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Molecular characterization for salinity tolerance in rice using microsatellite markers

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Abstract

A set of six rice cultivars including salt tolerant Narendra Usar Dhan-3 and CSR-30, moderately salt tolerant BPT-5204 and MTU-7029 and salt sensitive Rajendra Bhagwati and Pusa Basmati-1 along with their controlled callus and salt-stressed callus were characterized using a panel of fourteen salt tolerance related microsatellite markers distributed amongst seven chromosomes of rice. The largest amplicon was produced by marker RM242 and the smallest by RM253. A total of 100 allelic products were generated at 22 microsatellite loci with an average of 4.54 alleles per locus. The polymorphism information content values varied from 0.376 (RM4) to 0.827 (RM242) with an average 0.677. A similarity coefficient based dendrogram was constructed using un-weighted paired group method with arithmetic average (UPGMA) for entries classification. The first cluster accommodated salt tolerant cultivars CSR-30, Narendra Usar Dhan-3 and moderately tolerant cultivar MTU-7029, the second salt sensitive cultivar Rajendra Bhagwati, whereas the third moderately salt tolerant cultivar BPT-5204 and salt sensitive cultivar Pusa Basmati-1. Molecular marker based analysis confirmed the induction of salt tolerant calluses in three cultivars MTU-7029, Rajendra Bhagwati and BPT-5204. The work showed the utility of microsatellite markers in ascertaining the status of rice plants with respect to salinity stress tolerance.

Highlights

- Six rice cultivars including salt tolerant moderately salt tolerant and salt sensitive and along with their controlled callus and salt-stressed callus were characterized and evaluated for variation in different qualitative and quantitative traits.
- SSR analysis was performed to discriminate the different salt tolerant moderately salt tolerant and salt sensitive and along with their controlled callus and salt-stressed callus.

Keywords: Microsatellite markers, rice, salinity tolerance

Rice is the most important economic and nutritional cereal of the world. Its productivity is limited by the major abiotic stresses including salinity, drought, water logging and heat. Damages caused by these stresses are responsible for enormous economic loss world-wide. Salinity is a major abiotic stress affecting this crop adversely to a considerably greater extent. It is caused by poor water management, high evaporation, heavy irrigation, previous exposure to seawater and regular use of chemical fertilizers (Rakshit *et al.* 2010). Salinity is the biggest problem in rice growing areas of many countries (Senadhira 1987). It is estimated that 20% of the irrigated land in the world is affected by salinity (Yamaguchi and Blumwald, 2005). Moreover, it has been predicted that increasing salinity in agricultural fields will reduce the land available for cultivation by 30% within the next 25 years and up to 50% by the year 2050 (Wang *et al*. 2003). In India the salt affected area is around 8.6 million hectares, of which about 3.0 million hectares are coastal saline.

Excess salt in soil interferes with several physiological and biochemical processes resulting in problems such as ion imbalance, mineral deficiency, osmotic stress, ion toxicity and oxidative stress in crop plants. These conditions ultimately interact with several cellular components including DNA, protein, lipids and pigments in plants (Zhu, 2002) impeding the growth and development of a vast majority of crops. Plants have evolved many biochemical and molecular mechanisms to protect from the detrimental effects of salt-stress. The main biochemical strategies are induction of antioxidative enzymes, ion-homeostasis and synthesis of compatible organic solutes. The development and identification of salt-tolerant genotype that can tolerate high levels of salinity in the soils would be a practical solution of such problem in crops (Yamaguchi and Blumwald, 2005).

Molecular genetic studies have revealed that tolerance to salt stress in plants is controlled by interactions between several independently regulated but temporally and spatially controlled processes (Kawasaki *et al.* 2001; Ozturk *et al.* 2002; Seki *et al.* 2002). Using different mapping populations, quantitative trait loci (QTLs) for salt tolerance (Zhang *et al.* 1995) and seedling traits associated with salt tolerance have been mapped on different chromosomes (Prasad *et al.* 2000; Koyama *et al.* 2001; Gregorio *et al.* 2002; Lee *et al.* 2004; Ren *et al.* 2005; Lee *et al.* 2007). The chromosomal location of ion transport and selectivity traits that are compatible with agronomic needs have been mapped to reveal that QTLs for Na^+ and K^+ transport are likely to act through the control of root development and structure and the regulation of membrane localized transport compartmentalization. A major QTL designated as *saltol* has been mapped on chromosome one using microsatellite markers (Bonilla *et al.* 2002; Gregorio *et al.* 2002). The positive alleles for this QTL accounted for high K^+ and low Na^+ adsorptions and low Na^+ to K^+ ratio under salinity stress (Bonilla *et al.* 2002; Gregorio *et al.* 2002; Lin *et al.* 2004). A locus designated as *SKC1* has been mapped on chromosome one (Ren *et al.* 2005) that is responsible for maintaining K^+ homeostasis in the salt tolerant variety under salt stress. Similarly the major QTLs conferring salt tolerance at young seedling stage, designated as *qST1* and *qST3*, have been mapped on chromosome one and three (Lee *et al.* 2007).

