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Introgression of Transgenic Resistance for Rice Tungro Disease into Mega variety, ASD 16 of Tamil Nadu through Marker Assisted Backcross Breeding

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Rice Tungro is a viral disease caused by the joint infection of *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV). In order to obtain transgenic resistance against RTBV, *indica* rice cultivar, Pusa Basmati-1 (PB-1) was transformed to express the coat protein gene of an Indian isolate of RTBV. The transformed PB-1 showed a concomitant reduction of tungro symptoms. The present study was attempted to diversify this transgenic resistance against tungro disease from PB-1 into superior but highly susceptible variety, ASD 16 by following marker assisted backcross breeding. ASD 16, was crossed with transgenic PB1 and the F_1 plants were repeatedly backcrossed with the recurrent parent ASD 16 to obtain BC_3F_1 population. The transgenic plants that contained the RTBV resistant transgene were identified in BC_3F_1 and BC_3F_2 population by PCR analysis using functional marker associated with that gene. These foreground selected plants were subjected to background analysis and it revealed that there was 100 per cent recovery of the recurrent parent genome in BC_3F_2 plants.

Key words: Rice Tungro Disease, *Rice tungro bacilliform virus* (RTBV), Marker Assisted Backcross Breeding

Rice (*Oryza sativa* L.) is by far the most economically important food crop and the staple food for more than half of the world population. Cultivation of high yielding rice cultivars with reduced genetic variability, higher plant population per unit area and high fertilizer input farming coupled with staggered sowing and planting make rice more prone to diseases. Annually more than 40% of the world's rice crop is lost owing to biotic stresses like insects, pests, pathogens and weeds (Hossain, 1996).

Rice tungro disease (RTD), the most important viral disease of rice, is widespread in South and South-east Asia and is believed to be responsible for annual losses nearing 10_9 US $ worldwide (Herdt, 1991). More recent estimates reveal that the disease causes on an average about 2% losses in rice production in India, although at the regional level, losses can be more significant (Muralidharan *et al.,* 2003). Rice tungro is caused by the joint infection of two unrelated viruses *Rice tungro bacilliform virus* (RTBV), a double-stranded DNA-containing virus and *Rice tungro spherical virus* (RTSV), a single-stranded RNA virus (Jones *et al.,* 1991). RTBV and RTSV, also known as the ''Tungro virus complex'', are transmitted exclusively by the Green leafhopper (GLH), *Nephotettix virescens*. The most conspicuous symptoms of tungro are the stunting of plants and yellow-orange discolouration of leaves, both of which are believed to be caused by RTBV, as observed in symptomatic plants subjected to Agrobacterium- mediated inoculation of the virus (Dasgupta *et al.,* 1991).

Because of its role in limiting rice production, incorporation of tungro resistance has been an important breeding objective in rice improvement programs in Asia. Several sources of genetic resistance have been reported in rice against RTSV, but against RTBV, there are only a few (Azzam and Chancellor, 2002). Additionally, none of the host resistance sources have been genetically well-characterized. Thus, in order to ensure durability of the otherwise fragile resistance under field conditions, transgenic strategies for tungro resistance, targeting RTBV are promising.

Transgenic expression of genes encoding viral Coat Protein (CP) was the first successful illustration of the concept of pathogen derived resistance in plants and remains till date an effective method to obtain plants resistant to viruses (Malinowsk *et al.,* 2006). More recently, RNA-mediated methods have also been shown to be effective in generating virus-resistant plants (Tyagi *et al.,* 2008). However, CP-mediated resistance is considered to be more broadly applicable against variants of the same virus and related viruses than RNA-mediated approaches (Prins *et al.,* 2008). Considering the high variability in the RTBV nucleotide sequences, the use of CP-mediated resistance, rather than RNA-mediated

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one, may be more durable and effective at a greater number of locations across South and Southeast Asia against RTBV.

In order to obtain transgenic resistance against RTBV, *indica* rice cultivar Pusa Basmati- 1 was transformed to express the coat protein (CP) gene of an Indian isolate of RTBV and the transgenic lines expressing the gene showed moderate levels of resistance against the homologous virus (Ganesan *et al.,* 2009).

Recurrent backcrossing is a traditional breeding method commonly employed to transfer alleles at one or more loci from a donor to an elite variety. Molecular markers have been proved very useful in improving backcross breeding through precise transfer of target genomic regions. In addition, it can allow us to estimate the genomic composition and can speed up the recipient genome recovery *via* background selection (Hospital, 2001). Improvement of the elite cultivars through MAB has been intensively applied in rice breeding programs throughout Asian countries (Zhang, 2007).

