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DNA Marker-Assisted Analysis of Recombinant Inbred Lines Using Trait-Specific Markers and Candidate Genes in Rice (*Oryza sativa* L.)

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ABSTRACT

Rice (*Oryza sativa* L.) is the staple food for most people in Asia and is major caloric dietary source. As population and economy expand, the demand for consumption of rice will increase. To meet this growing demand it is required to increase the productivity levels of rice by combating biotic and abiotic stresses and other constraints. Early duration Recombinant Inbred Lines, 41 in total, developed using deep-rooted (Moroberekan) and high-yielding (IR50) parents were validated with 15 trait-specific SSR markers. These genotypes were validated by 4 candidate genes. Single marker analysis was done to study the association of markers with traits using the Student's *t*-test. RM242 and RM282 were associated with plant height, RM302 with days to 50% flowering, RM242 with number of tillers per plant, while RM257 was associated with seed weight per plant. Among the four candidate genes, Ext1L/1R, coding for extensin protein/leucine-rich repeats protein, was associated with days to maturity and MAPK for transcriptional control of stress response was associated with number of panicles.

Keywords: candidate genes, *indica* rice, *japonica* rice, RILs, single marker analysis, SSR markers
Abbreviations: QTL, quantitative trait locus; **RIL**, recombinant inbred line; **SSR**, simple sequence repeat

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for most people in Asia and is major caloric dietary source. It supplies 23% of global per capita energy and 16% of per capita protein (FAOSTAT 2003). Tropical *japonica* upland rice cultivars have thicker and deeper roots than *indica* cultivars (Courtois *et al.* 1996) whereas *indica* rice varieties have more ability to maintain osmoregulation than *japonica* rice (Robin *et al.* 2004). Hence in present study two genotypes, IR50 and Moroberekan were chosen. These contrasting characters in the two genotypes provide an opportunity to transgress *indica* and *japonica* alleles that could generate a range of recombinants which can provide genotypes with high tolerance to biotic and abiotic stresses and good yield.

Inbreeding from individual F_2 plants allows the construction of recombinant inbred lines (RILs), which consist of a series of homozygous lines each containing a unique combination of chromosomal segments from the original parents. The length of time needed for producing RI populations is the major disadvantage, because usually 6-8 generations are required. In present study such 41 RILs have been included for investigation.

The development of DNA markers has started a new era in crop improvement. Unlike morphological markers, these molecular markers are abundant and not affected by environment. The distribution of simple sequence repeat (SSR) sequences showed that regions of rice genome, which were richer in expressed genes also tended to be richer in SSR sequences, underscoring their usefulness as genetic marker (McCouch *et al*. 2000).

Recent advances in rice markers have made it possible to identify and map precisely a number of genes through linkage to DNA markers. Noteworthy examples of some of the genes tightly linked to markers are resistance or tolerance to blast, bacterial blight, virus disease, drought, submergence, salinity, low temperature, improved agronomic and grain quality traits (Jena and MacKill 2008). Chaitra *et al.* (2006) concluded that all deep-rooted plants amplified a 140-bp band while all shallow-rooted plants amplified a 158-bp band for RM201. Girish *et al.* (2006) carried out genetic variability, correlation, path coefficient analysis and test of normality was conducted in an F8 recombinant inbred aerobic rice population developed by single seed descent method to evaluate its potential as a mapping population. The subset of same population was used for scanning genomic regions for superior alleles. Kanagaraj *et al.* (2010) carried out BSA to identify markers linked to drought resistance using 23 RILs of IR20/Nootripathu, two *indica* ecotypes with an extreme drought response. Three primers, RM212, RM302 and RM3825 were found to co-segregated with RILs constituting the resistant bulk.

Drought-stress in plants stimulates the activity of several genes and the functions of their gene products have been predicted from sequence homology with known proteins. Candidate genes (CGs) are sequenced genes of known biological function associated with the manifestation of the trait. They may be structural genes or genes in a regulatory or biochemical pathway which affect trait expression. One CG hypothesis states that "a significant proportion of the QTL affecting trait variation are in fact CGs associated with that trait" (Rothschild and Soller 1997). Hence in present investigation early maturing genotypes were validated with trait-specific SSR markers and CG for their further selection in varietal development.

