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Identification of SCAR Marker Linked to Root Thickness in Rice (*Oryza sativa* L.)

N. M. Boopathi, R. C.Babu[1] , R. Chandirakala[1], S. C. Mathews[1], A. Singh[1], P. Chezhian[1] and P. Shanmugasundaram[1]

Centre for Plant Molecular Biology
Tamil Nadu Agricultural University
Coimbatore –641 003
India.

ABSTRACT. *Drought is a major abiotic stress limiting rainfed rice production. Breeding for drought resistance through conventional means is slow in attaining progress. Alternatively, secondary traits contributing in drought resistance could be selected for in breeding for drought resistance. However, phenotypic selection for several secondary traits is difficult and labour-intensive. Identifying molecular markers linked to these traits will facilitate indirect selection of such complex traits through marker-assisted selection (MAS). Several molecular markers linked to drought resistance traits have been identified in rice. But use of these markers in MAS is fraught with complex and time-consuming protocols. Hence, identifying simple PCR-based markers will improve the efficacy of MAS. This study has identified one RAPD marker putatively linked to root thickness. The RAPD fragment co-segregating with thick root lines was eluted, cloned and sequenced. The sequence information was used to design Sequence Characterized Amplified Regions (SCAR) primers. The SCAR marker produced a distinct polymorphic bands at 500 bp and 100 bp positions for thick root lines (CT993, thick DH lines and Norungan) and thin root lines (IR62266, thin DH lines and IR20) respectively.*

INTRODUCTION

Rice (*Oryza sativa* L.) is grown on more than 148 million hectares (Mha) and feeds more than one third of the world's population. It is cultivated in a wide range of ecosystems under varying temperatures and water regimes. About 39% of the world's rice (59 Mha) is grown in areas classified as rainfed low lands and uplands (IRRI, 1993). Of world's rainfed lowland rice area, 95% is in Asia. India has the largest rainfed lowland rice area, with about 16.5 Mha, which is about 40% of the total rice area in the country. In south and southeast Asia, future increases in the area under rice production would largely come from the rainfed ecosystem (Garrity *et al.*, 1986).

Drought stress is an important limitation to rice production in these areas, which seriously affects income and food security of subsistence rice farmers. Incorporation of drought resistance thus has great implications in boosting rainfed rice production. Conventional breeding methods are slow to show results owing to their inherent limitations.. Alternatively, secondary traits contributing in drought resistance could be identified and selected for in breeding programs. Several putative traits contributing in drought resistance in rice have been reported (Nguyen *et al.*, 1997). A

[1] Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore- 641 003, India.

deep and thick root system has been considered advantageous for improved drought tolerance in the rainfed ecosystem (Kamoshito *et al.*, 2002a), due to their greater penetration ability and access to moisture from deeper soil horizons. Further, thicker roots have a larger xylem diameter and consequently less axial resistance to water flow, thereby enhancing water uptake (Passioura, 1982). However, incorporation of the rice root system into elite lines by conventional breeding has met with little success due to laborious nature of measuring root traits and lack of *in situ* screening techniques. One promising approach is to identify molecular markers tightly linked to root trait(s). Upon identification of molecular markers, marker aided selection (MAS) can be performed as an alternate to difficult phenotypic screening. Molecular markers, mainly Restriction Fragment Length Polymorphisms (RFLPs), linked to various drought resistance component traits including root thickness have been identified in rice (Kamoshita *et al.*, 2002a, b). Though RFLP markers are robust, it involves time consuming protocols besides handling hazardous radioactive chemical thus limiting their application in routine MAS procedures.

