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Study of simple sequence repeat (SSR) polymorphism for biotic stress resistance in elite rice variety JGL 1798

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To provide ready to use markers for back ground selection in marker assisted breeding of rice, we used GPP 2 as donor parent for *xa13, Xa21*, *Gm*4 resistance to bacterial blight, gall midge and NLR 145 as another donor parent for *Pi-k*^{h} gene resistance to blast and JGL 1798 as recurrent parent was investigated using 128 simple sequence repeat (SSR) primers covered on chromosome number 1-12. The results reveal that 36 HRM primers showed distinct polymorphism among the donor and recurrent parents studied indicating the robust nature of microsatellites in revealing polymorphism. Based on this study, the large range of similarity values for related cultivars using microsatellites provides greater confidence for the assessment of simple sequence repeats (SSR) polymorphism.

Key words: Simple sequence repeats (SSR), parental polymorphism, JGL 1798, hyper variable markers.

INTRODUCTION

Rice (*Oryza sativa*. L), a member of the Graminae family, has a genome size of 0.45×10^{-9} bp (Arumunagathan and Earle, 1991), which is one tenth the size of the human genome and is a model system for cereal genome analysis. Biotic stresses, such as diseases (blast caused by the fungus *Magnaporthe grisea*, bacterial leaf blight caused by *Xanthomonas oryzae* pv. *Oryzae*, and insects (gall midge) account for significant yield losses annually. Resistance to these diseases and insect controlled either by dominant or recessive major genes (*xa13*, *Xa21, Pi-k*^{h}, *Gm4*) by QTL (Alam and Cohen, 1998; Himabindu et al., 2010). The DNA markers have been used effectively to identify resistance genes, and marker assisted selection (MAS) has been applied for integrating different resistance genes into rice cultivars lacking the desired traits. Jagityal Sannalu (JGL 1798), released by Acharya N G Ranga Agricultural University (ANGRAU), Rajendranagar, Hyderabad. This variety is being widely grown in Northern Telangana region of Andhra Pradesh during both *kharif* and *rabi* seasons. The cooking quality of this variety is on par with a premium rice variety, Samba Mahsuri, one of the parents [(Samba Mahsuri (BPT 5204) × Kavya (WGL 48684)] of this variety and is susceptible to bacterial leaf blight, gall midge and blast. As on date, 100 rice blast major resistance genes (R-genes) have been identified and mapped (Sharma et al., 2012) on different rice chromosomes, and tightly linked DNA markers have been developed. The polymerase

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Abbreviations: PCR, Polymerase chain reaction; **MAS,** marker assisted selection; **BB,** bacterial blight; **MABB,** marker assisted back cross breeding; **SSRs,** simple sequence repeats; **PIC,** polymorphism information content; **CTAB,** cetyl trimethylammonium bromide; **EDTA,** ethylenediaminetetra acetate.

chain reaction (PCR) based allele-specific and InDel marker sets are available for 9 blast resistance genes. And they provide an efficient marker system for MAS for blast resistance breeding (Hayashi et al., 2006). Eight blast resistance genes have been cloned and the genes have been used for their selective introgression into susceptible rice cultivar (Lin et al*.,* 2007). It is imperative to use DNA markers identified within the gene or from the flanking region of the gene as a tool for an efficient MAS strategy in rice improvement (Fjellstrom et al., 2004). Additionally, several blast resistance genes could be combined using MAS in a single genetic background to develop rice cultivars with broad-spectrum durable resistance to blast. Several bacterial blight (BB) resistance genes have been associated with tightly linked DNA markers, and some of them have been cloned (*Xa1, xa5, xa13, Xa21, Xa26, Xa27*) and used for breeding BB-resistant rice cultivars. Because of the availability of DNA markers derived from the resistance genes, it is now possible to pyramid several genes, into susceptible elite rice cultivars. The resistance genes *xa5, xa13, and Xa21* have been pyramided in to an indica rice cultivar (PR 106) using MAS that expressed strong resistance to BB races of India (Singh et al., 2001). It is the most effective way of transferring specific gene(s) to an elite susceptible cultivar. In rice, the feasibility of marker assisted back cross breeding (MABB) to pyramid BB resistance genes has been well demonstrated (Sundaram et al., 2008).