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Table 1 List of SSR trait-specific markers used for investigation.

Primer name	Sequence of forward primer 5′ to 3′	Sequence of reverse primer 5′ to 3′	Repeated motif	Expected product size (bp)	Chromo-some number	Trait associated
RM212	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG	$(CT)_{24}$	136	1	Deep root mass
RM 242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG	$(CT)_{26}$	225	9	Grain weight
RM 144	TGCCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG	$(ATT)_{11}$	237	11	Blast resistance
RM 234	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	$(CT)_{36}$	156	7	Root specific + drought tolerance
RM 282	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG	$(GA)_{15}$	136	3	Osmotic adjustment
RM 278	GTAGTGAGCCTAACAATAATC	TCAACTCAGCATCTCTGTCC	$(GC)_{17}$	141	9	Maximum root length
RM 201	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	$(GT)_{30}$ $(AT)_8$	158	9	Root length
RM 302	TCATGTCATCTACCATCACAC	ATGGAGAAGATGGAATACTTGC	$(GT)_{30}(AT)_8$	156	1	Drought tolerance
RM 80	TTGAAGGCGCTGAAGGAG	CATCAACCTCGTCTTCACCG	$(TCT)_{25}$	142	8	WBPH
RM224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	$(AAG)_8(AG)_{13}$	157	11	Blast resistance
pTA 248	AGACGCGAAGGGTGGTTCCCGA	AGACGCGGTAATCGAAGATGAAA	--------	700	11	Bacterial blight
RM 315	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	$(AT)_4(GT)_{10}$	133	1	Plant height
RM 257	CAGTTCCGAGCAAGAGTACTC	GGATCGGACGTGGCATATG	$(CT)_{24}$	147	9	Spikelet fertility
RM 164	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC	$(GT)_{16}TT(GT)_4$	246	5	Root penetration index
RM 264	GTTGCGTCCTACTGCTACTTC	GATCCGTGTCGATGATTAGC	$(GA)_{27}$	178	8	Tiller number + high amylase content

Table 2 List of candidate genes used for validation of early duration Recombinant inbred lines of IR 50/Moroberekan.

Name	Function of the gene	Primer name	Primer Sequence detail 5' to 3'	CHR No	Product size (bp)
Pp2a4	Protein phosphatase	pp2a4L - F	GGTTGGGGCATATCTCCTCGTGGT	10	928
		pp2a4RT - R	CCTAGGAGCTGGTTCAAACTGCAA	3	668
TPP	Trihalose 6 phosphatase	TPS-1L - F	CTATCTTGGGCTCATGGCGTGACTG	2	1000
		TPS-1R - R	CGCAGCATTTCGCAACCAACAAATA		
MAP kinase	Transcriptional control of stress response	MAPk-1L - F	CACCATCTCCTTCAGCCTCCGTTTC	7	975
		MAPk-1R - R	CACACCTCCACCCCAATCAAATTCC		
Extensin	Cell-wall protein for growth expansion	Ext-L - F	AGGAGAAGATGGCGATGGCCAATAA	10	977
		Ext-R - R	GAGCTCGAGATGCCTGATGATGTCC		

MATERIALS AND METHODS

The study included RILs developed from a cross between IR50 and Moroberekan at the Department of Genetics and Plant Breeding by Dr. Shailaja Hittalmani in field experiments conducted at K block (GKVK, Bengaluru, India) of Department of Genetics and Plant Breeding and selected 41 early duration genotypes were provided for this experiment have been evaluated under moisture stress situation from July to October of 2008 in a Randomized Complete Block Design (RCBD) with two replications (data not shown). Phenotypic data derived from this evaluation is used for further marker analysis.