Molecular markers are important tools for analysis of genetic composition, detection of desirable traits and somaclonal variation. Amongst the several classes of molecular markers, microsatellite marker is believed to be the most suitable for characterization of salt tolerant genotypes, callus and regenerated plants due to its highly polymorphic, highly reproducible, co-dominant and multiallelic nature. Because of several advantages, microsatellite markers have been widely used in rice for varietal identification, diversity analysis, identification of introgression in inter-specific crosses, tracing pedigrees, locating genes and quantitative trait loci and assisting in marker aided selection (Wong *et al.* 2009). Usefulness of microsatellite markers for germplasm characterization in rice is well established on the basis of the studies on landraces (Thomson *et al.* 2007), cultivars (Garris *et al.* 2005) and relatives (Brondani *et al.* 2003).

The present investigation was conducted to ascertain the nature of salt tolerance associated microsatellite markers based differentiation and divergence amongst some salinity tolerant and susceptible cultivars of rice and their controlled callus and salt stressed callus. This report will help to understand the genetic relationship among the rice genotypes having differential responses to salt stress and to formulate future strategies for the identification, development and characterization of salinity stress tolerant genotypes.

Materials and methods

Experimental materials

The leaves of 15-20 days old seedlings grown in pots, controlled callus and salt stressed callus of six rice cultivars including salt tolerant Narendra Usar Dhan-3 and CSR-30, moderately salt tolerant BPT-5204 and MTU-7029 and salt sensitive Rajendra Bhagwati and Pusa Basmati-1 as evidenced by seed germination, callus formation and callus growth under salt stress (Kumari *et al.* 2015) comprised experimental materials of the present study.

Isolation of genomic DNA

Total genomic DNA was isolated from leaves (L), controlled callus (CC) as well as salt stressed callus (CS) of rice cultivars using CTAB method as described by Doyle and Doyle (1990) with some modifications. The isolated DNA samples were stored at -20°C in TE buffer and the quality of extracted DNA samples was ascertained by agarose gel (0.8%) electrophoresis using 1kb DNA ladder (Gene ruler, Fermentas) as standard. The amount of DNA in the extracted DNA samples was determined by measuring the optical density at 260nm. Absorbance ratio (ratio of absorbance at 260 and 280 nm) was used as an indicator to check the purity of extracted DNA samples.

Amplification of genomic DNA

A panel of fourteen salt tolerance associated microsatellite primer pairs distributed among seven chromosomes (1, 6, 7, 8, 9, 10 and 11) of rice genome were used for targeted amplification of the genomic DNA. The information pertaining to chromosomal positions, primer sequences, repeat motifs of the microsatellite sites flanked by these primer pairs was obtained from rice genome database (http://www.gramene.org) and the most suitable annealing temperature for each of the primer pairs was experimentally determined (Table 1).

Table 1: List of 14 salt tolerance associated microsatellite primer pairs used in the present study

Sl No.	Locus	Chr. No.	Primer sequence (5′-3′)	Repeat Motif	Annealing temp (^{0}C)
1	RM2	1	(F) ACGTGTCACCGCTTCCTC (R) ATGTCCGGGATCTCATCG	$(GA)_{13}$	55
2	RM4	11	(F)TTGACGAGGTCAGCACTGAC (R)AGGGTGTATCCGACTCATCG	$GA)_{16}$	54
3	RM11	1	(F)TCTCCTCTTCCCCCGATC (R)ATAGCGGGCGAGGCTTAG	$(GA)_{17}$	53
4	RM14	1	(F)CCGAGGAGAGGAGTTCGAC (R)GTGCCAATTTCCTCGAAAAA	$(GA)_{18}$	55
5	RM20	11	(F)ATCTTGTCCCTGCAGGTCAT (R)GAAACAGAGGCACATTTCATTG	$(ATT)_{14}$	53
6	RM24	1	(F)GAAGTGTGATCACTGTAACC (R)TACAGTGGACGGCGAAGTCG	$(AG)_{22}$	56
7	RM204	6	(F)GTGACTGACTTGGTCATA (R)GCTAGCCATGCTCTCGTACC	$(AG)_{45}$	55
8	RM223	8	(F)GAGTGAGCTTGGGCTGAAAC (R)GAAGGCAAGTCTTGGCACTG	$(AG)_{20}$	56
9	RM242	9	(F)GGCCAACGTGTGTATGTCTC (R)TATATGCCAAGACGGATGGG	$(AG)_{11}$	55
10	RM253	6	(F)TCCTTCAAGAGTGCAAAACC (R)GCATTGTCATGTCGAAGCC	$(AG)_{22}$	56
11	RM292	1	(F)ACTGCTGTTGCGAAACGG (R)TGCAGCAAATCAAGCTGGAA	$(GT)_{10}$-G-$(TGA)_2$	55
12	RM302	1	(F)TCATGTCATCTACCATCACAC (R)ATGGAGAAGATGGAATACTTGC	$(AT)_{13}$	56
13	RM333	10	(F)GTACGACTACGAGTGTCACCAA (R)GTCTTCGCGATCACTCGC	$(TAT)_{19}(CTT)_{19}$	54
14	RM336	7	(F)CTTACAGAGAAACGGCATCG (R)GCTGGTTTGTTTCAGGTTCG	$(AAG)_{18}$	56