Randomly Amplified Polymorphic DNA (RAPD) markers (Williams *et al.*, 1990), also have the power to detect DNA polymorphism similar to RFLPs. RAPD markers have been employed to screen rice cultivars for salt sensitivity/tolerance (Erickson *et al.*, 1995). However, a major limitation in RAPD is repeatability, because PCR reactions are very sensitive to factors such as annealing temperature, template DNA concentration *etc.* Under such circumstances, cloning and further sequence characterization of the RAPD fragments and designing longer and specific oligonucleotide primers (Sequence Characterized Amplified Regions - SCARs) to verify its linkage to the targeted trait in a segregating progeny would be more reliable (Paran and Michelmore, 1993) and will facilitate its application in MAS (Tanksley *et al.*, 1989) since, SCARs are codominant in nature and are highly reproducible and stable. In an earlier study, a RAPD marker, $OPAK02_{500}$ was found to be associated with certain rice doubled haploid (DH) lines that were tolerant to drought stress in the field (Boopathi *et al.*, 2001). These tolerant DH lines were found to have thicker roots. Whereas, the DH lines with thinner roots were drought sensitive and did not have this marker indicating that the marker $OPAK02_{500}$ may be linked to root thickness. However, bulked segregant analysis (BSA) using rice lines differing only in root thickness will further verify the association of the marker with root thickness in rice. BSA is used to rapidly identify a marker linked to gene or genomic region that regulates the trait of interest (Michelmore *et al.*, 1991). Hence, the present study was conducted with the objective of identifying a marker linked to root thickness in rice DH lines.

MATERIALS AND METHODS

A DH line population of 220 lines has been developed from a cross involving CT 9993-5-10-1-M and IR 62266-42-6-2 (abbreviated as CT9993 and IR62266, respectively) at International Rice Research Institute (IRRI), Philippines. CT 9993 is a *japonica* from uplands (International Centre for Tropical Agriculture, Columbia). It has deep and thick roots with high root penetration ability and is drought tolerant. IR 62266 is an *indica* adapted to rainfed lowlands (IRRI, Philippines). It has shallow and thin roots with low root penetration ability and drought susceptible (Babu *et al.*, 2001). A subset of 154 DH lines of this population had been phenotyped for root thickness (Zhang *et al.*, 2001) and out of these ten DH lines with thicker root and another ten lines with thinner root were chosen. In addition, Norungan, a land race traditionally

grown under rainfed uplands of Tamil Nadu state, India and the drought sensitive check, IR20 were also included. Norungan has a deep and thick root system and is highly drought tolerant. IR20 on the other hand has shallow and thin root system and is highly drought sensitive (Babu *et al.,* 2001). The root thickness and other particulars of the selected DH lines, their parents, Norungan and IR20 are given in Table 1. Seeds of these rice accessions were obtained from IRRI, Philippines and Agricultural Research Station, TNAU, Paramakudi, India.

The genomic DNA was extracted from the etiolated leaf samples and purified according to Gawel and Jarret (1991). The DNA was quantified and diluted to a concentration of 25 ng μl^{-1}. BSA was done following the method described by Michelmore *et al.* (1991). Thicker root bulk and thinner root bulk were prepared by pooling equal quality of DNA from ten DH lines of each category separately. The DNAs of two bulks, parents, land race and check variety were used for RAPD analysis using OPAK02. The cocktail for the amplification was prepared and agarose gel electrophoresis was done as described by Williams *et al.* (1990).

The SCAR marker, $OPAK02_{500}$ was identified by loading the RAPD products on a 1.8 % low melting agarose gel and the fragment was cut along with the agarose. This excised agarose slice was crushed in a sterile eppendorf and dissolved in 400 µl of TE buffer. This suspension was incubated at -20°C for 2h and after incubation it was centrifuged at 10000 rpm. The supernatant was dissolved in 70 µl of 3 M sodium acetate and 1 ml of ethanol and incubated overnight at -20°C. At the end of incubation it was centrifuged for 15 min at 10000 rpm. The pellet was dissolved in 10 µl TE buffer and rechecked in 1.8% agarose gel for its purity.

The purified RAPD fragment was ligated into pUC57/T vector and transformed into *E. coli* DH5α (InsT/Aclone™ PCR Product Cloning Kit, Fermentas, Lithuania). Recombinant clones were identified by blue/white selection and the white colonies were used for PCR reactions. The PCR program is same as that of RAPD, except that the reaction mixture volume used was 25 µl. After confirming the insert in the recombinant clone, it was pure cultured in separate plate and sent for sequencing (Microsynth, Switzerland) using M13 forward and reverse primer. The sequence information obtained from the pUC57/T of the recombinant clone was analyzed for the sequence of the insert.

