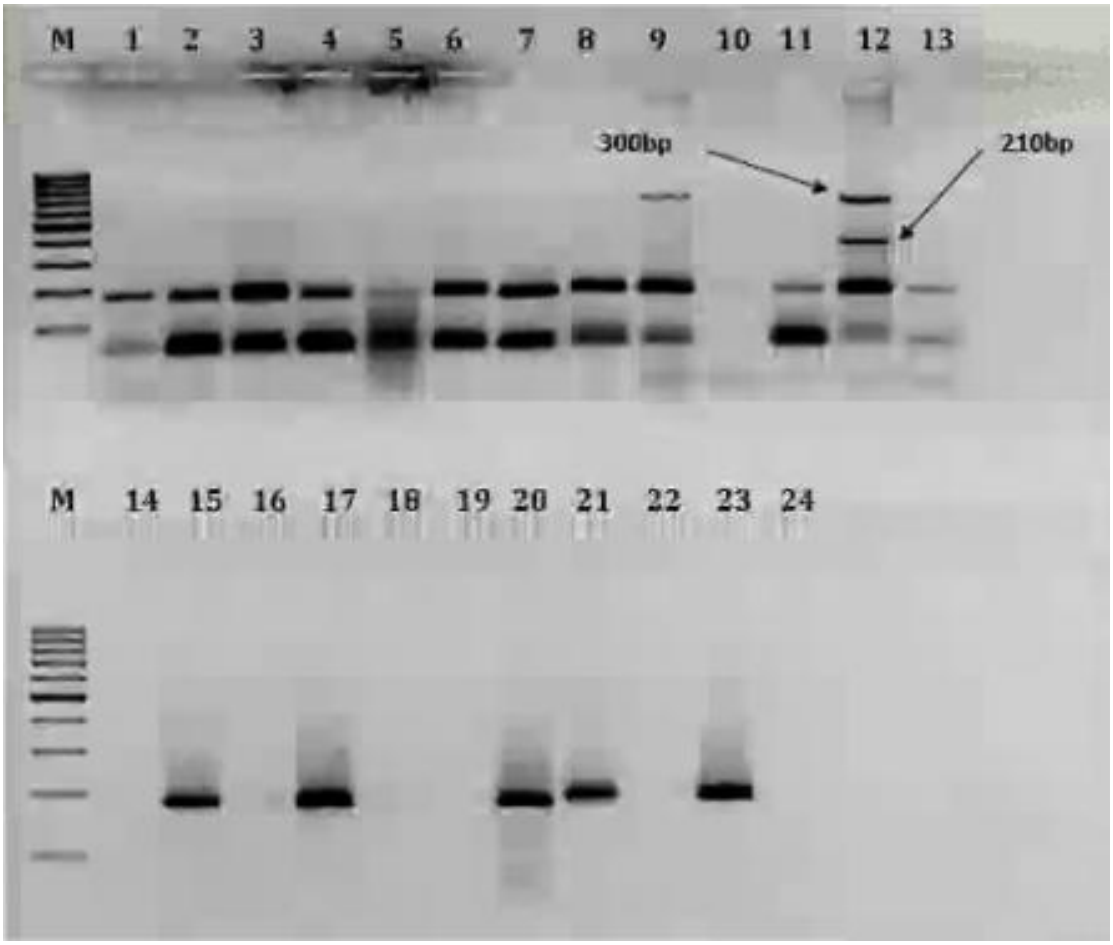

Fig 5: DNA bands amplified from leaves of different rice genotypes using microsatellite marker RM145. M=100bp ladder