Genomic DNA was extracted from leaf tissues of 25-days-old seedlings of the 41 RILs and two parents by modified cetyl trimethyl ammonium bromide method. The DNA was quantified at 260 nm using a UV spectrophotometer (Labmed Inc. UV-2602). After determining polymorphism between parents 15 SSR markers *i.e.* RM80, RM 212, RM 242, RM 144, RM 224, RM 201, RM 278, RM 302, RM234, pTA 248, RM 264, RM164, RM 257, RM 315 and RM 282 were used in the study for validation of RILs. These SSR markers (**Table 1**) were downloaded from Gramene website (www.gramene.org/). These are mapped and associated with drought-tolerant characters in different mapping populations (unpublished data). The PCR reaction mixture contained 40 ng of template DNA, 0.2 mM of each primer pair (Sigma Aldrich, USA), 1 mM dNTPs (Bangalore Genei), 10 X PCR buffer (1 X is 10 mM Tris Hcl pH 8.8 at 25°C, 1.5 mM KCl and 0.1% Triton X-100 all chemicals purchased from Sigma Aldrich, USA), 1 unit of *Taq* polymerase (Bangalore Genei) and deionized water to get a total reaction volume of 20 ml. The PCR profile involved an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final extension for 5 min at 72°C. The amplified PCR products were resolved on 3% agarose gels in 1 X TAE buffer.

Candidate genes are sequenced genes of known biological function associated with the manifestation of the trait. Four candidate genes Pp2a4 (protein phospatase), TPP (trihalose 6 phosphatase), Extensin (Ext 1L/1R) and MAPkinase were used to validate RILs along with parents. These candidate genes have been found to express in response to abiotic stress (Wang *et al.* 2004). The PCR reaction mixture consisted of 1 µl of template DNA, 25 ng each of left and right primers (**Table 2**) (Sigma Aldrich, USA), 100 µM each of dNTPs (Bangalore Genei), 1 U of Taq polymerase (Bangalore Genei), and 1X PCR buffer (10 mM Tris pH 8.0, 50 mM KCl, 1.8 mM $MgCl_2$ and 0.01 mg/ml gelatin, all chemicals purchased from Sigma Aldrich) in a volume of 20 µl. One drop of mineral oil was put on reaction mixture. The PCR profile involved an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 56°C for 0.3 min, 72°C for 1 min and a final extension for 5 min at 72°C. The amplified PCR products were resolved on 9% denaturing polyacrylamide gel in 1X TBE buffer.

Single marker analysis was done with the help of "Student's" *t*-distribution as given by Gosset in 1908.

$$t = \frac{\overline{X_1} - \overline{X_2}}{\sqrt{Sp^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where,

$\overline{X_1}$ = mean of trait of interest under investigation in individuals of marker class I;

$\overline{X_2}$ = mean of trait of interest under investigation in individuals of marker class II;

Sp^2 = pooled variance

n_1 = number of genotypes in marker class1

n_2 = number of genotypes in marker class 2

$$Sp^2 = \frac{S_1^2(n_1 - 1) + S_2^2(n_2 - 1)}{n_1 + n_2 - 2}.$$

RESULTS AND DISCUSSION

A small set of early duration genotypes were selected from a population of RILs developed from the drought susceptible IR50 and drought-tolerant Moroberekan parents. Fifteen trait specific SSR markers and four candidate genes were used for validation of genotypes. These SSR markers and candidate genes have been found to be associated with different characters.

It has been found that RM302 is closely associated with phenotypic trait of days to 50% flowering (**Tables 3, 4**). The same marker has been associated with drought resistant by Kanagaraj *et al*. (2010). RM242 is linked to the number

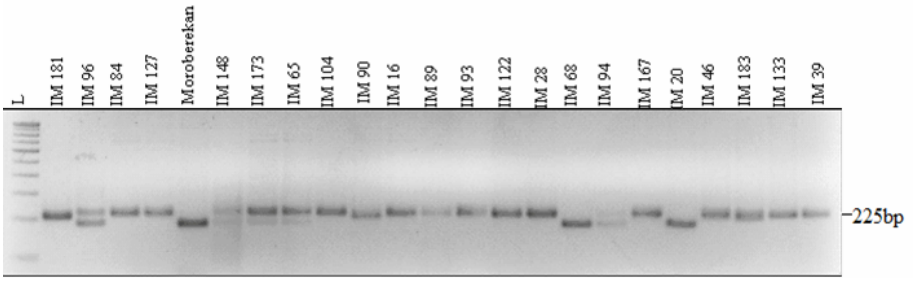

Fig. 1 Agarose gel of 22 RILs and Moroberekan (male parent) for SSR marker RM 242. L, 100 bp ladder; IM181, IM96, IM84, IM127, IM148, IM173, IM65, IM104, IM90, IM16, IM89, IM93, IM122, IM28, IM68, IM94, IM167, IM20, IM46, IM183, IM133, IM39, RILs; Moroberekan, male parent.