Having these concepts in mind, the present study was undertaken to diversify transgenic resistance for tungro disease from Pusa Basmati- 1 into superior but tungro susceptible popular south Indian variety, ASD 16 through marker assisted backcrossing. The genotypic selection was done using functional maker. Later the recurrent parent genome recovery of the genotypes was identified through background analysis using SSR markers spanning across rice genome.

Materials and Methods

Pusa Basmati-1 transformed to express the RTBV resistant coat protein gene was used as a donor parent. The recipient varietiey was ASD 16, widely grown cultivar in south India but susceptible to tungro disease. ASD 16 was crossed with Pusa Basmati- 1. Wet cloth method of emasculation was practiced and pollination was done manually. The crossed seeds were harvested separately and used for raising F_1 generation. The F_1 plants were backcrossed with the recurrent parent ASD 16 continuously up to three generations with the selection of PCR positive transgenic plants in each generation. The BC_3F_1 seeds were raised in portrays in greenhouse and were transplanted into pots at 20 DAS and thereafter allowed to self. Samples were collected from these plants at two leaf stage, DNA was extracted and the plants containing the RTBV resistant gene were identified using PCR based functional marker.

The seeds of the identified PCR positive transgenic plants were harvested at physiological maturity stage, dried and were used to raise the BC_3F_2 generation. Thirty progenies from each plant were planted separately with a spacing of 30 x 30 cm. Foreground analysis was done for the progenies of all the plants and RTBV gene resistant plants were identified. Different morphological traits like days to 50 % flowering, plant height, number of tillers per plant, number of productive tillers per plant, panicle length, panicle exertion percentage, spikelet fertility, 100 grain weight and plant yield were measured for the PCR positive plants along with normal ASD 16 as control. The mean performance of the identified transgenic lines was compared with the non-transgenic ASD 16.

From the identified PCR positive plants in the BC_3F_2 generation, 10 random plants were selected based on their phenotypic performance to raise the BC_3F_3 generation in order to identify the homozygous positive plants for the recipient allele. 30 seeds from each of the 10 plants were sown in portrays in greenhouse. PCR analysis was carried out for all the plants and the plants whose progeny was completely positive for RTBV resistant gene were considered as homozygous plants and were selected for background analysis.

Foreground selection

To identify the presence of RTBV resistant gene, oligonucleotide primer, Hyg forward and reverse was used. The plants which showed amplification for the functional primer were considered as positive plants and those plants were forwarded to next generation.

Primer used

Hyg forward-5' GCCTGAACTCACCGCGACG 3'

Hyg reverse- 5' CTCATCGAGAGCCTGCGCG 3'

Background Analysis

Parental Polymorphism

About 100 SSR markers that span the whole Rice genome were used to screen for the parental polymorphism. PCR was carried out using 25 ng DNA as template for amplification, 100 mM dNTPs, 1 ìl each of forward and reverse primer, and 0.33 ìl of Taq DNA polymerase. The template DNA was initially denatured at 94^0C for 5 min. followed by 35 cycles of PCR amplification with the following cycling conditions of 45 sec denaturation at 94^0C, 1 min primer annealing at the prescribed temperature for the given primer (or as given in Gramene database), 1.5 min extension at 72^0C and a final extension of 72^0C for 7 min. The PCR products were separated on 3% agarose gel and visualized under UV light.

Progeny screening

The SSR markers that showed polymorphism between donor (PB1) and recurrent parent (ASD 16) were used to screen the identified RTBV resistant homozygous plants to recover the recipient genome. Plants were scored as whether they contain the ASD 16 allele (+) or not (-).

Estimation of recurrent parent genome contribution (G) was calculated as

$$G = x/n$$

Where,

n = total number of amplified parental polymorphic markers screened

x = number of markers showing homozygosity for recurrent parent allele

Results and Discussion

In the present study efforts were made to transfer RTBV resistant coat protein gene (RTBV-2ds gene) from the transgenic Pusa Basmati-1 into agronomically superior but tungro susceptible variety, ASD16 through backcrossing assisted with markers. In addition, genetic similarity of backcross population with their recurrent parent was also studied to get superior line that look nearly closer to parental genotype. In this direction, the results obtained from the current research are presented here under.

Marker assisted selection

Marker assisted selection (MAS) is an approach that involves the use of known molecular markers linked to traits of interest to aid and speed up the normal selection process in a breeding program. Often MAS changes the selection criteria of a breeding programme as selection is no longer being based on phenotype but rather more directly towards selection of specific genes (Francia *et al.,* 2005).

One of the strengths of MAS is the fact that markers are not environmentally influenced. With the increasing availability of a large number of different molecular markers, MAS can be used for selection of simple as well as quantitative traits (Mohan *et al.,* 1997). Following successful marker development, