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Identification of Microsatellite Markers Associated with Drought Tolerance in Rice (*Oryza sativa* L.) using Bulked Line Analysis

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ABSTRACT. In the present study, bulked line analysis (BLA) was used to identify microsatellite markers associated with drought tolerance in rice. Thirty-eight rice accessions from diverse genetic backgrounds were first evaluated for water stress under field conditions. A considerable variation was noticed among the rice accessions for various physio-morphological traits under stress. Based on field performance, 13 drought tolerant and 13 drought sensitive rice lines were selected. Drought tolerant bulk (DTB) and drought susceptible bulk (DSB) were constituted by pooling equal quantities of DNAs from the selected genotypes. The microsatellite analysis was performed on the two DNA pools using a total of 174 rice microsatellite primer pairs. Out of seven polymorphic primers identified between the bulks, two primers - RM223 and RM263 co-segregated in all the individual rice accessions constituting the bulks. These SSR markers are linked to drought resistance and may be useful in marker-assisted selection (MAS) for improvement of rainfed rice.

INTRODUCTION

Rainfed rice accounts for more than 45% of the world area planted in rice (IRRI, 1997). As the crop relies solely on rainfall for its water supply, drought stress is a serious constraint to rice production due to insufficient and uneven rainfall during the growing period. Therefore, improving drought tolerance is one of the important objectives of rice breeding for rainfed ecosystems. With limited success in conventional breeding due to low heritability of yield under stress, identification and selection of secondary traits contributing in drought tolerance have been suggested (O'Toole, 1989). However, genetic improvement of putative traits contributing in drought tolerance has been difficult due to labour-intensive techniques for screening large germplasm for these traits. The advent of DNA-based markers aids in breeding of complex traits such as drought tolerance through marker-assisted selection (MAS). Identification of DNA markers associated with drought tolerance is usually carried out with large mapping populations, where each progeny of the population is genotyped. This is highly demanding in resource, time and often cost ineffective. Several strategies have been reported to reduce the number of plants to be genotyped. Bulked segregant analysis (BSA) is one such strategy for identifying DNA markers linked to the trait of interest against a randomized genetic background of unlinked loci (Michelmore *et al.*, 1991). It involves bulking of DNAs from selected individuals based on their phenotype. Phenotype based DNA pools (*e.g.*, BSA) might be successful in identifying new DNA markers for the regions of genome known to contain QTLs (Wang and Paterson, 1994). BSA

is commonly used for identification of linkage between simply inherited traits and DNA markers. However, if cultivars or advanced lines sharing same phenotype are used for bulking, identification of DNA markers associated with a target trait will be more flexible for genetic stock preparation. This method termed as "bulked line analysis" (BLA) has been used for the identification of the DNA markers associated with fertility restorer (R_f) gene in rice (Tan *et al.*, 1998). Quarrie *et al.* (1999) reported that bulking in population of plants with diverse genetic background, such as variety mixes or composite populations of out-breeding species can be done as a variant of BSA and bulked maize composites were used to identify markers linked to yield components under drought. Bulking of different accessions has been done to identify markers linked to female sex in pointed gourd, *Trichosanthes dioica* Roxb. (Singh *et al.*, 2002). The maximum distance between the target gene and an informative marker that can be detected by BLA method is expected to be shorter than the limit of detection of approximately 25 cM by the BSA method (Michelmore *et al.*, 1991). Although this method cannot localize the gene directly, it is useful for identification of DNA markers that are associated with the target trait.

PCR-based DNA markers are the favorite selection in molecular breeding because of their simplicity and low cost. However, high level of allelic diversity, technical efficiency and multiplex potential of microsatellites make them preferable for many forms of high throughput mapping, genetic analysis and marker assisted plant improvement strategies (Coburn *et al.*, 2002; McCouch *et al.*, 1997). In addition, highly polymorphic nature of many microsatellites is of particular value when analyzing closely related genotypes, as is often the case in breeding program working within narrowly adapted gene pools. With approximately one microsatellite after every 157 kb in rice (McCough *et al.*, 2002), these markers provide important codominant landmarks. Thus, the objective of this study was to identify microsatellite marker(s) associated with drought tolerance in rice accessions from diverse genetic backgrounds using BLA.

MATERIALS AND METHODS

Evaluation of rice accessions under water stress

Thirty-eight rice accessions including landraces and improved cultivars from diverse genetic background and hydrological habitats were selected. The selected accessions were evaluated for drought response under upland condition in an experimental field of Tamil Nadu Agricultural University, Coimbatore, India during the dry season (March-July, 2004). The accessions were evaluated under water stress and control (non-stress) conditions with three and two replications, respectively, in a randomized block design. The plot size was 2.0 x 0.2 m^2 with 20 and 10 cm spacing between and within rows, respectively. Seeds were hand dibbled into dry soil. Fertilizer application, insect and weed control measures were applied periodically as required. All plots were surface irrigated to field capacity once a week, except when water stress was imposed by withholding irrigation to stress plots on 87^{th} day after sowing (DAS). During the initial two weeks after withholding irrigation, there were intermittent rains. Afterwards there was no rain continuously for three weeks, when data on water stress indicators were recorded as described below.

Leaf rolling and drying scores were taken at midday on 16 days after the last rainfall using 1 to 7 scale standardized for rice (IRRI, 1996). Leaf relative water content (RWC) was determined at midday on 16 days after rainfall (Barrs and Weatherley, 1962). Canopy temperature was measured 17 days after rainfall using a hand-held infrared thermometer (model AG - 42, Telatemp Corporation Inc, Fullerton, CA, USA) as described by Garrity and O'Toole (1995). Stress was relieved on 37th day after imposing stress by irrigating the plots to field capacity and drought recovery score was made 2 days after irrigation using 1 to 7 scale, where the higher number indicates greater recovery. Plant height was recorded at maturity. The plants were harvested 128 DAS and relative biomass (biomass under stress as a percentage of biomass under control) was calculated. Data were subjected to standard statistical analysis.

Bulked line analysis with microsatellite primers

Leaf samples were collected from 20 day old seedlings and extraction of the DNA was done as described by Gawel and Jarret (1991). DNA concentration was determined by spectrophotometric analysis at 260 and 280 nm. Equivalent amount of DNA from 13 drought tolerant and 13 drought susceptible lines were pooled into two separate bulks, drought tolerant bulk (DTB) and drought susceptible bulk (DSB), respectively. A total of 174 rice microsatellite (RM pairs) primers (Research Genetics Inc., USA) representing different chromosomes were selected randomly and used to amplify the SSR regions among the bulked DNA samples. PCR reactions were performed in a volume of 15 µl with final concentrations of dNTPs 100 µM each, forward and reverse primers 0.2 µM each, Taq polymerase 0.02 U and DNA 37.5 ng. The reaction in thermal cycler (PTC-100^{TM} MJ Research, Massachusetts, USA) was programmed as 95^{0}C for five minutes and then 35 cycles of 94^{0}C for 45 seconds, 57^{0}C or 55^{0}C (depending on the individual microsatellite) for 45 seconds and 72^{0}C for one minute. Final extension was at 72^{0}C for five minutes. Poly Acrylamide Gel Electrophoresis (PAGE) was done with an aliquot of 5 µl from amplified product mixed with 3 µl loading buffer (98% formamide, 10mM EDTA, bromophenol blue and xylene cyanol). The sample was denatured for five minutes at 95^{0}C, and separated on a 5% polyacrylamide (acrylamide:bisacrylamide in 19:1)/9M urea sequencing gel at 1800 V for 1.5 hours with 1X TBE (Tris-borate-EDTA) buffer in Hoefer^{TM} SQ3 sequencer (Amersham Pharmacia Biotech, USA). Amplification products were detected by silver staining according to the manufacturer's instructions. Band sizes were determined by comparison with a 100bp ladder size standard from Gene Craft, Germany. Putative polymorphisms between bulks were checked for co-segregation in individuals constituting the bulks.

RESULTS AND DISCUSSION

Significant variations in physio-morphological traits associated with drought tolerance were observed among the rice accessions studied (Table 1). Among the 38 rice accessions had higher per se performance for all the traits. The two *japonica* subspecies, Azucena and CT9993 also performed better. Among improved cultivars, TKM 1 performed better in all the traits. Leaf rolling, leaf drying and canopy temperature were negatively correlated with RWC, stress recovery, plant height and relative biomass under stress (data

not shown). Similar results were reported in rice by Blum *et al.*, (1999); Babu *et al.*, (2003); Lanceras *et al.*, (2004).

Thirteen drought tolerant and 13 drought susceptible rice accessions were selected for BLA based on field performance under water stress. The selection was made on the basis of mean values of different physio-morphological traits of individual rice accession compared to grand mean value of the respective trait. Accessions were given scores either positive (+) or negative (–) for each trait based on deviation of their mean value from grand mean value for that trait. Accessions, which scored comparatively higher in all the parameters, were characterized as drought tolerant and those with low scores were characterized drought susceptible (Table 1). Accessions with mixed scoring were given intermediate status and were not considered for BLA.

Out of 174 primer pairs used for screening the DTB and DSB, seven *viz.* RM5, RM107, RM223, RM242, RM257, RM263 and RM595 were found to exhibit polymorphism between bulks. Out of these seven primer pairs, RM263 and RM223 co-segregated completely in all the individual accessions constituting the bulks (Figures 1 and 2). With RM263, all the 13 individuals constituting DTB produced uniform bands of less molecular weight than individuals of DSB. However, among individuals of DSB, IR8 and IR20 produced bands of slightly higher molecular weight than others. This may be due to allelic difference in IR8 and IR20 from other individuals of DSB. With RM223, all the 13 individuals constituting DSB produced uniform band of higher molecular weight than individuals of DTB. However, among individuals of DTB, Azucena and CT9993 produced bands of slightly less molecular weight than others. This may be due to allelic difference in Azucena and CT9993 from other individuals of DTB, since these two are *japonica* ecotypes while rest are *indica* ecotypes of square of error.)

RM263 has been mapped on chromosome 2 of rice (Figure 3) and found to be linked with capacity for osmotic adjustment under water stress in CT9993/IR62266 doubled haploid (DH) line population of rice (Zhang *et al.*, 2001). Boopathi (2004) reported linkage of RM263 with leaf drying, stress recovery, canopy temperature and plant height under drought stress in IR20/Nootripathu recombinant inbred (RI) line population of rice. Markers flanking RM263 have been associated with several drought resistance traits. Yadav *et al.*, (1997) reported that the QTL region, RZ318 - RZ58 flanking RM263, linked to maximum root length, root thickness and deep root - shoot ratio in IR64/Azucena DH population of rice. Hemamilini *et al.*, (2000) also reported QTLs for total root number and root thickness in this region.

RM223 corresponds to chromosome 8 (Figure 3). Though RM223 has not been reported to be associated with drought tolerance traits, the flanking regions of RM223 have been reported to be associated with several drought tolerance traits in rice. Champoux *et al.*, (1995) reported QTLs associated with leaf rolling under water stress in this region in CO39/Moroberekan RI population. Ray *et al.*, (1996) also reported this region to be associated with number of tillers and total number of roots in this RI population.

Table 1. Variation in physio-morphological traits under water stress among rice accessions from land races and improved cultivars (varieties)

Accessions	LR	LD	RWC (%)	CT (°C)	SR	PH (cm)	RB	CH
Nootripathu#	3.6	1.6	55.0	37.7	5.0	79.1	0.93	DT
Varappu								DT
Kodanchan#	4.3	3.0	45.1	36.1	5.0	74.0	0.87	
Puzhidikar#	3.0	1.6	57.0	36.1	7.0	55.0	0.78	DT
Kallurundaikar#	5.0	3.0	72.8	36.3	5.6	77.6	0.89	DT
Norungan#	5.0	4.3	61.4	36.2	5.6	75.0	0.91	DT
Kuliadichan#	5.0	3.0	59.0	36.3	7.0	72.3	0.89	DT
Mattaikar#	4.3	4.3	61.5	35.3	6.3	82.3	0.92	DT
Sivappu								DT
Chittaraikar#	5.0	3.0	60.0	36.2	6.3	76.3	0.89	
Kuruvai								DT
Kalangiam#	5.0	4.3	65.2	36.3	5.6	78.0	0.88	
Vandana	5.6	4.3	62.0	35.7	3.6	57.0	0.86	DT
TKM 1£	5.6	4.3	55.1	35.9	5.6	67.7	0.94	DT
Azucena*	3.0	3.0	67.6	37.7	7.0	74.0	0.83	DT
CT 9993*	3.0	1.0	77.1	38.0	7.0	51.2	0.79	DT
ASD 15$	6.3	5.0	45.6	43.0	3.0	29.4	0.56	DS
ASD 19$	5.6	4.3	46.6	41.5	3.0	31.3	0.64	DS
ADT 44$	7.0	5.6	53.8	42.7	2.3	33.8	0.69	DS
ADT 46$	7.0	5.0	59.6	39.8	4.3	42.0	0.6	DS
CO 44$	7.0	5.0	36.9	38.8	3.0	37.6	0.48	DS
CO 45$	7.0	5.0	49.3	39.5	5.6	46.3	0.75	DS
CO 47$	7.0	7.0	56.3	40.2	1.6	28.6	0.76	DS
IR 36$	6.3	6.3	49.6	41.9	3.0	33.3	0.58	DS
IR 50$	6.3	5.0	52.6	39.0	4.3	45.3	0.71	DS
IR 64$	6.3	5.0	47.6	41.5	3.0	32.9	0.67	DS
IR 20$	7.0	6.3	46.5	42.5	1.6	21.3	0.63	DS
IR 72$	7.0	5.0	47.4	40.1	4.3	36.9	0.68	DS
IR 8$	7.0	6.3	36.5	40.4	5.6	24.0	0.71	DS
ASD 16$	5.0	4.3	67.4	39.4	5.0	50.2	0.7	INT
ASD 17$	4.3	3.0	56.5	39.0	3.0	55.3	0.72	INT
CO 31$	7.0	4.3	57.7	37.0	5.0	49.3	0.68	INT
CO 43$	6.3	3.0	42.5	38.9	6.3	41.1	0.7	INT
MDU 5£	7.0	6.3	36.9	34.8	1.6	42.6	0.86	INT
PMK 2£	7.0	5.0	53.4	38.6	6.3	61.9	0.83	INT
TKM 11£	6.3	5.3	45.0	35.2	3.6	48.6	0.77	INT
TRY 2£	6.3	6.3	66.8	37.3	6.3	60.5	0.81	INT
TPS 1£	7.0	7.0	48.1	38.3	1.6	45.6	0.77	INT
RM 96019	5.6	6.3	67.5	40.6	1.6	41.6	0.59	INT
Sonamani	7.0	5.0	44.3	40.2	3.0	50.0	0.85	INT
Pokkali#	7.0	5.3	43.1	38.7	1.0	37.8	0.78	INT
Mean	5.8	4.5	54.1	38.5	4.4	51.2	0.76	
Standard Deviation	1.28	1.48	10.0	2.29	1.79	17.53	0.12	
CV (%)	12.1	16.6	4.6	1.4	15.2	3.7	2.9	
F value	12.6**	11.6**	49.0**	55.8**	22.7**	257.8**	85.5**	

Landraces adapted to upland, $: Improved cultivars for lowland. *: Japonicas adapted to upland, £ : Improved cultivars for upland, ** Significant at 1% level

LR: Leaf rolling, LD: Leaf drying, RWC: Relative water content, CT: Canopy temperature, SR: Stress recovery, PH: Plant height, RB: Relative biomass, CH: Characterization for drought resistance, DT: Drought tolerant, DS: Drought susceptible, INT: Intermediate, SD: Standard deviation, CV: Coefficient of variation, 'F' Value: Ratio of mean sum of square of variation between rice accessions and mean sum

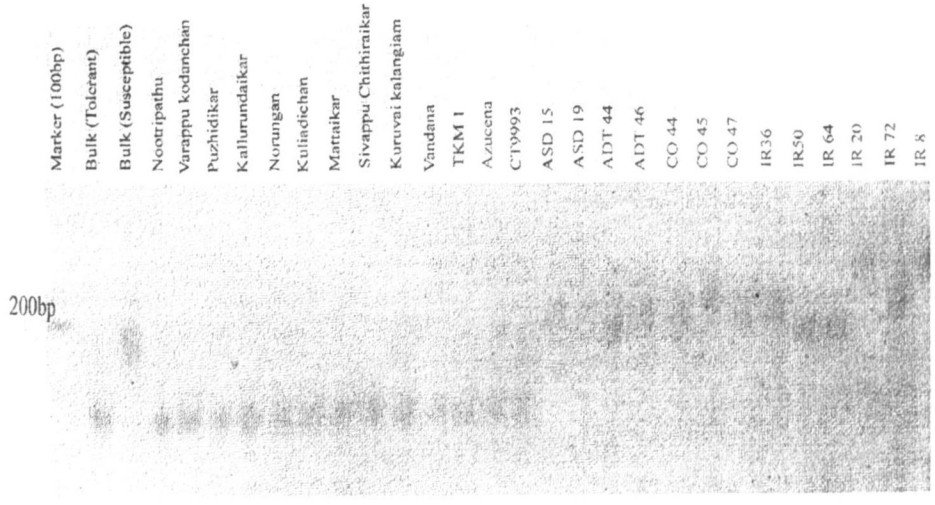

Fig. 1. Simple Sequence Repeats profile for pimer RM263

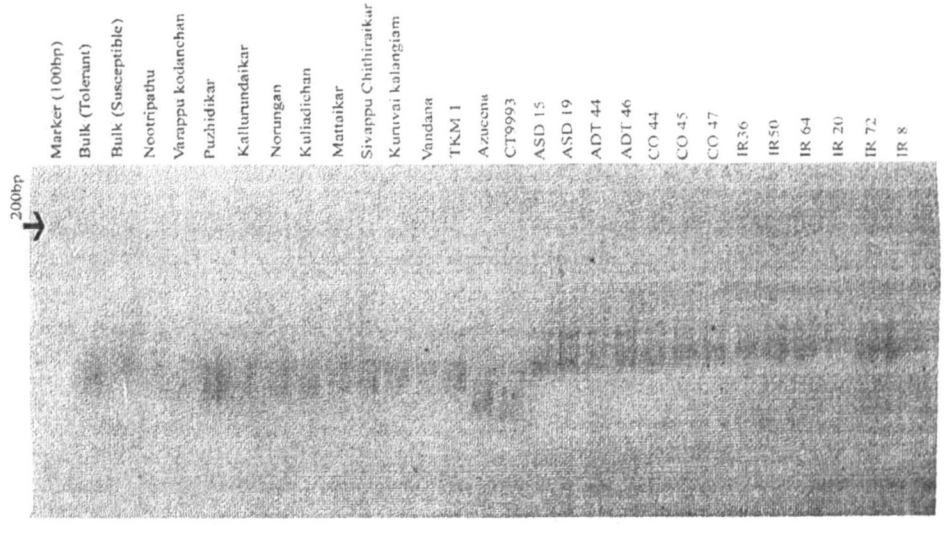

Fig. 2. Simple Sequence Repeats profile for primer RM223

Lilley *et al.*, (1996) reported the region between RG978 and RG1, which flanked RM 223, to be associated with osmotic adjustment. Yadav *et al.*, (1997) reported the region to be linked with deep root - shoot ratio in IR64/Azucena DH population of rice. Co-segregation of RM263 and RM223 among individuals of DTB and DSB indicated that these

markers are linked to genomic regions contributing in drought tolerance in rice accessions from diverse genetic backgrounds and may be useful in MAS for rainfed rice improvement.

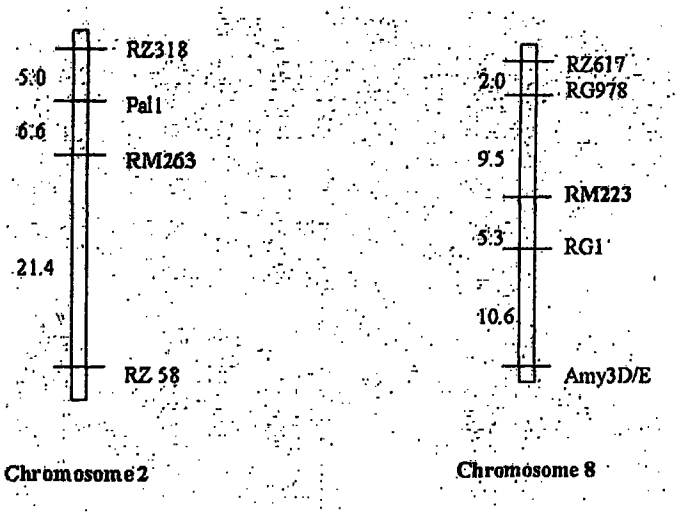

Fig. 3. Chromosomal locations of the RM263 and RM223 primers associated with drought resistance/susceptibility among the rice accessions in this study. This linkage map was adapted from Temnykh *et al.*, (2001). Numerals in the left indicate the centiMorgans distance between the markers

CONCLUSION

A considerable variation was noticed among the rice accessions for various physio-morphological traits under stress. Bulked line analysis identified two SSR markers *viz.*, RM223 and RM263 linked to drought resistance and may be useful in marker aided selection for rainfed rice improvement.

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