Designing longer primers of 24 bp length was done by analysing the sequence of RAPD fragment OPAK02 using Oligo software. The criteria considered here were the melting temperature (Tm), the GC content and absence of palindromic and repetitive sequences (Newton and Graham, 1990). Accordingly, forward and reverse primers were designed for OPAK02 with Tm of 70 and 68°C and GC content of 45.83 and 41.66%, respectively. Both the primer sequences (5'GGTAGCCTCCTTGTATATGGTAA3' and 5'GGTAGCCTCCCGATTTATATTAAC3') had no palindromic or repetitive sequences and the difference between the Tm for these two primers was 2°C. PCR was carried out in a reaction volume of 25 µl containing 100 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 200 µM each of the four dNTPs, 15 pmoles of each primer, 15 ng of the template DNA and 1.0 unit of *Taq* polymerase. The same PCR profile used for RAPD analysis was used except that the annealing temperature of 55°C. The amplified products were run on 2% agarose gel.

Table 1. Root thickness and pedigree of the parents, rice DH lines, their parents, landrace and IR20.

S. No.	Description	Pedigree	Root thickness*(mm)
	Parents		
1	CT9993-5-10-1-M	*Japonica*	1.08
2	IR62266-42-6-2	*Indica*	0.74
	DH lines with thicker roots		
3	IR68586-F2-CA-13	Interracial derivative	1.12
4	IR68586-F2-CA-52	-do-	1.09
5	IR68586-F2-CA-36	-do-	1.06
6	IR68586-F2-CA-72	-do-	1.06
7	IR68586-CA-148	-do-	1.04
8	IR68586-F2-CA-126	-do-	1.03
9	IR68586-F2-CA-114	-do-	1.02
10	IR68586-F2-CA-104	-do-	1.02
11	IR68586-F2-CA-4	-do-	1.01
12	IR68586- CA-6	-do-	1.01
	DH lines with thinner roots		
13	IR68586-CA-30	Interracial derivative	0.71
14	IR68586-CA-11	-do-	0.74
15	IR68586-F2-CA-14	-do-	0.75
16	IR68586-CA-19	-do-	0.78
17	IR68586-F2-CA-136	-do-	0.78
18	IR68586-CA-27	-do-	0.79
19	IR68586-CA-8	-do-	0.79
20	IR68586-CA-21	-do-	0.80
21	IR68586-CA-17	-do-	0.81
22	IR68586-F2-CA-96	-do-	0.81
23	Norungan	*Indica*	1.48
24	IR20	*Indica*	0.63
	LSD (5% P)		0.22

≥1.0 mm thicker roots; < 1.0 mm thinner roots *Zhang *et al.*, 2001

RESULTS AND DISCUSSION

Four polymorphic bands were generated by the primer OPAK02 in CT 9993 and out of these four bands, the band which was approximately 500 bp in length was also present in thick root bulk and the land race Norungan. It was absent in IR 62266, thin root bulk and IR 20. Then OPAK02 was used for co-segregant analysis i.e., screened with the individuals constituting the bulks. The $OPAK02_{500}$ was present in all the DH lines with thick root and was completely absent in all the DH lines with thin root (Plate 1). In an earlier study, this marker was associated with certain DH lines that were tolerant to water stress under field conditions and also have thicker roots. The marker was absent in DH lines that were drought sensitive and have thinner roots (Boopathi *et al.*, 2001). Hence this fragment was chosen for development of SCAR marker. As a first step, the fragment was eluted and inserted into a PCR cloning vector