Resistance to gallmidge is under the control of at least 11 resistance genes (*Gm1, Gm2, gm3, Gm4, Gm5, Gm6, Gm7, Gm8, Gm9, Gm10, Gm11*), 8 of which have been tagged and mapped. (Himabindu et al., 2010). The usefulness of resistant cultivars for protection against gall midge infestation suggests that MAS will be a highly useful tool for breeders in areas where the pest is prevalent.

According to the study of Frisch et al. (1999a), molecular markers are used in backcross breeding for two purposes: (1) To trace the presence of a targ*et* allele for which the term `foreground selection' was suggested for this selection of targ*et* allele by Hospital and Charcosset (1997); and (2) to identify individuals with a low proportion of undesirable genome from the donor parent. This approach is called `background selection' and was first proposed by Tanskley et al*.* (1989) and then by Hillel et al*.* (1990) and was further investigated by Hospital et al*.* (1992) and later reviewed by Viescher et al*.* (1996). The main advantage of using DNA markers is to accelerate the fixation of recipient alleles in non-target regions and to identify the genotypes containing crossovers close to target genes (Tanksley et al*.,* 1989).

Among different classes of available molecular markers, simple sequence repeats (SSRs) are useful for a variety of applications in plant genetics and breeding because of their reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and good genome coverage. SSR markers have been more useful for parental polymorphism study. It is the basic step for MAS. The polymorphism information content (PIC) value ranged from 0.064 (RM 274) to 0.72 (RM 580) with an average of 0.46. The Jaccard's similarity coefficient ranged from 0.42 to 0.90 reported by Seetharam et al. (2009). In rice, microsatellites have been classified into two groups based on length of SSR motif and their potential as informative markers: Class I microsatellites contain perfect SSRs >20 nucleotides in length and Class II contains perfect SSRs >12 nucleotides and <20 nucleotides in length. Class I markers are reported to be highly variable (Cho et al., 2000), whereas Class II SSRs are less variable owing to limited expansion of microsatellite repeat motif during slipped-strand mispairing over the shorter SSR template (Temnykh et al., 2001). As of now, 18 828 Class I microsatellite markers have been identified (IRGSP, 2005) and a high-density SSR map with genome coverage of approximately two SSRs per centimorgan (cM) has been constructed in rice (McCouch et al., 2002). The present study was undertaken with the objective of identifying polymorphic SSRs for further background selection of JGL 1798.

MATERIALS AND METHODS

Plant material

Three rice genotypes constituted the experimental material JGL 1798, NLR 145 collected from ARI, Rice section, ANGRAU and GPP 2 collected from DRR, Rajendranagar, Hyderabad, India.

DNA extraction and SSR analysis

Genomic DNA was extracted by modified cetyl trimethylammonium bromide (CTAB) method (Sambrook and Russel, 2001). 15 - 20 days rice leaves were extracted with DNA extraction buffer (2% CTAB, 100 mM Tris, 20 Mm ethylenediaminetetra acetate (EDTA), 1.4 M NaCl) preheated at 60 UC and 200 mg. The quality and quantity of extracted DNA was judged by comparing it with λ-DNA in agarose gel electrophoresis. DNA quantification and purity was checked by measuring the O.D at 260 and 280 nm using a UV visible spectrophotometer. For marker assisted-multiple gene introgression, 4 gene specific primers were used for fore ground selection and 128 HRM primers for back ground selection. These information regarding chromosomal location and sequences of primers were obtained from www.gramene.org.