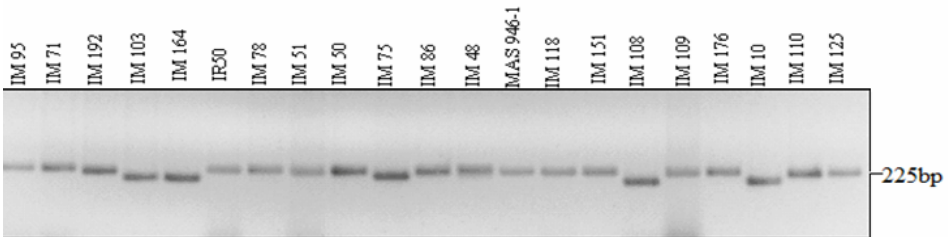

Fig. 2 Agarose gel of 19 RILs along with IR50 and MAS946-1 for SSR marker RM 242. IM95, IM71, IM192, IM103, IM164, IM78, IM51, IM50, IM75, IM86, IM48, IM118, IM151, IM108, IM109, IM176, IM10, IM110, IM125, RILs; IR50, Female parent; MAS946-1, local check.

Table 3 List of SSR markers showing association with different phenotypic characters.

Marker	Calculated *t* value	Table *t* value 5%/1%	Trait associated
RM302	2.181*	1.703 2.473	Days to 50% flowering, Days to maturity
RM242	1.92*	1.689 2.432	Number of tillers
RM257	2.106*	1.711 2.492	Grain yield per plant
RM242	3.224**	1.689 2.432	Plant height
RM282	2.946**	1.690 2.440	Plant height
RM234	2.863**	1.685 2.422	Plant height

Table 4 List of candidate genes showing association with different phenotypic characters.

Candidate gene	Calculated *t* value	Table *t* value 5%/1%	Trait related
Ext 1L/1R	2.016*	1.6827 2.419	Days to 50% flowering and days to maturity
MAPK	3.048**	1.689 2.432	Number of panicles

* Significant at 5%; ** Significant at 1%

of tillers. Zhao *et al.* (2008) reported that the marker interval RM242-RM278 is associated with QTL loci governing grain weight. RM257 was associated with panicle exsertion by Yue *et al*. (2008), is found to be associated with seed weight per plant. For plant height which is a major quantitative trait three SSR markers i.e., RM242, RM282 and RM234 have been found to be linked (**Table 3**).

Along with trait-specific markers, four candidate genes were validated for the early duration RILs of the cross IR50/Moroberekan (**Figs. 1, 2**). It has been noticed that out of four candidate genes chosen for the investigation three candidate genes i.e., Pp2a4, TPP, Extensin (Ext 1L/1R), were amplified for both the parents but the extent of amplification was varied in the progenies. This may be because of recombination that has occurred between the regions of candidate gene and thus transgressive segregants were produced with absence of candidate gene sequence as reported by Song *et al.* (2007). Only one candidate gene i.e., MAP kinase was amplified in IR50 (*indica*) where as in Moroberekan (*japonica*) no amplification was found. MAP Kinase is a protein kinase produced in response to stress. It is involved in transcriptional control of stress response. This gene get expressed in stress condition and the protein produced gets accumulated in panicles by producing more number panicles as reported by Chao *et al.* (2007). According to Robin *et al.* (2004), *indica* rice varieties have more ability to maintain osmoregulation than *japonica* rice. In present investigation the MAP kinase has been amplified in IR50 which is a *indica* rice variety has ability to maintain higher osmoregulation than *japonica* rice variety Moroberekan and it is done with the help of proteins such as MAP kinase and thus giving more number of panicles but this MAP kinase gene amplified product was absent in Moroberekan which gives less number of panicles per plant.