Polymerase chain reaction (PCR) based **a**mplification was carried out using 15 µl reaction mixture containing 5X PCR buffer, 1mM deoxyribonucleotide triphosphates (dNTPs), 10mM of $MgCl_2$, 5µM of each forward and reverse primer, 1 unit Taq DNA polymerase (Fermentas) and 20 ng of template DNA. The PCR amplification was carried out with the help of a Thermocycler (Eppendorf) programmed to 1 cycle of 4 min at 94^0C as an initial strand separation step. This was followed by 35 cycles of 1 min at 94^0C for denaturation, 1 min for primer annealing with varying annealing temperature depending on the primer pairs used (53^0C-56^0C) and 2 min at 72^0C for primer extension. Finally 1 cycle of 10 min at 72^0C was used for final extension and then products generated from amplification reactions were stored at 4^0C till separation and resolution through electrophoresis.

The products of amplification reactions were separated using 2% Top vision Agarose (Fermentas) gels. Gels were stained in ethidium bromide solution. A 50 bp DNA ladder (Gene ruler, Fermentas) was used as size marker to determine the molecular size of amplified products. Electrophoresis was done at 100V for 90 minutes in 0.5X TBE buffer. After electrophoresis, the gels were documented by using a gel documentation system (Alpha Innotech).

Scoring of alleles

The products of amplification reaction, which appeared in the form of bands on the gel, were analyzed by determining their position and size in relation to the position and size of the ladder. The different bands produced by each one of the fourteen primer pairs utilized for targeted amplification of specific region of genomic DNA in the present study were compared and classified into the two different categories of shared and unique bands. Allelic diversity and the suitability of the microsatellite sites based polymorphism for identification of polymorphic and informative markers to characterize and differentiate the experimental materials was assessed on the basis of comparison of polymorphism information content (PIC) of the primer pairs. The data on polymorphism information content (PIC) of the primer pairs was generated by calculating the value according to the formula given by Anderson *et al.* (1993) as follows:

$$PICi = 1 - \sum_{j=1}^{k} P^2ij$$

Where, P_{ij} is the frequency of the j^{th} allele for i^{th} marker and summation extends over k alleles

The information regarding informativeness of the marker was obtained by computing the polymorphism per cent as follows:

PP = (Number of unique alleles/Total number of alleles) × 100

Clustering of entries

The polymorphism in respect of microsatellite sites was recorded on the basis of presence or absence of the bands in different entries used in the present study. All the entries used during molecular characterization were scored for the presence and absence of the microsatellite sites based bands. The data were entered into binary matrix as discrete variables and this data matrix was subjected to further analysis. Genetic similarities among entries were calculated on the basis of presence and absence of common bands. The genetic associations among entries were analyzed by calculating the similarity coefficients (Dice 1945) for pair-wise comparisons based on the proportions of shared bands produced by primers.

Similarity coefficient =2a/(2a+b+c)

Where,

a = Number of bands between J^{th} and K^{th} genotypes

b = Number of bands present in J^{th} genotype but absent in K^{th} genotype

c = Number of bands absent in j^{th} genotype but present in K^{th}genotype

Cluster analysis was performed using the data on similarity coefficients. The method used for tree building in the cluster analysis involved sequential agglomerative hierarchical non-overlapping (SAHN) clustering based on similarity coefficients. The dendrogram based on similarity indices was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). Analysis was performed with the help of NTSYS-pc software (Rohlf 1997). The nature of differentiation and divergence amongst salt tolerant, moderately salt tolerant and salt sensitive cultivars and their controlled callus

and salt-stressed callus under evaluation in the present investigation was assessed by identifying the clusters at appropriate phenon levels.

Results

Polymorphism survey

Fourteen salt tolerance associated microsatellite markers generated polymorphic bands in 18 entries evaluated. The largest band size was produced by marker RM242 (227-281 bp) and the smallest by RM253 (116 -152 bp). A total of 100 allelic variants were detected at 22 primer binding sites with an average of 4.54 alleles per locus (Table 2). The number of loci ranged from one in the cases of markers RM2, RM4, RM24, RM204, RM223, RM292, and RM333 to three in RM20. In the cases of remaining primer pairs, namely, RM11, RM14, RM242, RM253, RM302 and RM336, two loci were detected. The number of alleles per locus ranged from three in the cases of RM14 and RM302 to eight in the cases of RM24 and RM204. Average number of alleles per locus was recorded to be 4.54. Microsatellite primer pairs RM11, RM14, RM20, RM242, RM253, RM302 and RM336 generated amplified products due to amplification of more than one locus. A total of 41 unique and 59 shared allelic variants were generated. The number of unique alleles per locus ranged from one in the cases of RM223 and RM292 to six in the cases of RM336 and RM20. Similarly, the number of shared alleles per locus ranged from two in the cases of RM4 and RM336 to ten in the case of RM20. The primer pairs RM336 and RM242 exhibited easily recognizable polymorphism amongst the entries evaluated in the present study (Figures 1 and 2).

