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IDENTIFICATION OF MOLECULAR MARKER(S) FOR ROOT LENGTH IN RICE (*ORYZA SATIVA* L.)

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ABSTRACT
An investigation was carried out to find the molecular markers associated with root length of rice using 271 Recombinant Inbred Lines (RILs) developed from Danteshwari X Dagad Deshi. Based on root pulling resistance and core sampling root scan data for root length, extreme DNA bulks of root length (11 lines in each bulk) were prepared. These bulks were subjected to Bulk segregant analysis with 186 SSR markers, followed by Co-segregation analysis with 14 SSR markers. From this analysis, it can be concluded that the markers HvSSR 01-80, HvSSR 01-87, HvSSR 05-31, RM242, RM135, RM499, RM232, RM296 and RM26334 are associated with the trait. Among them RM242 located on chromosome 9 can be considered as good marker for root length in marker assisted selection.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important world food crops, serving as the staple food for over one-third of the world's population (Deepti Davla *et al.*, 2013) and it is referred as "Global Grain" (Shalini and Tulasi, 2008) because of its use as prime staple food in about 100 countries of the world. Although, rice is cultivating in large area but the final yield gain per unit area is very less due to biotic and abiotic stresses (Bala Krishna and Satyanarayana, 2013). About half the total world rice area is rainfed, where drought is major production constraint (Anon. 2004). Maximum root length and root dry weight were good indicators of drought avoidance in upland rice and plants having deeper root system should colonize a large soil volume and improve the water uptake from the lower layers (Kanbar *et al.,* 2009). Root length is one of the characteristic feature responsible for adaptability to drought stress in rice (Abd Allah, 2009). For development of lines suitable for moisture stress condition root volume and root length was also reported as better combinations for selection. (Lakshmi Hijam *et al.,* 2012). So, we aimed in this investigation to find out the molecular markers associated with root length of rice, which can offer an excellent opportunity to develop varieties for drought tolerance by improving root length trait using marker assisted selection.

MATERIALS AND METHODS

Plant material

A Recombinant inbred line population in F_{12} generation having 271 lines was developed from Danteshwari and Dagad Deshi, a drought tolerant land race (Verulkar *et al.,* 2004) as parents by using modified single seed descent method.

Methods

These lines were grown in rain fed direct sown condition under Randomized Block Design (RBD) with two replications during *wet* season 2011 at research cum instructional farm of Indira Gandhi Krishi Vishwavidyalaya, Raipur. The field selected for the study was upland in topology with sandy loam soil. Each genotype was sown in three rows of 2m length with spacing of 15cm between the rows. All normal packages of practices were followed to raise a good crop.

Root Pulling Resistance

Root pulling resistance (RPR) was measured from irrigated control plots at flowering stage as described by O'Toole and Soemartono (1981). Based on root pulling resistance, two groups, twenty lines with high RPR and twenty lines with low RPR were identified. From those of each twenty, first eleven lines were selected in each group for further core sampling from the rainfed direct sown condition (16, 78, 80, 89,149,156,191, 220, 229, 259, 269 lines are from low RPR group and 10, 26, 70, 72, 74,105,106,139,140,174, 245 lines are from high RPR group).

Core sampling and Root scanning

Root studies were followed according to the root scanning protocol given by International Rice Research Institute (IRRI), Philippines. In each line (having 3 rows), between two rows samples were selected for root scanning. It is having three steps (a) core sampling (b) root washing and (c) root scanning.

Core sampling

From selected line two sub replications were sampled up to 45 cm. Cores were inserted in soil by hammer and then removed from the soil by pulling with a chain block suspended above the core. Soil core was divided into 15cm segments.

Root washing

Three segments of core sample were added with water, stirred well to loosen the soil and roots were collected by pouring the mixed solution through fine sieve. Collected roots were taken into small tubes and filled with 25%ethanol.

Root scanning

It was done with help Win Rhizo root scanner. Collected roots from samples were spread over Plexiglas trays (care was taken during spreading to avoid overlapping).

