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ASSESMENT OF ROOT PULLING STRENGTH AND MOLECULAR MAPPING FOR ROOT TRAITS IN RICE(*ORYZA SATIVA* L.)

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ABSTRACT

Rice (*Oryza sativa* L.) is a highly drought sensitive crop, and most semi-dwarf high yielding varieties suffer severe yield losses from reproductive stage drought stress. In such case rice roots play an important role to sustain the plant in water stress condition. In this study the 83 elite rice population was used for assessment of root pulling strength (RPS) which showed the positive correlation with root length.RPS varied from 21.5 (minimum) for rice genotype SLO-16 to 40 for cross 116 and bhataphool (maximum). Greater the RPS reflects the dense root system or greater the root length/ volume.In the present investigation, an attempt has been made to assess the genetic diversity among 83 rice genotypesusing morphological traits and the population was genotyped using molecular markers across the whole genome. For root length, the marker RM242 on chromosome #9 was statistically significant with p-value 0.0081 and percent of total phenotypic variation (R^2) for a trait was 8.52% and also the *in-silico*analysis revealed that the marker is present on putative QTL CQH31 and CQH33 responsible for enhancement of root traits. Thus we identified that RM242 (CH#9) was the peak marker for the screening of root traits in rice.

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INTRODUCTION

Rice (*Oryza sativa* L.), an important agricultural crop and a model species, has been cultivated for more than 7,000 years (Yun Fei *et al.*, 2007; Zong *et al.*, 2007). Asian rice, *Oryza sativa* is a cultivated, inbreeding species and as a staple cereal crop, rice feeds more than 50% of the world's 61 population (Mather *et al.* 2007)"Global grain'' (Shalini and Tulsi, 2008 and Reddy *et al.*, 2013). But its productivity is often reduced by drought, especially when grown under rainfedconditions (Muthukumar *et al,* 2015). Identification of molecular markers associated with plant production traits under drought, especially in the target populations of the environment (TPE) presents an opportunity to improve rainfed rice production using genomics tools (Muthukumar et al, 2015).Identification of rice varieties having good root architecturesystem is necessary to obtain the drought resistance rice cultivars. Early recognition of the importance of roots for drought resistance, and the diversity in rice root architecture, provided a strong foundation for drought research. To make advances in rice breeding itis important to understand the relatedness andancestry of introduced rice accessions, and identifymolecular markers associated with root traits.

For unraveling complex genetic traits and for marker-assisted selection in plant breeding, using elite germplasm for quantitative trait loci (QTL) mapping provides advantages over traditional linkage analysis. Molecular markers have been used extensively in genetic analysis of root trait. Roots play a key role in absorbing water and nutrients essential for the plant (Devendra et al, 2015)Deep rooted plants have also shown to be better productive under water limited conditions (Reynolds and Tuberosa, 2009). For instance a deep, thick and branched root system is correlated with better survival under adverse condition. Genetic control of root development has been studied mainly through molecular mapping.

Besides the molecular analysis for identification of genes or QTLs govern by root trait the other technique was also used in this study to identify the cultivar having deep, branched root system. A technique based on phenotype described as Root Pulling Resistance (RPR) was used to evaluate genotypic differences in root growth and development of 83 elite rice cultivars. This technique has been widely used to assess the nature of root development in cereal crops (Nass and Zuber, 1971; Arihara and Crosbie, 1982). The method appears sensible for root depth phenotyping in rice, especially when other direct field methods for root selection are not very forthcoming. Several root characteristics in rice are associated with drought tolerance and other biotic-abiotic stresses and avoidance capability of plants. The RPR measurements showed a significant positive correlation with maximum root length, root thickness, branching number, and root dry weight, number of tips and forks. Rice genotypes that had a high RPR value were identified as having longer, thicker, and denser root systems. Furthermore, the data demonstrated that the RPR technique is ideal for selecting superior root systems and potential drought tolerant rice cultivars. Now days it is necessary to identify the rice cultivars having better root system based on precise phenotyping and molecular mapping techniques used. Hence the present study aims to screenthe rice genotypes

that have the greater RPS resistance and molecular markers linked to root traits.

MATERIALS AND METHODS

Phenotypic evaluation

Assessment of root pulling strength, root length of elite rice lines

For field studies, the experimental breeding population of 83 elite rice genotypes was sown in field and observations were record at maximum tillering stage for root pulling strength (RPS) by selecting two plants from each line using root puller machineas describe by O'Toole and Soemartono (1981). Also the observation for agronomic trait Root Length (RL) was recorded using standard scaling method for the same lines.