Polymerase chain reaction (PCR)

The polymerase chain reaction was carried out in Eppendorf thermal cycler using 128 primers. The PCR reaction mix includes the following: DNA 10 ng/µl; 10 X buffer; 10 mM dNTPs; 50 mM $MgCl_2$, 10 µM each of forward and reverse primers. The PCR profile starts with initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, primer annealing 55°C for 30 s (for gene specific primers like *xa13, RM 547, RM 206* at 55°C and *pTA248* 58°C), extension 72°C for 1 min, final extension 72° C for 10 min, and cooling 4°C for α was included. These steps were repeated for 35 cycles for amplification of DNA. After completion of amplification, PCR products were stored at –20°C and the amplified products were analyzed by electrophoresis using 2% (for *xa13, pTA248*) and 3% (*RM 547, RM 206* and for background primers) agarose gels. Ethidium bromide

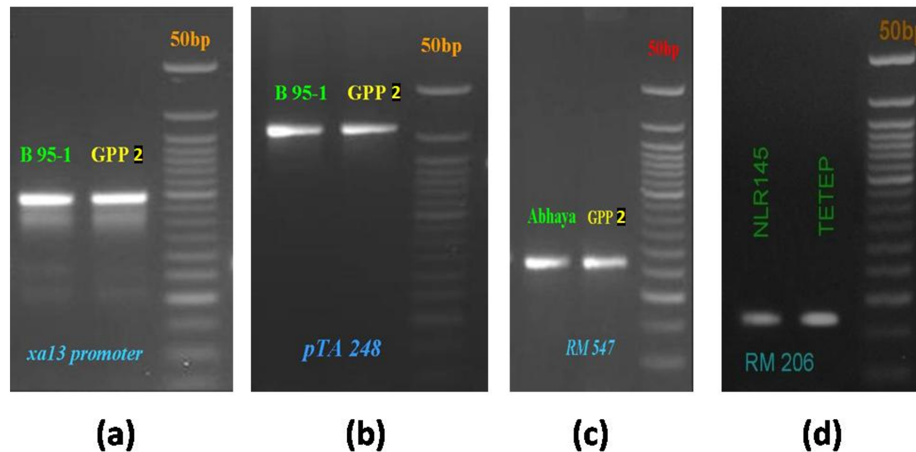

Figure 1. Validation of target genes in donor parents.

was added while pouring the gel so that the DNA fluoresces when gel was exposed to UV light. The DNA fragments was then visualized under UV transilluminator and the banding pattern was observed and recorded using gel documentation unit (Gene flash) which was stored for further scoring and permanent records.

Data analysis

The 3 genotypes were scored for the presence and absence of the SSR bands. And the data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis.

RESULTS

Validation of *xa13, Xa21, Pi-k^h* and *Gm4* genes in the donor parents

In the present study, Jagityal Sannalu (JGL 1798) variety was chosen as recurrent or recipient parent, while GPP 2 was used as donor parent for *xa13, Xa21* and *Gm4* resistance genes. NLR 145 is another donor parent for blast resistance gene, *Pi-k^h*. Before starting marker assisted back crossing (MABC) breeding, these donors are to be confirmed for the resistance genes by comparing with their respective source materials. Once the parents were confirmed for the resistance genes, then the parental polymorphism study was carried out between recurrent and donor parental lines.

GPP 2 is the donor parent for three resistance genes viz., *xa13*, *Xa21* and *Gm4.* Hence this donor parent is to be validated for the presence of three genes by comparing with their respective check materials B 95-1(BLB resistance genes *xa13* and *Xa21*) and Abhaya (gall midge resistance gene *Gm4*).

The results reveal that an allele of 500 bp was amplified with *xa13 promoter* in the donor parent GPP 2. This band was exactly identical to the band that was amplified in the check material, B 95-1. The marker *pTA-248* amplified an allele of 916 bp (Figure 1), which was similar with that of B 95-1 confirming that the donor parent was carrying *Xa21* gene. Huang et al. (1997) also used *pTA248*, a gene sequence based marker for *Xa21* gene in marker assisted selection. Amplification pattern of *RM547* was observed at 270 bp in GPP 2 and it was identical to the band that was observed in Abhaya. Arundathi et al., 2010 also observed *Gm4* gene in PTB10 by using gene specific primers. These results revealed that the donor, GPP 2 was carrying two BLB resistance genes and one gall midge resistance gene. These results were identical with the findings of Sundaram et al. (2008, 2009).

NLR 145, another donor parent was verified for the presence of blast resistance gene, *Pi-k^h* by using the gene specific primer, *RM206* along with check material, Tetep. The result confirmed that NLR 145 was carrying *Pi-k^h* gene since RM206 primer pair amplified an allele of 140 bp in NLR 145 and this allele was exactly similar to that of Tetep. The result is in confirmation with the findings of Sharma et al. (2005).