Genomic DNA isolation

A mini prep method (Doyle and Doyle, 1987) was used to extract genomic DNA from selected lines along with parents. Approximately 2g of young leaf material cut into the small pieces was transferred to 2ml centrifuge containing 500 μL of DNA extraction buffer along with small stainless steel beads. These tubes were fixed in tissue homolyzer (MO. BIO. *powerlyzer* 24) and it was operated in two cycles at 2400 rpm about 2 minutes with 5 seconds pause between two cycles. After removing stainless steel beads from tubes, 400 μL of 24:1 chloroform: Iso amyl Alcohol was mixed. Centrifugation of these tubes at 14000rpm for about four minutes gave super aqueous which was taken into new centrifuge tube. To the double of the super aqueous taken 100% chilled ethanol was added and it was kept at -20ºC for about 30 minutes to precipitate the DNA. After that it was centrifuged at 14000rpm for about four minutes to settle the DNA as a pellet and later it was washed with 70% ethanol. At the end it was air dried and 100μl TE buffer was added to dissolve the DNA pellet. Each DNA sample was quantified and diluted to 20sg/µL to proceed for PCR. Diluted DNA (20sg/µL) from each eleven lines which were showing more and less root lengths in root scan data were mixed to form More Root Length Bulk (MRLB) and Less Root Length Bulk (LRLB) respectively as suggested by Michelmore *et al*. (1991).

PCR and electrophoresis

For amplification, SSR and HvSSR markers were used. For DNA amplification, reaction mixture consisted of following in 20µL volume (Table 1) and temperature profile used for PCR amplification (Table 2). To each completed reaction 2µL of loading dye was added and they were electrophoresed in 5% PAGE. After electrophoresis gels were stained with Ethidium Bromide (EtBr) for 4 minutes, washed with distilled water and photographed using gel doc unit (*BIO RAD*).

Selective genotyping

A total of 186 markers (76 HvSSR and 110 SSR) were used for genotyping. Primarily both the bulks along with parents were subjected to amplification using 186 markers. Among those primers, which were showing polymorphic along with parents were selected for Co-segregation analysis. Single marker analysis was used to validate these markers.

Statistical analysis

Single marker analysis by Chi square analysis with Yates correction was used for the identification markers linked with these root traits.

RESULTS AND DISCUSSION

Root pulling force is dependent on root length density of the portion of the root system that remains in the soil (Ekanayake *et al*., 1986). It was reported that a high pulling force is associated with the plant's ability to develop deeper and larger diameter roots with great penetration ability (Gowda *et al.,* 2011). In present investigation, according to root scan data obtained from samples collected from field condition showing more root pulling resistance had more root length and root volume (Table 4). Two bulks prepared each with eleven lines, selected based on the root scan data. Root scan data of core sampling revealed that differences in length and volume of roots for the lines selected based on the root pulling resistance are significant.

Bulked Sergeant Analysis (BSA)

Primarily, extreme bulks along with parents were subjected to PCR and electrophoresis with 186 markers. Of the 186 markers, fourteen markers produced alleles that exhibited parental polymorphism along with similar polymorphic pattern for bulks (Fig. 1). They were HvSSR 01-80, HvSSR 01-87, HvSSR 01-89,HvSSR 03-40, HvSSR04-35, HvSSR 05-31, RM 242, RM 135, RM 499, RM 278, RM 232 and RM 296. Only fourteen (7.5%) markers out of 186 markers used, exhibited polymorphism. The low level of polymorphism may be probably the indica x indica cross used in this study. Low level of polymorphism between Vandana and Way Rarem was also reported by Bernier *et al*. (2007). The relatively low recovery of parental polymorphism under this study was attributable to the narrow genetic variation between the parents as both of these were *indica* type and adopted to grow in the same rice ecosystem. The advantage of using BSA is that the approach relies on the dramatic reduction in the number of marker assays when compared to building a genetic map for the purpose of identifying markers associated with a phenotype (Wenzl *et al.,* 2007).