Genotyping of elite rice lines with selected SSR markers

Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from the leaf tissue for each rice cultivar/genotype following the miniPrep method described by Doyle and Doyle, 1987.The DNA samples were quantified using Nanodrop Spectrophotometer (ND 100) and the absorbance ratio (A260/A280) was recorded for each sample to find out the purity of DNA. A total of five polymorphic microsatellite markers, approximately evenly distributed on the 12 chromosomes of rice, were used for genotyping. PCR amplification was performed in a 20 µL reaction mixture, consisting of 30ng/µL template DNA, 10x PCR buffer, 1 mMdNTPs, 10 µM forward and reverse primers, and 1 unit of *Taq*DNA polymerase. The temperature profiles used as Initial denaturation 95°C, denaturation 95°C, annealing 55°C extention 72ºC and store at 4ºC. The primer sequences used for study given in Table 3.

Resolving PCR product on PAGE

The PCR products were detected using the optimized silver staining method in denaturing polyacrylamide gel (Bangalore genei Sequencing gel). The individual bands were scored for further analysis. Primer sequences and PCR amplification conditions for each set of primers and the physical positions of marker loci were based on published Nipponbare sequence databases (http://www.gramene.org)

Data analysis

All the genotypes were scored for the different allelic forms for each marker. The phenotypic and genotypic data were used for marker trait association analysis. Also the data was prepared as per the requirement of molecular mapping and graphical genotyping tool (GGT V2.0). The resultant data file was employed to generate graphical images indicating allelic frequency and different allelic forms of each marker on respective chromosome.

Molecular mapping

Association between agronomic trait and markers were calculated using ANOVA: single marker analysis (SMA). The significant marker trait associations were indicated by a P-value (<0.05) with corresponding R^2 for each marker as the percent of the total variation explained by the markers.

RESULTS AND DISCUSSION

Assessment of root pulling strength of elite rice lines

Root length and diameter distribution are important characteristics to be considered when describing and comparing root systems.Ekanayake*et al* (1985) found a significant positive correlation across diverse rice genetic materials between root pulling force and dehydration avoidance as expressed in leaf water status maintenance and visual scored of drought resistance under severe drought stress in the field. Long fibrous roots have long been recognized as an important dehydration avoidance mechanism in rice and such roots evidently also ascribe stronger anchorage and greater resistance to pulling force. In the present investigation we evaluated 83 rice line / genotypes for root pulling strength and root length (Table 1). The observation were recorded for root length, the mean root length varied from 9.67 cm for a rice genotype IR83381-B-B-137-3 (minimum RL) to 23.83 for IR84859-B-41-1-2 (maximum RL). Also the observations of root pulling strength were recorded with the puller machine by pulling the complete one hill of rice plant at maximum tillering stage and it was observed that Root Pulling Strength (RPS) varied from 21.5 (minimum) for rice genotype SLO-16 to 40 for cross 116 and bhataphool(maximum). The frequency distribution for 83 rice genotypes in different range of root pulling strength indicated the phenotypic variation in elite rice line population(Table 2). Observation on only root length did not correlated with the root pulling strength indicating many other root parameters affect stronger anchorage and greater resistance to pulling force. The maximum lines/genotypes *i.e* 15 rice lines out of total population showed the root pulling strength (RPS) 32 to 32.5 and two rice genotypes showed root pulling strength 40 which was the highest RPS observed in whole population used in this study.

The rice genotype/line IR 84859-B-41-1-2 measured the root length 23.83cm which was highest among the all population and this genotype having the root pulling strength (RPS) 31. This shows the co-relation between root length and root pulling strength. But to find out marker trait co-relation is important than correlating root length and root pulling strength. So the marker trait association was done to analyze the marker-trait association for these traits.

Genotyping of the rice genotypes with molecular markers associated with drought tolerance/root traits

The association between trait and markers were calculated using single marker analysis (SMA). DNA markers that show specific molecular weight bands in the selected genotypes across the total population were genetically linked to the loci determining the quantitative trait. The primers RM201, RM202, RM242, HvSSR01-80, HvSSR01-87 were tested for co-segregation analysis to conform the linkage of the marker on putative QTLs associated with the particular traits. Association analysis was done on 83 elite rice lines for three different agronomic traits. We detected a total of 09 significant marker-trait association ($p<0.05$) (Table 4). All of the 09 significant SSR loci were identified for the agronomic traits, with the R^2, percentage of the total variation explained ranging from 5.72 to 27.23%.