The candidate gene Ext 1L/1R coding for extensin protein/leucine-rich repeats protein is expressed in root hair cells and the protein is specifically localized in the wall of the hair proper, where it becomes insolubilized during development. It has been reported that LRX1 is an extracellular component of a mechanism regulating root hair morphogenesis and elongation by controlling either polarized growth or cell wall formation and assembly (Baumberger *et al.* 2001). This candidate gene has shown varied expression levels in progenies even though it is present in both the parents. This gene was found to be associated with days to 50% flowering and days to maturity. This indicates that the genotypes which possess extensin gene will contain protein in their roots and produce good root hairs with long root length and thus will be able to grow deep and reach the water source if available at greater depths and combat drought by tolerance mechanism but the genotypes in which the candidate gene is absent, instead of wasting photosynthetic material for root growth it will orient it for grains thus escaping drought by completing its life cycle in short period of time thus escapes the drought.

The detection of genes or QTLs controlling traits is possible due to genetic linkage analysis, which is based on the principle of genetic recombination during meiosis. This permits the construction of linkage maps composed of genetic markers for a specific population. Segregating populations such as F_2, F_3 or backcross (BC) populations are frequently used. However, populations that can be maintained and produced permanently, such as recombinant inbreds and doubled haploids, are preferable because they allow replicated

and repeated experiments. Hence the RILs used in present study provide good opportunity to study the trait association. Using statistical methods such as single-marker analysis or interval mapping to detect associations between DNA markers and phenotypic data, genes or QTLs can be detected in relation to a linkage map (Kearsey 1998). The identification of QTLs using DNA markers was a major breakthrough in the characterization of quantitative traits (Paterson *et al*. 1988).

A large number of rice studies have used markers as a tool to identify major genes, QTLs, or to introduce new characters in elite germplasm. Knowing the location of these genes/traits and specific alleles offers the possibility to apply marker-assisted selection (MAS) in rice, because one of the main objectives of crop improvement is the introgression of one or more favourable genes from a donor parent into the background of elite variety. Marker-assisted selection allows plant selection at the juvenile stage from an early generation. For simply inherited traits, conventional PCR, which requires a small amount of DNA, is becoming very useful for screening large populations of segregating progenies. Unfavourable alleles can be eliminated or greatly reduced during the early stages of plant development through MAS, focusing the selection in the field on reduced numbers of mature plants (Karzun *et al*. 2003).

SSR markers have been successfully employed in different experiments on genetic variation, mapping, tagging of economically important traits, for generating high density linkage map, for monitoring introgression of genomic regions and for marker-assisted selection. Yue *et al*. (2008) associated RM 257 with panicle exsertion; the same marker was associated with seed weight per plant in our investigation. Nematzadeh *et al.* (2010), who conducted linkage analysis on F2 recessive class, showed that RM258 and RM171 flanked to restorer gene *Rf4* at 3.1 and 6.3 cm, respectively. Selvaraj *et al.* (2011) performed single marker analysis and simple regression analysis to identify the marker phenotype association which resulted in the identification of 23 SSR markers putatively associated for the six traits studied. Three SSR markers (RM 5757, RM 451 and RM 492 from chromosomes four and two) were linked for leaf blast resistance. Aliyu *et al.* (2011) used SSR markers for the tagging salt tolerance and related traits. They found that RM 493 and RM 3412 was found to be associated with leaf diameter ($P < 0.05$) under salt stress. Selva Pirabu *et al.* (2011) screened RILs of the cross IR50* Rathu Heenati to assess the resistance to brown plant hopper based on the standard seedbox screening test (SSST). They performed F test to assess the association of SSR markers with the trait. They found that the SSR marker RM2346 was identified to be associated with the BPH resistance based on the damage score from SSST. In the present study, identification of trait specific marker will help in further screening of genotypes and effective introgression of the genomic regions governing important traits.

CONCLUSION

Marker-assisted selection (MAS) can be used for monitoring the presence or absence of genes in breeding populations and can be combined with conventional breeding approaches. The use of effective DNA markers derived from the genes for important agronomic traits will help in detection of presence of the genes and validate the genotypes for various traits.

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