Table 1: PCR mix for one reaction (Volume 20μL)

Reagent	Stock concentration	Volume (μL)
1) Nanopure H_2O	-	13.3
2) PCR buffer	10 X	2.0
3) $MgCl_2$	15 mM	0.5
3) dNTPs (Mix)	10 Mm	1.0
4) Primer (forward)	5 pmol.	0.5
5) Primer (reverse)	5 pmol.	0.5
6) *Taq* polymerase	5 unit/mL	0.2
7) DNA template	2.0 ng/µL	2.0
Total	20	

Table 2: Temperature profile used for PCR amplification using microsatellite markers

Steps	Temperature (ºC)	Duration (min.)	Cycles	Activity
1	95	5	1	Denaturation
2	94	1	↑	Denaturation
3	55	1	34	Annealing
4	72	2	↓	Extension
5	72	10	1	Final Extension
6	4	24 hrs	1	Storage

Table 3: Chi square (χ^2) analysis with Yates correction

Markers	Observed values for LRL lines		Observed values for MRL lines		Chi square value withYates correction
	A-type (a)	B-type (b)	A-type (c)	B-type (d)	
HvSSR 01-80	11	0	2	9	12.03*
HvSSR 01-87	11	0	6	5	4.14*
HvSSR 01-89	8	0	6	5	3.00
HvSSR 03-40	7	2	3	7	2.78
HvSSR 04-35	8	1	2	4	2.94
HvSSR 05-31	8	0	2	5	6.60*
RM 17	7	1	5	6	2.01
RM 242	8	0	2	8	9.65*
RM 135	11	0	3	7	8.88*
RM 499	9	1	2	5	4.87*
RM 232	7	3	2	9	3.91*
RM 296	10	1	3	7	6.03*
RM 278	7	3	3	8	2.35
RM 26334	7	0	0	7	13.23*

Here (a), (b), (c), (d) are taken as variables in 2 x 2 contingency tables to substitute in the formula. χ^2 value at 0.05 level of probability

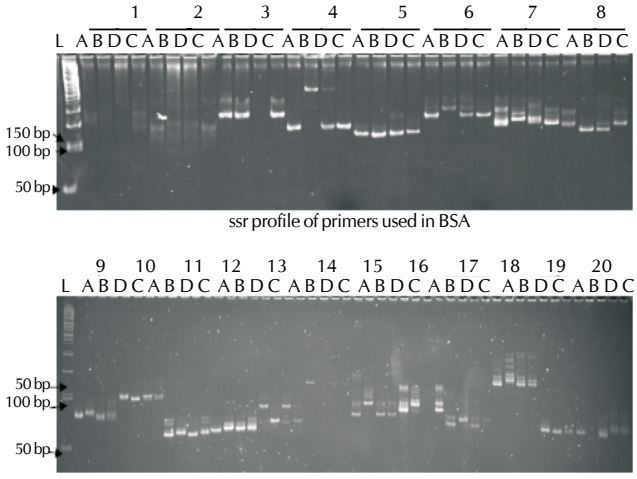
Table 4: Root scans data of MRLB lines and LRLB lines

S. no.	MRLB lines	Root length (cm)	Root volume (cm^3)	S. no.	LRLB lines	Root length (cm)	Root volume (cm^3)
1	70	114.26	0.0215	1	16	13.24	0.0025
2	26	148.84	0.042	2	220	36.13	0.0115
3	106	161.35	0.0465	3	229	45.18	0.007
4	245	165.03	0.0475	4	80	49.56	0.012
5	139	182.31	0.0475	5	259	51.82	0.0105
6	74	186.24	0.048	6	269	78.59	0.018
7	105	205.30	0.057	7	78	107.81	0.0215
8	140	206.44	0.068	8	149	115.10	0.0285
9	174	235.11	0.07	9	89	123.06	0.0235
10	10	253.32	0.07	10	156	152.27	0.04
11	72	298.67	0.0745	11	191	156.06	0.02

Co-segregation analysis

The population used in the study includes the fixed homozygous lines F_{12} generation Therefore, theoretical expected ratio between A and B banding pattern of lines should be 1:1. Any significant deviation from this ratio indicates that marker is closely located/associated to the gene of interest. In this analysis each line of bulk were subjected to PCR with fourteen markers along with parents. The amplification pattern is presented in Fig. 2. Based on the banding pattern obtained, scoring was done and data was analysed using Chi-square analysis with Yates correction to identify the markers significantly associated with the traits.The result of Chi-square(?^{2}) test is presented (table 3).From the analysis, it found that out of fourteen markers used in the study, nine markers HvSSR 01-80, HvSSR 01-87, HvSSR 05-31, RM 242, RM 135, RM 499, RM 232, RM 296 and RM 26334 were deviated from the normal ratio and found significant. It is indicating association of these markers with root length and root volume trait.Among nine markers identified to be putatively linked to gene of interest, HvSSR 01-80 on chromosome 1, RM242 on chromosome 9 and RM26334 on chromosome 11 exhibited higher Chi square values. To date, 675 QTLs related to root traits have been detected (summarized by Courtois *et al*., 2009). Among them, 103 QTLs for maximum root length have been identified on the 12 chromosomes (reported by Uga *et al*., 2011). From the Gramene QTL Database (2010), a numbers of associations with these fourteen markers were found out. Among them, RM242 and RM296 on chromosome 9 at locus 73.3cM and 20.4cM was found to be associated with more number of root related traits. Steele *et al*. (2006) reported that an NIL with a chromosome segment containing a QTL for root length (between RM242 and RM201) on chromosome 9 significantly increased root length and plant height under irrigated and water stress conditions. Ramya *et al*. (2010) also reported a region between RM160 - RM215 on chromosome 9 contribute to maximum root depth under both control and drought stress condition. In this present study two markers RM242 and RM296 identified to be linked with root related traits on chromosome 9, lie between marker intervals RM160 – RM215. Uga *et al*. (2011) reported a new major QTL *Dro*1, for RDR (Ratio of Deep Rooting) which may be under the 30 QTLs for 12 root traits summarized by Courtois *et al*. (2009) on chromosome 9 between the interval 15Mb and 20Mb.

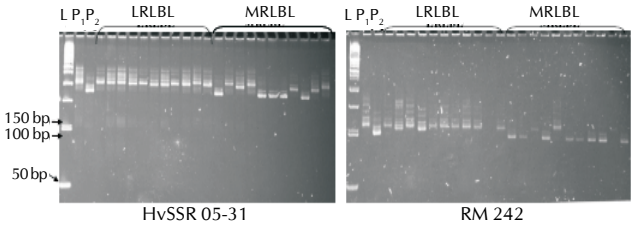
Deeper rooting is a key strategy associated with avoiding drought stress (Uga *et al.,* 2011). Yoshida and Hasegawa (1982) concluded that the root length density in deeper soil was one of the factors that determined drought resistance in rice. Molecular identification of genes controlling root traits will contribute to discovery of new functional alleles and to marker-assisted selection by the introgression of target genes (Collins *et al*. 2008). Kanbar *et al*. (2009) reported that among the root traits studied, total root length is strongly related to drought tolerance under rainfed upland conditions. So, the present investigation resulted with the marker associated with the root length can be exploited in pyramid breeding by combining with other useful genes to improve rice grain yield (Ashikari *et al*., 2005).



ssr profile of primers used in BSA

L - Ladder, A - Danteshwari, B - Dagad Deshi, C - Less root bulk length, D - More root bulk length,
1 - HvSSR 01-49, 2 - HvSSR 01-55, 3 - HvSSR 01-80, 4 - RM 259, 5 - RM 273, 6 - RM 296, 7 - RM307, 8 - HvSSR01-10, 9 - HvSSR 01-24, 10 - HvSSR 01-30, 11 - HvSSR 01-33, 12 - HvSSR 01-34, 13 - RM 341, 14 - RM 323, 15 - RM 340, 16 - RM 231, 17 - RM 232, 18 - RM 517, 19 - RM 239, 20 - RM 234

Figure 1: Gel images showing bulk segregant analysis



L - Ladder, P_1 - Danteshwari, P_2 - Dagad Deshi, LRLBL - LRL bulk lines, MRLBL - MRL bulk lines

LRL bulk lines (from left to right) - 16, 78, 80, 89, 149, 156, 191, 220, 229, 269
MRL bulk lines (from left to right) - 10, 26, 70, 72, 74, 105, 106, 139, 140, 174, 245

Figure 2: Gel images Co- segregation analysis with primers Hv SSR 05-31 and RM 242

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