Association between RM 242 on chromosome #09 and root

Table 1: Total phenotypic data for Root Length (RL) and Root Pulling Strength (RPS) of 83 rice genotypes/ lines used in study

S.No.	Line	Root length (RL)	Root Pulling Strength (RPS)	Shoot length (RL)
1	R-RF-69	14.33±4.04	27.00±1.41	26.33±5.53
2	R-RF-84	15.00±4.27	31.00±1.41	30.17±0.76
3	R-RF-75	14.53±1.05	32.00±0.00	30.57±4.76
4	R-RF-95	16.77±1.94	31.00±1.41	36.43±7.10
5	R-RF-78	14.67±2.08	27.50±0.71	26.73±3.27
6	R-RF-81	23.10±5.91	30.00±2.83	43.73±5.43
7	R-RF-65	16.83±2.02	32.50±3.54	37.33±1.89
8	R-RF-82	10.00±2.00	34.00±2.83	38.50±1.80
9	IR 83376-B-B-150-3	13.37±2.87	26.50±0.71	28.70±0.96
10	IR 84882-B-120	13.20±1.59	32.00±0.00	29.83±1.04
11	IR 84887-B-15	18.73±1.67	23.50±2.12	34.70±2.71
12	IR 33929-B-B-132-2	15.00±0.00	37.00±1.41	39.53±2.16
13	IR 86857-46-1-1-2	11.53±1.36	27.00±1.41	31.80±4.20
14	IR 86781-3-3-1-1	16.50±1.32	38.00±0.00	34.50±4.77
15	IR 83372-B-B-133-2	11.70±2.21	29.50±0.71	21.60±3.11
16	R-RF-45	16.17±0.29	33.00±1.41	30.50±6.95
17	IR 83381-B-B-55-4	14.17±2.29	31.50±3.54	25.03±1.94
18	IR 83381-B-B-6-2	13.23±2.42	32.00±5.66	32.67±1.44
19	IR 83383-B-B-140-2	13.60±0.44	29.50±2.12	26.03±1.18
20	IR 83383-B-B-129-3	21.00±8.23	32.00±0.00	26.90±3.48
21	CB-09-510	14.27±2.02	37.50±2.12	32.33±3.35
22	PM 6004	11.53±0.90	35.00±1.41	27.87±7.94
23	CR 2642-52	15.03±5.56	37.00±1.41	32.00±9.51
24	IR 83381-B-B-137-3	9.67±0.29	33.00±1.41	32.17±3.88
25	Sahbhagidhan (CH)	14.60±4.65	39.00±4.24	29.33±1.61
26	IR 88287-677-60-3	16.57±0.64	29.00±1.41	31.73±4.75
27	IR 84878-B-60-4-1	14.20±1.47	33.00±1.41	38.37±2.32
28	IR 84852-B-12-1-4	12.20±2.86	35.00±1.41	29.50±2.75
29	IR 88287-677-53-7	13.63±2.83	30.00±5.66	35.10±2.60
30	IR 83381-B-B-38-3	14.00±0.50	28.00±8.49	31.97±3.16
31	IR 83383-B-B-129-4	18.10±4.37	32.00±0.00	39.07±6.93
32	IR 84859-B-41-1-2	23.83±1.04	29.00±1.41	35.17±1.53
33	IR 83383-B-B-140-3	18.20±2.82	31.00±1.41	27.90±2.98
34	IR 83372-B-B-133-2	15.20±5.63	34.00±2.83	21.00±9.26
35	IR 84882-B-120-CRA-6	16.53±1.76	37.50±3.54	26.57±6.02
36	Annnada	18.30±2.15	27.00±1.41	41.00±0.78
37	MTU 1010	12.57±1.21	32.50±4.95	26.33±1.89
38	IR 64	13.27±2.74	29.00±4.24	38.63±9.84
39	Mahamaya	17.47±1.42	31.00±1.41	32.90±5.15
40	Poornima	15.80±1.47	32.50±0.71	33.97±0.64
41	Samleshwari	17.27±2.93	33.00±1.41	34.50±5.41
42	Vandana	23.20±1.99	29.00±1.41	36.30±0.98
43	Danteshwari	16.50±2.18	23.00±1.41	24.17±3.18
44	Swarna	12.53±2.00	37.00±4.24	23.80±3.36
45	Swarna sub 1	17.83±1.61	34.00±2.83	25.17±1.61
46	ARB8	15.83±2.02	31.00±1.41	34.33±5.80
47	Abhaya	9.90±4.60	33.00±4.24	25.60±3.65
48	Azucina	14.07±2.29	35.00±7.07	40.20±0.20
49	ARB6	13.83±2.25	29.00±4.24	22.50±16.48
50	Bamleshwari	12.70±0.82	28.00±2.83	30.93±3.86
51	Budda	18.23±7.60	36.00±2.83	51.67±2.75
52	Bakal	16.37±0.15	37.00±7.07	42.17±2.15
53	Bas 370	16.33±1.15	35.00±1.41	37.33±2.36
54	Bhataphool	15.67±3.67	40.00±2.83	42.63±1.17
55	Batroo	13.33±2.89	33.00±1.41	43.50±3.04
56	Bhatajhooli	14.00±3.04	33.00±1.41	43.73±4.24
57	CT 9993	10.67±0.76	31.50±2.12	33.33±5.69
58	Cross116	15.67±1.26	40.00±2.83	45.30±14.41
59	ChaptiGurmatiya	19.47±4.50	38.00±8.49	53.33±5.53
60	DesiLaldhan	19.30±3.80	23.00±4.24	35.40±5.39
61	Desi no.17	11.17±2.02	34.00±11.31	51.40±2.25
62	DagadDesi	14.47±1.00	31.00±1.41	40.67±2.08
63	IC267982	14.83±1.15	26.50±0.71	32.77±5.06
64	IR36	23.00±5.07	32.00±2.83	38.00±6.00
65	IR42253	13.83±2.57	32.50±0.71	39.57±3.40