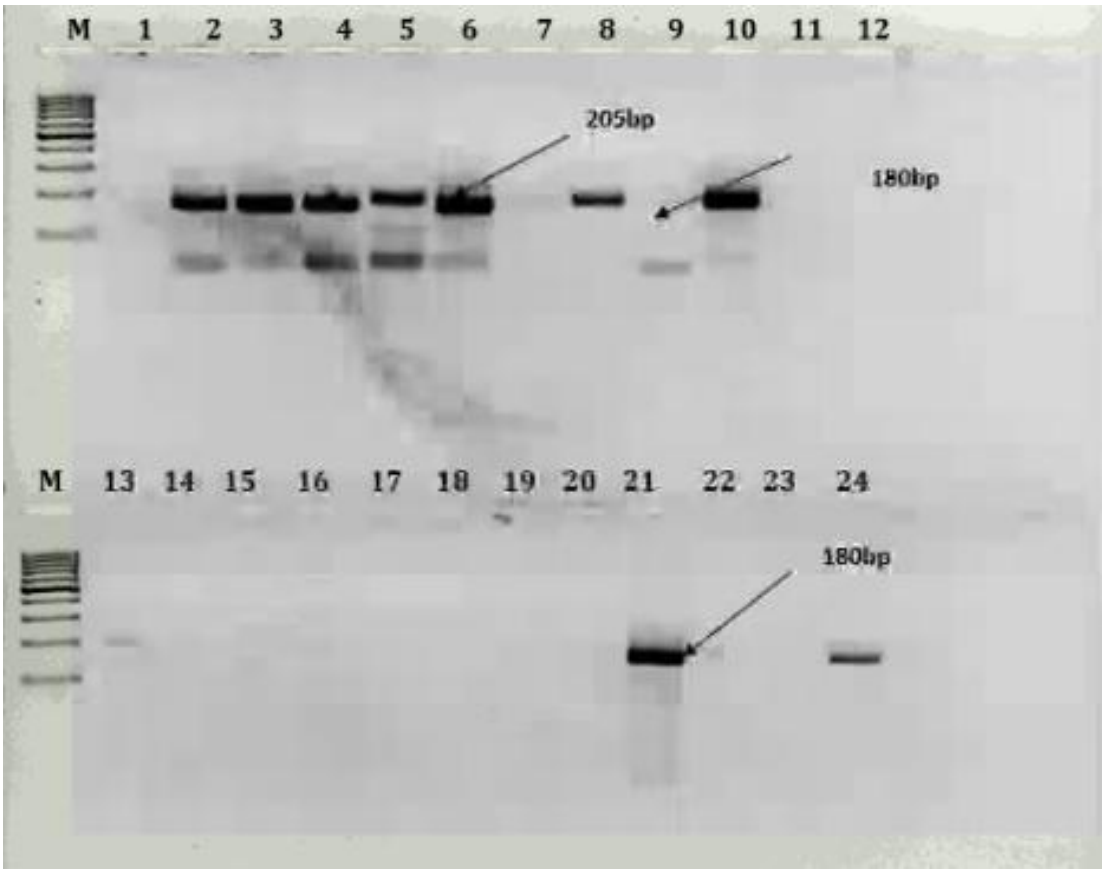

Fig 6: DNA bands amplified from leaves of different rice genotypes using microsatellite marker RM10745. M=100bp ladder

Conclusion

The selected molecular markers (RM 145, RM160, RM7075 and RM 253) demonstrated polymorphism in 24 rice cultivars. These markers have been able to discriminate against tolerant genotypes from susceptible ones. Since the markers used in this study showed polymorphism with these genotypes, these makers could be used to identify salt tolerant genes in the Marker Assisted Selection and quantitative trait loci (QTL) mapping after the necessary validation.

Acknowledgements

I especially want to thank my major advisor, Dr. Subhash N, Professor and Head, Plant Tissue Culture Lab, Department of Biotechnology, AAU, Anand, for his guidance during research at Anand Agricultural University. His perpetual energy and enthusiasm in research had motivated all, including me. In addition, he was always accessible and willing to help his students with their research. As a result, research life became smooth and rewarding for me Hence, sincerely my first and foremost thanks are to him.

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