Polymorphism information content (PIC) and polymorphism percent (PP)

The level of polymorphism exhibited among the entries using 14 markers was assessed by calculating polymorphism information content (PIC) of each marker. The PIC values revealing allele diversity and frequency among the entries varied from 0.376 in the case of marker RM4 to 0.827 in the case of RM242 with an average of 0.677. The markers having polymorphism information content (PIC) higher than the average value of 0.677 were RM242, RM204, RM11, RM2, RM14, RM24, RM253 and RM333. The polymorphism per cent was recorded to be the maximum (75) in the case of RM336 and the minimum (25) in the case of RM223 and RM292. The markers RM336, RM2, RM204, RM4 and RM253 recorded higher polymorphism per cent than the average value of 40.26 (Table 2).

Table 2. Analysis of primer pairs used for the amplification of genomic DNA extracted from eighteen entries

Sl No.	Primer	No. of locus	Size of alleles (bp)	No. of alleles	No. of unique alleles	No. of shared alleles	PP	PIC
1	RM2	1	150.00-172.00	06	03	03	50.0	0.703
2	RM4	1	161.36-181.82	04	02	02	50.0	0.376
3	RM11	2	143.48-185.00	08	03	05	37.5	0.802
4	RM14	2	184.78-206.25	06	02	04	33.33	0.685
5	RM20	3	205.94-288.24	16	06	10	37.5	0.623
6	RM24	1	152.94-197.06	08	03	05	37.5	0.814
7	RM204	1	126.00-182.64	08	04	04	50.0	0.820
8	RM223	1	143.10-156.52	04	01	03	25.0	0.648
9	RM242	2	227.78-281.25	09	03	06	33.33	0.827
10	RM253	2	116.07-152.27	07	03	04	42.85	0.688
11	RM292	1	156.52-167.39	04	01	03	25.00	0.512
12	RM302	2	120.37-207.89	06	02	04	33.33	0.623
13	RM333	1	185.71-207.90	06	02	04	33.33	0.722
14	RM336	2	131.48-205.26	08	06	02	75.00	0.645

PP: Polymorphism per cent; PIC: Polymorphism information content

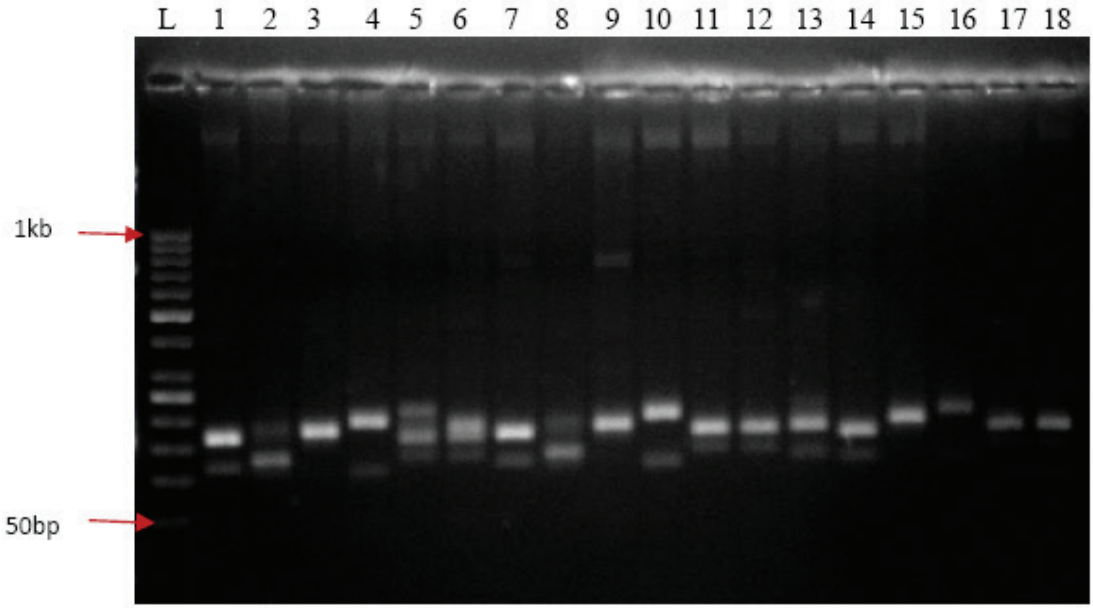

Fig. 1: Amplification pattern of genomic DNA extracted from the leaves, controlled callus and salt stressed callus of rice cultivars by primer RM336