Parental polymorphism for resistance genes

Study of parental polymorphism is a pre requisite to begin marker assisted selection or marker assisted back cross breeding. Unless the parents are polymorphic for the traits of interest, the further selection of plants carrying the traits of interest is not possible in the progenies. In the present study, parental polymorphism survey was taken up between JGL 1798 and GPP 2 and also between JGL 1798 and another donor parent, NLR 145. The two donor parents were tested along with the recurrent parent, JGL 1798 for all the four target genes. The results indicated that a clear polymorphism was observed between the parents, JGL 1798 and GPP 2 for *xa13* gene when the primer pair, *xa13* promoter was used for amplification. *xa13* specific marker amplified a clear band of 260 bp in JGL 1798, while another band of 500 bp was amplified in the donor parent, GPP 2. Similarly, polymor-

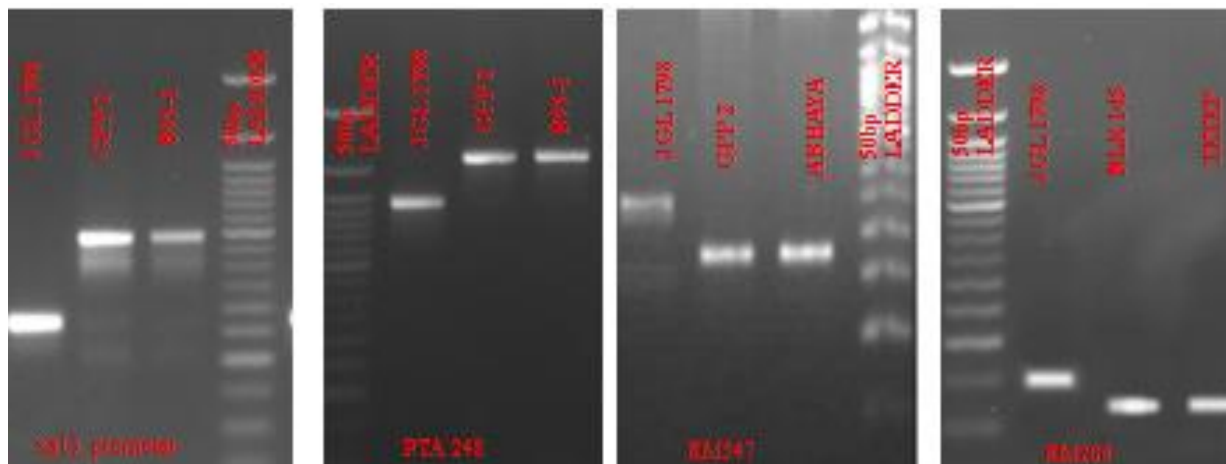

Figure 2. Polymorphism between parental lines for four target genes using gene specific primer pairs.

phism was observed between GPP 2 (916 bp) and JGL 1798 (700 bp) when *pTA-248* primer was used for *Xa21* gene. *RM547*, a gene specific primer was used for observing the parental polymorphism for the target gene, *Gm4* showed that an allele of 290 bp was observed JGL 1798, while the resistance allele of 270 bp in the donor parent, GPP 2. These results clearly showed that there was a clear polymorphism between JGL 1798 and GPP 2 for three target genes viz., *xa13*, *Xa21* and *Gm4.* Nair et al. (1996) also observed polymorphism between resistant and susceptible parents by using random amplified polymorphic DNA (RAPD) primers.

SSR primer pair of RM206 was used to study the polymorphism between JGL 1798 and NLR 145 for *Pi-k^h^*, a blast resistance gene. A susceptible allele of 170 bp in JGL 1798, while the resistance allele of 140 bp was observed in NLR 145 (Figure 2). The allele that was observed in JGL 1798 was clearly different from resistance allele that was observed in NLR 145. The present investigation clearly stated that three resistance genes including one for gall midge and two for BLB were presented in GPP 2, while NLR 145 was carrying a blast resistance gene, $Pi\text{-}k^h$. The recurrent parent, JGL 1798 was carrying all the four corresponding susceptible alleles.