Table 1: Cont....

S.No.	Line	Root length (RL)	Root Pulling Strength (RPS)	Shoot length (RL)
66	IR55419	13.20±2.52	27.00±1.41	40.73±3.65
67	Kranti	21.37±4.01	28.00±2.83	46.67±5.84
68	Laloo 14	12.77±1.36	27.50±0.71	39.00±1.99
69	Ramjiyawan	15.70±0.44	26.00±5.66	46.37±1.65
70	Safri 17	11.13±2.44	35.00±1.41	35.50±5.96
71	Shennong89366	14.33±2.31	28.00±2.83	45.17±2.89
72	IBD 1	13.37±2.90	32.50±3.54	33.67±1.68
73	SLO 16	16.83±2.93	21.50±2.12	44.50±3.61
74	Kalokuchi 223	14.77±0.64	25.50±4.95	38.17±1.37
75	Kalia	10.40±3.38	29.00±1.41	30.33±9.07
76	PRATAO	12.87±2.12	32.50±0.71	44.43±6.99
77	Chua Dau130	14.50±0.87	32.00±2.83	53.67±4.01
78	IR 55419	14.77±3.01	32.00±2.83	32.13±0.29
79	CHENGRI 2	12.50±2.50	31.00±1.41	39.67±5.03
80	CR5272	16.23±0.55	27.50±0.71	32.80±0.44
81	EPAGRI 109	13.57±2.80	29.50±0.71	32.17±1.04
82	PIN KAEO	11.20±2.25	32.00±5.66	31.30±1.90
83	DJOGOLON-DJOGOL	14.50±3.50	26.00±0.00	26.83±5.51

Table 2: Frequency distribution of 83 rice genotypes/ lines for root pulling strength

S. No.	Range of root pulling strength	Frequency	Elite lines/ genotypes
1	21.5	1	SLO 16
2	23 to 23.5	3	IR 84887-B-15, Danteshwari, DesiLaldhan
3	25.5	1	Kalokuchi 223
4	26 to 26.5	4	IR 83376-B-B-150-3, IC267982, Ramjiyawan, DJOGOLON-DJOGOL
5	27 to 27.5	7	R-RF-69, Annnada, R-RF-78, IR55419, CR5272, IR 86857-46-1-1-2, IR55419
6	28	4	Shennong89366, Kranti, Bamleshwari, IR 83381-B-B-38-3
7	29 to 29.5	9	IR 83372-B-B-133-2, IR 83383-B-B-140-2, IR 88287-677-60-3, IR 84859-B-41-1-2, IR 64, Vandana, ARB6, Kalia, EPAGRI 109
8	30	2	R-RF-81, IR 88287-677-53-7
9	31 to 31.5	9	R-RF-84, R-RF-95, IR 83381-B-B-55-4, IR 83383-B-B-140-3, Mahamaya, ARB8, CT 9993, DagadDesi, CHENGRI 2,
10	32 to 32.5	15	PIN KAEO, IR 84882-B-120, IR 83381-B-B-6-2, Chua Dau130, R-RF-75, IR 55419, IR 83383-B-B-129-4, IR 83383-B-B-129-3, IR36, MTU 1010, PRATAO, IBD 1, IR42253, Poornima, R-RF-65
11	33	7	R-RF-45, IR 83381-B-B-137-3, IR 84878-B-60-4-1, Samleshwari, Abhaya, Batroo, Bhatajhooli
12	34	4	R-RF-82, IR 83372-B-B-133-2, Swarna sub 1, Desi no.17
13	35	5	PM 6004, IR 84852-B-12-1-4, Azucina,Bas 370, Safri 17
14	36	1	Budda
15	37 to 37.5	6	IR 33929-B-B-132-2, CB-09-510, CR 2642-52, IR 84882-B-120-CRA-6, Swarna, Bakal
16	38	2	IR 86781-3-3-1-1, ChaptiGurmatiya
17	39	1	Sahbhagidhan (CH)
18	40	2	Bhataphool and Cross116

length, this time the result is statistically significant because the p-value is well below 0.05. The 200bp band show association with higher root length in the given breeding population of elite rice lines (Fig 1). The p-value obtained after analysis was 0.008171 with percent of total phenotypic variation for a trait that is accounted by the marker (R^2) was 8.5254% for RM242. Also the bands of molecular weights 230bp and 250bp shows significant association for a quantitative trait shoot length with the p-value 0.01 for both the base pairs bands and percent of total phenotypic variation was 7.61% and 7.07% for respective molecular weight bands.After the *in-silico* analysis (gramene.com) this marker was present on putative QTL CQH31 and CQH33 responsible for enhancement of root traits. Reference for band size (www.gramene.org) was taken from Gramene (www.gramene.org) shows the bands between molecular weight 200bp-235bp was responsible for root traits. Here is also the conformation in this study that the band of 200bp is associated with root traits. Therefore the conclusion is that there is a gene in the vicinity of RM242 that affect root traits.

HvSSR01-80 marker, on chromosome #01 show significant association with two trait shoot length (SL) and root pulling strength (RPS) having p-value 0.002, 0.036 with percent of the total phenotypic variation (R^2) was 10.86% and 5.28% respectively.The *in-silico* analysis revealed that this marker was present on putative QTL AQHJ009 and CQH3, the QTL responsible for root branching and penetrate root thickness. Association of HvSSR01-87 marker on chromosome #01 (www.gramene.org) with three different quantitative traits, the output obtained for this marker in this study was show

Table 3: Marker information used for genotyping in the study

S NO	Primers	Chro. No.	Position in cM		Sequence (5′-3′)
1	RM242			F	GGCCAACGTGTGTATGTCTC
		9	72.1	R	TATATGCCAAGACGGATGGG
2	RM201			F	CTCGTTTATTACCTACAGTACC
		9	78	R	CTACCTCCTTTCTAGACCGATA
3	RM202			F	CAGATTGGAGATGAAGTCCTCC
		11	49.1	R	CCAGCAAGGCATGTCAATGTA
4	HvSSR 1-80			F	TTTGAGCAAATAAACTTGACG
		1	139.1	R	GCTTCTACTTCCACAAGGC
5	HvSSR 1-87			F	TTGGTACACGACCATGATTA
		1	151	R	ATGGATCTGTGTGTGCGT

Table 4: Association between SSR markers and three agronomic traits with P < 0.05.

S. No	Marker	bp band associated	Trait	p-val(< 0.05)	% R^2
1	HvSSR 1-80	260	SL	0.002343	10.86422
2	HvSSR 1-87	340	SL	4.22E-07	27.23302
3	HvSSR 1-87	350	RPS	0.034252	5.725408
4	HvSSR 1-87	400	SL	4.22E-07	27.2302
5	RM 202	180	SL	0.0197	10.86422
6	RM 202	200	SL	0.001737	11.47085
7	RM 242	200	RL	0.008171	8.525464
8	RM 242	230	SL	0.015047	7.079262
9	RM 242	250	SL	0.015047	7.079262

Note: SL- Shoot Length, RPS- Root Pulling Strength, RL-Root Length

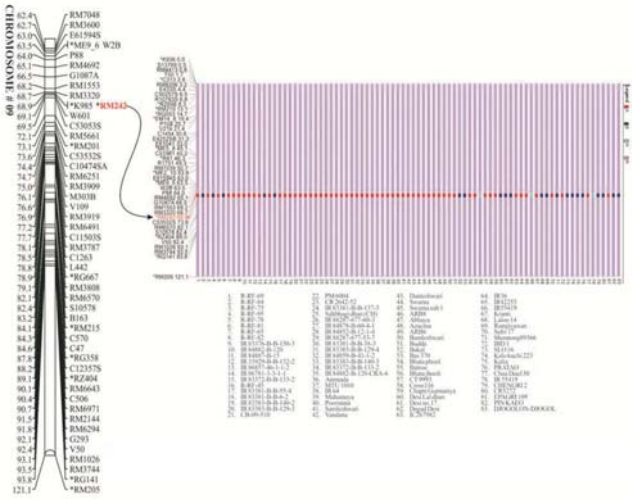

Figure 2: Visualisation of marker alleles of RM242 (CH#09) through Graphical Genotyping resolved in 83 elite rice genotypes

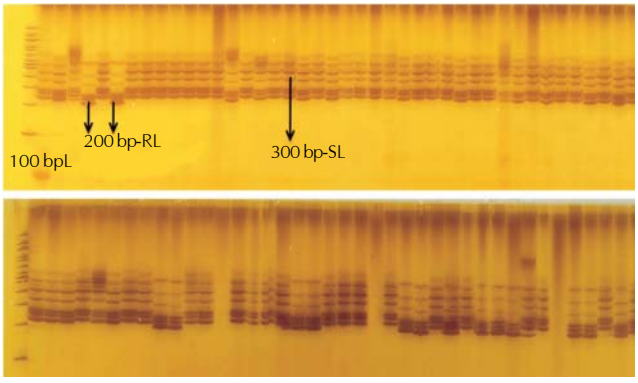

Figure 1: Molecular mapping of marker RM 242 with Root Length (RL-200bp band) and Shoot Length (SL-300bp band)

association with two traits, shoot length as well as root pulling strength having p-value 4.22E-07 with R^2 value was 27.23% for 340bp band and for same trait the 400bp band with same R^2 value show association. This marker showed a strong association with shoot length out of other four markers because the p-value obtained after t-test was very less. Lesser the P-value then there was higher the total phenotypic variation (% R^2) which was useful for diversity identification in germplasm collection. Also this marker show association with root pulling strength (RPS) on 350bp band with calculated p-value 0.034 and R^2 value 5.72%. This shows co-segregation of marker with gene responsible for the respective traits and therefore the conclusion is that there is the gene in the vicinity of HvSSR01-87 that affects the respective trait.

Graphical genotyping of rice genotypes using SSR maker data

Graphical genotypes are diagrammatic representations of the genetic composition of the chromosomes of segregating population by utilizing DNA marker data. As stated by researchers (1989), graphical genotypes reduce discrete locus data into a concise graphic image of individual linkage groups. These linkage groups are expressed in terms of the chromosomal regions inherited from the parents, reflecting chromosomal recombination and assortment. Graphical genotypes can only be constructed if marker positions on chromosomes are known from linkage (or physical) maps. Graphical outputs of genotyping data in this study were generated using GGT version 2.0 tool.

The study showed genomic constitution analysis (marker allele contribution) of rice lines/genotypes based on chromosome-

wise distribution of polymorphic SSR loci. Marker alleles for each locus were marked in different colors and incorporated in ascending order of position of markers (in cM) on the chromosomes. Some simple graphical genotypes are those indicated in Fig 2(RM242) which represents different allelic forms of same marker, the linkage map was illustrated to the left of graph. However strictly speaking, graphical genotypes are derived from DNA marker data and these are typically indicated the variation in allelic forms of same molecular marker in the population. The analysis revealed that the two alleles more or less contributed equally/unequally in case of markers located on chromosome for 83 rice genotypes.

Thus the phenotyping base on root pulling strength using root pulling machine to compare the root traits is one of the best phenotyping method. Genotyping based on molecular markers and identification of marker trait association helpful in screening out rice cultivars/genotype that has good root architecture. Out of five markers used in this study related to root traits, the marker RM242 co-segregate with Root Length (RL). The region was reconfirmation using *in-silico* analysis on chromosome #09. Once you identify the rice cultivars having dense or longer root length that will be use in future to transfer such traits from these cultivars into in local cultivars (most grown rice variety) for specific regions.

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