1. BPT-5204 (L) 4. R.Bhagwati (L) 7. BPT-5204 (CC) 10. R.Bhagwati (CC) 13. BPT-5204 (CS) 16. R.Bhagwati(CS)
2. MTU-7029(L) 5. CSR-30 (L) 8. MTU-7029(CC) 11. CSR-30 (CC) 14. MTU-7029(CS) 17 CSR-30 (CS)
3. N.UsarDhan3(L) 6. P.Basmati -1(L) 9. N.Usar Dhan-3(CC) 12. P.Basmati-1 (CC) 15. N.Usar Dhan3(CS) 18. P.Basmati-1CS)

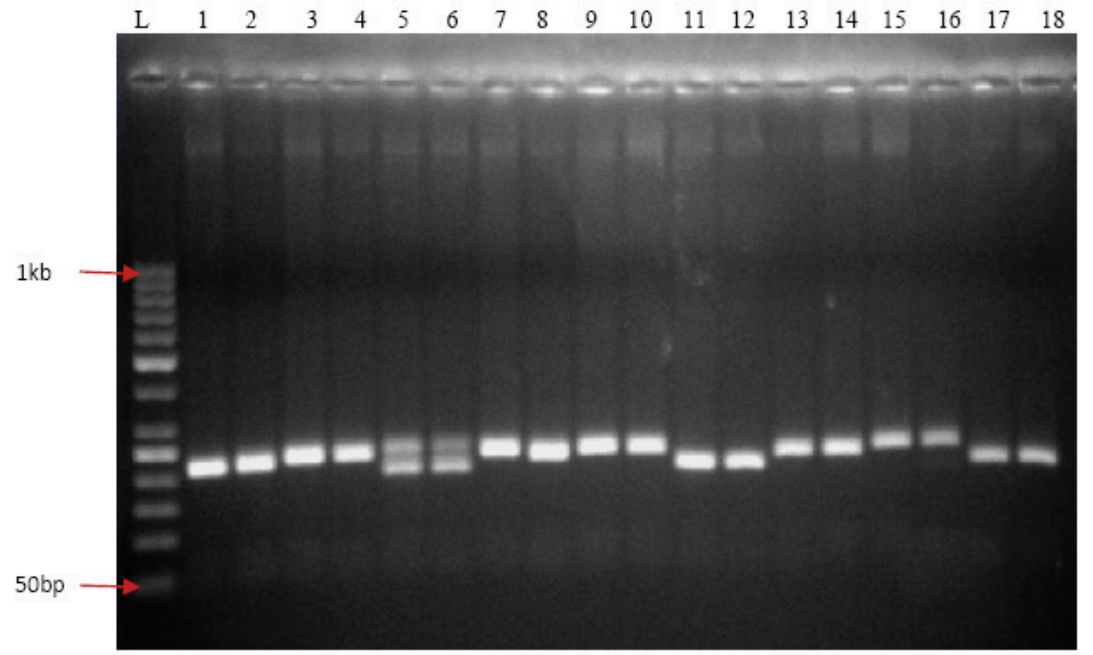

Fig. 2. Amplification pattern of genomic DNA extracted from the leaves, controlled callus and salt stressed callus of rice cultivars by primer RM 242

1. BPT-5204 (L) 4. R.Bhagwati (L) 7. BPT-5204 (CC) 10. R.Bhagwati (CC) 13. BPT-5204 (CS) 16. R.Bhagwati(CS)
2. MTU-7029(L) 5. CSR-30 (L) 8. MTU-7029(CC) 11. CSR-30 (CC) 14. MTU-7029(CS) 17. CSR-30 (CS)
3. N.UsarDhan3(L) 6. P.Basmati -1(L) 9. N.Usar Dhan-3(CC) 12. P.Basmati-1 (CC) 15. N.Usar Dhan3(CS) 18. P.Basmati-1CS)

The lane (L) represents 50 bp molecular size marker; Lanes 1 to 6 represent leaves, 7 to 12 represent controlled callus, 13 to 18 represent salt stressed callus of six rice cultivars

Table 3: Estimates of 14 salt tolerance associated microsatellite markers based Dice's similarity coefficients among 18 rice entries used in the present study