Since the polymorphism was very clear among the parents for all the four target genes (Table1), further selection of plants carrying the target genes in the successive backcrossing generations is referred as foreground selection in MABC (Hospital and Charcosset., 1997). Hence these markers will be used for foreground selection. Similarly Yang et al. (1994) and Mc Couch et al. (1997) used SSRs to study the polymorphism between and the rice varieties. The gene-specific markers viz., *xa13 promoter, pTA-248, RM547and RM206* have been reported to be tightly linked to BLB, gall midge and blast resistance genes *xa13*, *Xa21, Gm4*, $Pi\text{-}k^h$, respectively. Naveed et al. (2010) performed similar type of molecular survey for *xa5* gene.

Parental polymorphism survey between recipient parent, JGL 1798 and the donor parents GPP 2 and NLR 145

For effective use of MAS or MABB, polymorphic markers between the parents are highly useful to exercise background selection. Background selection is highly useful to identify the plants carrying desirable genome of interest. Among the 128 hyper variable primers, di nucleotide primers were maximum in number (80) followed by tri- (40) and tetra nucleotide repeats (8) (Figure 4) di nucleotide (AT) repeats were abundant on all chromosomes, followed by AAT repeats in most of the chromosomes. Among the 128 primer pairs, 82 primer pairs were recognized as monomorphic and 36 primer pairs exhibited polymorphism between recurrent parent JGL 1798 and donor parents GPP 2 and NLR 145 (Figure 3).

Twenty two primer pairs viz., HRM 10936, HRM 10167, HRM 11111, HRM 13659, HRM 14250, HRM 15855, HRM 16153, HRM 15679, HRM 17405, HRM 16652, HRM 18939, HRM 20583, HRM 20710, HRM 21881, HRM 22622, HRM 23146, HRM 23578, HRM 25310, HRM 27323, HRM 28110, HRM 28202 and HRM 28800 were detected as polymorphic in nature between recurrent parent JGL 1798 and donor parent GPP 2.

Thirty one primer pairs viz., HRM 10167, HRM 11111, HRM 13154, HRM 12469, HRM 13659, HRM14250, HRM 15626, HRM 15831, HRM 15855, HRM 16153, HRM 15337, HRM 17405, HRM 16913, HRM 16652, HRM 16649, HRM 18939, HRM 18770, HRM 19697, HRM 21539, HRM 21881, HRM 22622, HRM 23146, HRM 23578, HRM 24481, HRM 24542, HRM 24199, HRM 27323, HRM 25970, HRM 28110, HRM 28202 and HRM 28800 were observed as polymorphic markers between recurrent parent JGL 1798 and donor parent NLR 145. Five primer pairs viz., HRM 22622, HRM 23146, HRM 23578, HRM 27323 and HRM 25970 were observed

Table 1. Amplification pattern of gene linked primer pairs in donor and recipient parents.

Primer name	Linked gene	Chromosome number	Donor parent	Resistant allele size(bp)	Recipient parent	Susceptible allele size (bp)
xa13promoter	*xa13*	8	GPP 2	500	JGL 1798	260
pTA-248	*Xa21*	11		916		700
RM547	*Gm4*	8		270		290
RM206	*Pi-k^h^*	11	NLR 145	140		170

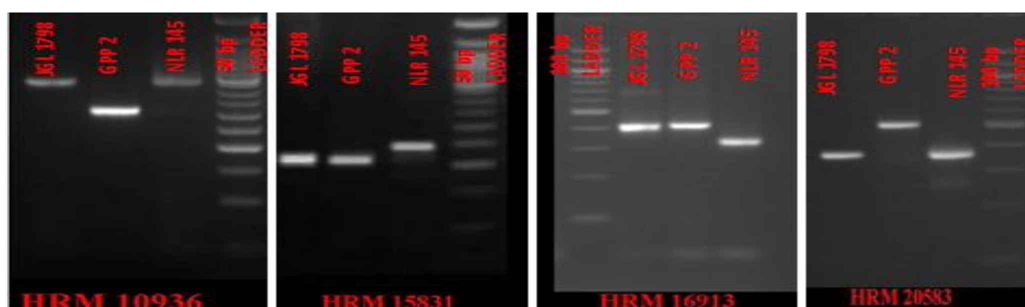

Figure 3. Polymorphism between recipient parent JGL 1798 and donor parents GPP 2 and NLR 145 with by using HRM primers pairs.