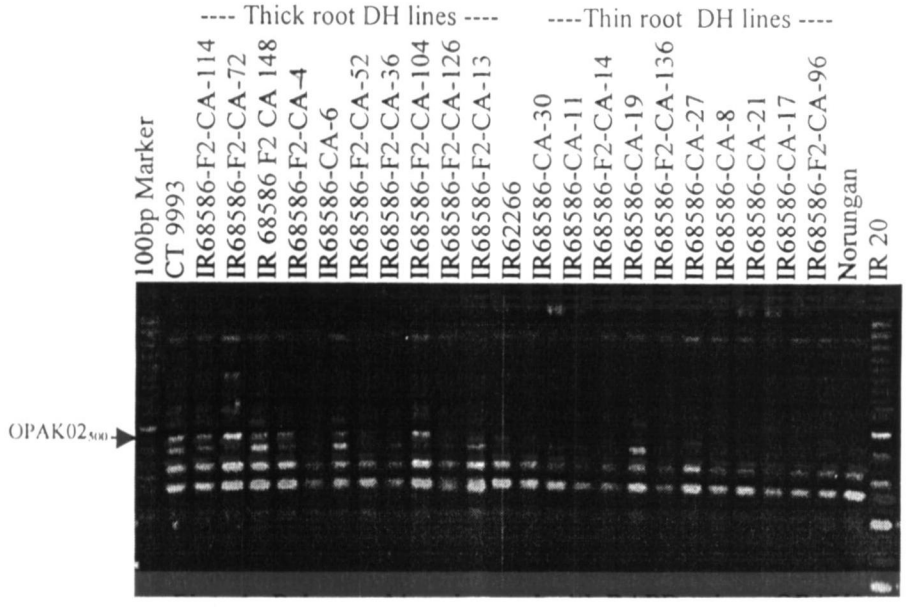

Plate 1. Polymorphism detected with RAPD primer OPAK2.

and the clone was sequenced. The insert was having 447 bp, including the OPAK02 primer sequence (5' CCATCGGAGG 3') and the complementary OPAK02 primer sequence (5' CCTCCGATGG 3') at both ends, which was flanked by the multiple cloning sites of pUC57/T. The *Taq* polymerase has the ability of adding 'A' at the end of PCR reactions (Clark, 1998). The presence of 'AA' sequence at the end of the insert sequence confirmed the sequence was from the PCR product. The BLASTN 2.2.2 search of this sequence information (http://www.ncbi.org) reveals that the identified sequence has more than 80% identities with the already sequenced rice genome. Based on the sequence information the forward and reverse primers were designed. The PCR analysis of this SCAR primers revealed that it produced ~ 500bp band in CT9993, all individual DH lines constituting thick root bulk and the land race, Norungan. Besides it also produced a distinct band of ~ 100bp in length in IR62266, all individual DH lines constituting thin root bulk and IR20 (Plate 2). This confirms the codominant nature of SCAR marker.

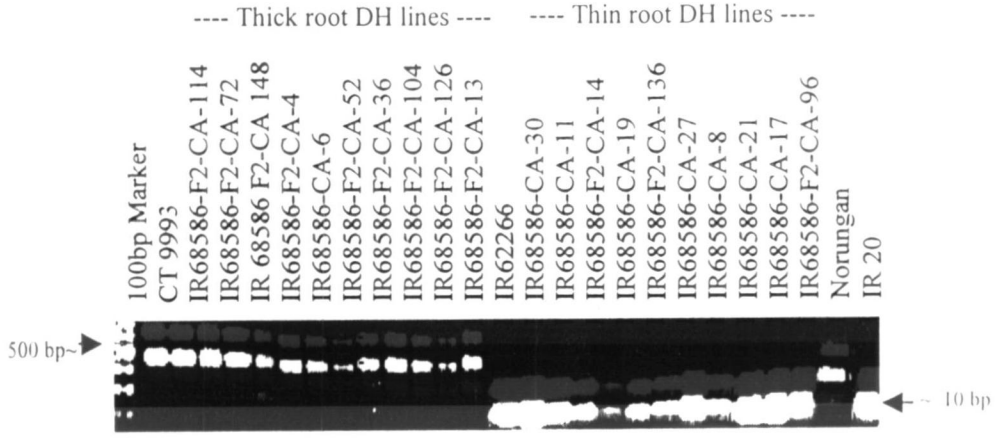

Plate 2. Polymorphism detected with SCAR primer

CONCLUSION

The results have thus identified a SCAR marker co-segregating with root thickness in DH lines and Norungan. Since identified marker differentiated thick root DH lines and Norungan (a thick root landrace) from thin root DH lines and IR20 (a thin root cultivar), this may be linked to root thickness. Further studies on mapping of the SCAR marker on chromosome linkage map by genotyping the entire DH population segregating for root thickness will validate the significance of the marker for its use in MAS for drought resistance improvement in rice.

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