	CSR30 (L)	MTU-7029(L)	N.Usar Dhan-3 (L)	R. Bhagwati (L)	BPT-5204 (L)	P.Basmti-1 (L)	CSR-30 (CC)	MTU-7029 (CC)	N.Usar Dhan-3 (CC)	R.Bhagwati (CC)	BPT-5204 (CC)	P.Basmti-1(CC)	CSR-30 (CS)	MTU-7029 (CS)	N.Usar Dhan-3 (CS)	R.Bhagwati (CS)	BPT-5204 (CS)
MTU-7029 (L)	0.428																
N.Usar Dhan-3(L)	0.500	0.285															
R.Bhagwati (L)	0.125	0.125	0.333														
BPT-5204 (L)	0.222	0.100	0.111	0.111													
P.Basmti-1 (L)	0.250	0.111	0.285	0.285	0.375												
CSR-30 (CC)	1.000	0.428	0.500	0.125	0.222	0.250											
MTU-7029 (CC)	0.428	1.000	0.285	0.125	0.100	0.111	0.428										
N.Usar Dhan-3(CC)	0.500	0.285	1.000	0.333	0.100	0.285	0.500	0.2857									
R.Bhagwati (CC)	0.111	0.111	0.285	0.800	0.100	0.250	0.111	0.111	0.285								
BPT-5204(CC)	0.222	0.100	0.111	0.111	1.000	0.375	0.222	0.100	0.111	0.100							
P.Basmti-1 (CC)	0.250	0.111	0.285	0.285	0.375	1.000	0.250	0.222	0.285	0.250	0.375						
CSR-30 (CS)	1.000	0.428	0.500	0.125	0.222	0.250	1.000	0.428	0.500	0.111	0.222	0.250					
MTU-7029 (CS)	0.428	0.666	0.285	0.125	0.100	0.111	0.428	0.666	0.285	0.111	0.100	0.111	0.428				
N.Usar Dhan-3 (CS)	0.500	0.285	1.000	0.333	0.111	0.285	0.500	0.285	1.000	0.285	0.111	0.285	0.500	0.285			
R.Bhagwati (CS)	0.100	0.100	0.250	0.615	0.090	0.222	0.100	0.100	0.250	0.615	0.090	0.222	0.100	0.222	0.250		
BPT-5204 (CS)	0.181	0.083	0.200	0.200	0.750	0.300	0.181	0.083	0.200	0.181	0.750	0.300	0.181	0.083	0.200	0.166	
P.Basmati-1(CS)	0.250	0.111	0.285	0.285	0.375	1.000	0.2500	0.111	0.285	0.250	0.375	1.000	0.250	0.111	0.285	0.222	0.300

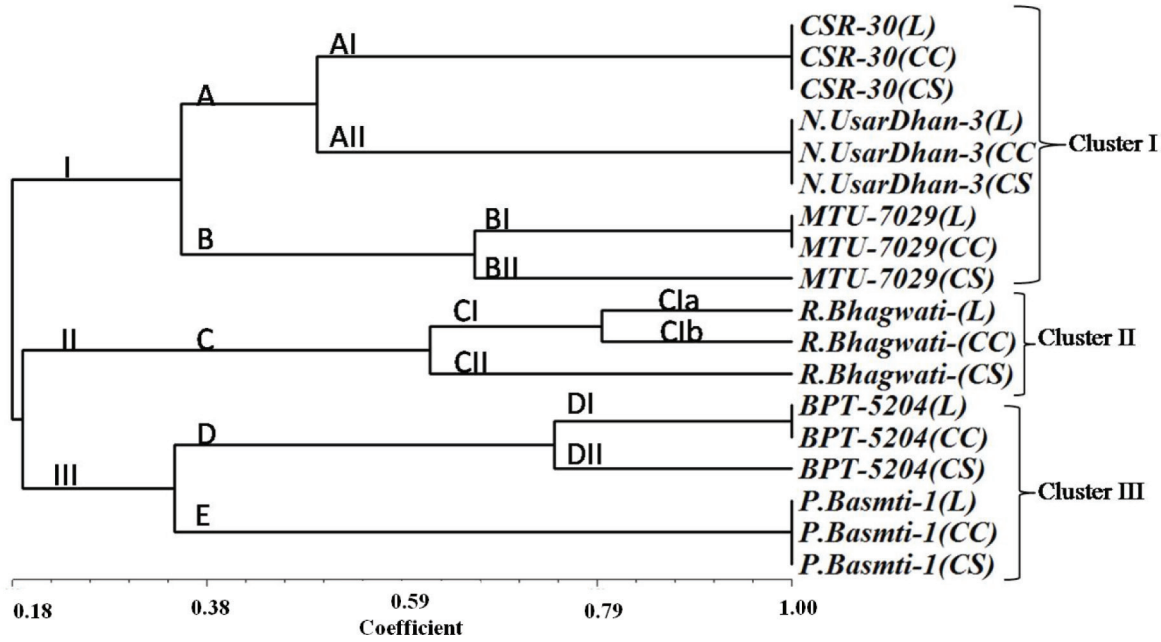

Fig. 3: Dendrogram based on Dice similarity coefficient among 6 rice cultivars evaluated using 14 salt tolerance linked SSR markers

Similarity coefficients between entries

The magnitude of similarity coefficients ranged from 0.083 to 1.00. The highest similarity coefficient (1.00) was observed between three entries each of CSR-30, Narendra Usar Dhan-3, Pusa Basmati-1 (L, CC and CS) and two entries of MTU-7029 (L and CC) and BPT-5204 (L and CC). This was followed by similarity coefficient of 0.80 between two entries of Rajendra Bhagwati (L and CC). The next higher similarity coefficient (0.75) was observed between the entries of BPT-5204 (L/CC, CS). The similarity coefficient of 0.66 was observed between entries of MTU-7029 (L/CC, CS). The entry of Rajendra Bhagwati (CS) showed a similarity coefficient of 0.61 in pair-wise combinations with two other entries of this cultivar (L and CC). Among the six rice cultivars evaluated, the maximum similarity coefficient (0.50) was obtained between CSR-30 and Narendra Usar Dhan-3, the two salt tolerant cultivars and this was followed by a similarity coefficient of 0.42 between CSR-30 and MTU-7029, the former being salt tolerant while the later as moderately salt tolerant cultivars (Table 3). The two moderately salt tolerant cultivars MTU-7029 and BPT-5204 had the minimum similarity coefficient (0.083) in their pair-wise combination.

Clustering of entries based on markers

Considering broad classification of entries, as indicated by dendrogram, basically the entries were divided into three groups. Cluster-I, the first multi-genotypic group consisted of nine entries including three entries of each of the two salt tolerant cultivars CSR-30 and Narendra Usar Dhan-3 and three entries of moderately tolerant cultivar MTU-7029 (L, CC and CS). Cluster-II, the second multi-genotypic group consisted of three entries of salt sensitive cultivar Rajendra Bhagwati. Cluster-III, the third multi-genotypic group consisted of three entries each of moderately tolerant cultivar BPT-5204 and salt sensitive cultivar Pusa Basmati-1(Fig. 3). Thus, the 14 salt tolerant associated microsatellite markers were able to reveal recognizable genotypic differences, which existed at the molecular level between salt tolerant and salt susceptible cultivars.

By drawing the phenon line at twenty-five similarity units in order to allow the entries with comparatively more similar pattern for markers to be clustered together, each of the first and third multi-genotypic clusters was further divided into two sub-clusters. Therefore, five relatively smaller groups (A, B, C, D and E) were obtained when phenon line was drawn at twenty-five similarity units. The sub-

cluster A consisted of six entries, which were further accommodated into two sub-sub clusters AI and AII at higher phenon level. The sub-sub cluster AI consisted of CSR-30 (L), CSR-30 (CC) and CSR-30 (CS) with genetic similarity coefficient equal to unity in pair-wise combinations, indicating no variation between the leaves, controlled callus and salt stressed callus of cultivar CSR-30. Similarly, the sub-sub cluster AII comprised Narendra Usar Dhan-3 (L), Narendra Usar Dhan-3 (CC) and Narendra Usar Dhan-3 (CS) with genetic similarity coefficient equal to unity in pair-wise combinations. The sub-cluster B was further divided into two sub-sub clusters BI accommodating MTU-7029 (L) and MTU-7029 (CC) with genetic similarity coefficient equal to unity, while BII consisting of only one entry MTU-7029 (CS). Therefore, dendrogram revealed genetic differentiation of salt stressed callus of MTU-7029 from the cultivar MTU-7029 and its controlled callus.

Sub-clustering of the entries belonging to cluster C yielded two sub-sub clusters CI and CII. The sub-cluster CI consisted of Rajendra Bhagwati (L) and Rajendra Bhagwati (CC) with genetic similarity coefficient of 0.80, indicating tissue culture induced variation in controlled callus, while the sub-sub cluster CII accommodated Rajendra Bhagwati (CS), thereby revealing the induction of further genetic variation in salt stressed callus of cultivar Rajendra Bhagwati (Table 3). The sub-cluster D was further dissociated into sub-sub cluster DI that included BPT-5204 (L) and BPT-5204 (CC) with genetic similarity coefficient equal to unity and sub-sub cluster DII accommodating BPT-5204 (CS). Therefore, clustering pattern of entries clearly reflected genetic differentiation of salt stressed callus of BPT-5204 from the cultivar BPT-5204 and its controlled callus. The sub cluster E comprised Pusa Basmati-1 (L), Pusa Basmati-1 (CC) and Pusa Basmati-1 (CS) with genetic similarity coefficient equal to unity in pair-wise combinations, clearly indicating the absence of genetic variation between leaf, controlled callus and salt stressed callus of Pusa Basmati-1.

Going by the results of the present study, it is evident that the use of a panel of 14 salt tolerance associated microsatellite primer pairs did not reveal the existence of any genetic variation between leaf, controlled callus and salt stressed callus of three cultivars, namely, CSR-30, Narendra Usar Dhan-3 and Pusa Basmati-1. Similarly, any genetic variation between leaf and controlled callus of the cultivars MTU-7029 and BPT-5204 was not revealed. However, salt stressed callus showed variation in the cases of both these cultivars. It was interesting to find out that genetic variation existed among leaf, controlled callus and salt stressed callus of the cultivar Rajendra Bhagwati, as revealed by the analysis of microsatellite markers based polymorphism surveyed in the present study.

Discussion

Salt tolerance associated 14 microsatellite primer pairs based molecular analysis of the normal leaf, controlled callus and salt stressed callus of the six rice cultivars with differential responses to salt stress revealed remarkably higher level of genetic polymorphism which allowed unique genotyping of the entries and somaclonal variants. Some of the primer pairs generated several allelic variants but some generated only few. The differences noticed in respect of the number of alleles per primer indicated the existence of appreciably greater extent of allelic diversity amongst the materials included in this investigation. The average number of alleles (4.54) observed in the present study corresponded well to some of the earlier reports showing it to be 4.2 (Faridul Islam *et al.* 2012) and 4.9 (Zeng *et al.* 2004). Contrarily the number of alleles detected in the present study was considerably higher than the average number of alleles (3.33) reported by Sajib et al. (2012). This could be due to inclusion of the genotypes of diverse origin in the materials under evaluation in the present study. However, the number of alleles noted in this study was relatively lower than 5.69 as reported earlier by some research workers (Shanthi *et al.* 2012). The allelic variants revealed by the markers used in the present study showed a considerably greater degree of polymorphism, suggesting that the genotypes selected for this study harboured enough genetic divergence. The inconsistency among reports might be due to the genotypes used and selection of primers with scorable alleles.

All the markers utilized in the present study generated unique and shared alleles. The number and proportion of unique alleles varied considerably with the primer pairs. In general, marker detecting

greater number of alleles per locus detected more number of unique alleles in accordance with the earlier reports (Bajracharya *et al.* 2006; Brondani *et al.* 2006; Joshi and Behera, 2006; Lapitan *et al.* 2007; Ebana et al. 2008; Herrera et al. 2008; Borba et al. 2009; Pervaiz et al. 2010; Rabbani *et al.* 2010; Vanaza *et al.* 2010; Singh *et al.* 2011). The presence of unique alleles indicated that the materials used in this study are useful and represent good source of genetic diversity for their purposeful and effective utilization in rice breeding for salt tolerance.

The markers differed in their ability to determine variability among different entries based on their polymorphism. The polymorphism information content (PIC) values, which reflect allele diversity and frequency of the markers among the cultivars, were not uniform for all the primer pairs tested. The value varied from one primer to another primer. Numerically, the value was found to vary from 0.376 in the case of primer RM4 to 0.827 in the case of primer RM242 with an average value of 0.677 across all the primers. The PIC values observed in the present study are more or less comparable to previous estimates reported on the basis of analysis of microsatellite markers in rice. The mean PIC value obtained in the present study was higher than 0.57, 0.57 and 0.48 as reported by Zeng et al (2004), Faridul Islam et al (2012) and Sajib et al (2012) respectively. Contrarily, the value obtained in the present study was lower than the value of 0.732 as obtained in an earlier study (Shanthi et al 2012). Findings of these high PIC value might be due to inclusion of more diverse set of rice germplasm as observed in present investigation. Higher PIC value of a marker indicates higher probability of detecting the number of allele among cultivars. Considering the number of alleles generated by different primer pairs in conjunction with level of polymorphism detected in the present study, the primers RM2 and RM204 appeared to be the most informative primers for the purpose of molecular characterization and grouping of rice cultivars on the basis of their salt tolerance.

The estimates of similarity coefficients clearly indicated a considerably greater extent of variation among the entries under evaluation in the present study and provided greater confidence for the classification of entries and assessment of genetic relationships. A perusal of the data on similarity coefficients further indicated the existence of complete similarity and absence of any somaclonal variants in the cases of three cultivars, namely, CSR-30, Narendra Usar Dhan-3 and Pusa Basmati-1. However, somaclonal variation was observed in normal callus of the cultivar Rajendra Bhagwati and salt stressed callus of the cultivars MTU-7029, Rajendra Bhagwati and BPT-5204. Analysis of allelic data generated on the basis of amplification profile obtained with microsatellite markers allowed differentiation of salt tolerant entries from the rest comprising moderately tolerant and susceptible entries. The markers utilized in the present study were sufficient for discrimination and unambiguous differentiation of salt tolerant cultivars CSR-30 and Narendra Usar Dhan-3 from the most salt sensitive cultivar Pusa Basmati-1 and moderately salt tolerant cultivars MTU-7029 and BPT-5204. Salt tolerance related genetic architecture of Rajendra Bhagwati, as revealed by microsatellite primer pairs used in the present study appeared to be intermediate between that of the salt tolerant and most salt sensitive cultivars. Faridul Islam et al (2012) differentiated stress tolerant rice cultivars on the basis of SSR marker. However, Sudharani et al (2013) assessed 8 rice genotypes with different levels of salt stress tolerance by 85 primers. They could get salt tolerant genotypes mixed with salt sensitive ones. In the present investigation, salt tolerant cultivars were differentiated from salt sensitive ones because only salt tolerance associated markers were used. The information about the genetic differentiation and divergence of these rice cultivars, as revealed on the basis of their molecular characterization using salt tolerance associated microsatellite markers, will be useful to breeder for proper identification and selection of appropriate parents and genotypes in future breeding program for developing new salt stress tolerant rice varieties.

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