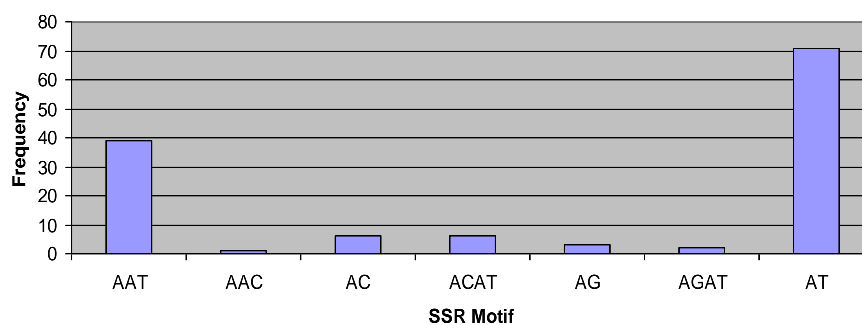

Figure 4. Frequencies of total 128 hyper variable microsatellite markers with different repeat motifs

on target chromosomes 8 (*xa13* & *Gm4*) and 11 (*Xa21* & *Pi-k^h^*) carrying the target genes.

DISCUSSION

From application angle, DNA markers are widely used in marker-assisted breeding/selection. In MAS, markers are used at two levels, that is, foreground selection and background selection. For foreground selection, gene-specific or tightly linked markers of target traits are used. A recent review by Jena and Mackill (2008) provided the list of DNA markers that are tightly linked with some major quantitative trait loci (QTLs) or genes relating to agronomic traits. For effective use of MAB, identification of appropriate markers for background selection is equally important. It facilitates speedy and reliable recovery of recurrent genome.One hundred and twenty eight SSR primers that are distributed all over twelve rice chromosomes were selected to assess the polymorphism between the recurrent and donor parents. These primer pairs were selected based on repeat number and location on different chromosomes. Preference was given to the primers that are with more number of repeats, tri and tetra nucleotides in addition to di nucleotides (Narasimhulu et al., 2010). Since the relative frequencies of di-, tri- and tetra nucleotide repeat motifs of the chosen markers were compared chromosome wise.

During the amplification of genomic DNA of three rice genotypes, the number of amplified fragments that are polymorphic in nature were ranged between 2 and 3, with an average polymorphic fragment per primer was 2.5, the

PIC values were ranged between 0.72 (HRM 10936) 0.88 (HRM 28800) with an average of 0.75; A high level of polymorphism was observed when compared to the low level of polymorphism in earlier studies. Similarly high PIC values were reported in barley wheat and rice (Gu et al., 2005 and Seetharam et al., 2009).

The results revealed that out of 36 polymorphic primer pairs, a maximum of seven primer pairs were observed on chromosome 3, followed by four primer pairs on chromosome 4, three each on chromosomes 1, 2, 6, 8, 9 and 12, two each on chromosomes 5, 7 and 11 and one on chromosome number 10. Among 36 primer pairs, AT repeats were maximum of 18 in number followed by 14 AAT, 2AGAT and 2 AC repeats. Similarly, Temnykh et al. (2001), Grover et al. (2007) and Narsimhulu et al. (2010) observed maximum polymorphism with AT repeat primers. This richness in polymorphism is due to the presence of transposable sequences that target $(AT)^n$ repeats (Akagi et al., 1997; Grover et al., 2007).

In the present investigation, 92% of markers showed clear amplification, while 31% of markers showed polymotphism. The present study further revealed that the selected primers belongs to Class I microsatellites that are highly polymorphic in nature because of low PCR failure rate with increased probability of SSR expansion when compared to Class II microsatellites (Cho et al., 2000; Temnykh et al., 2001; Narsimulu et al., 2010).

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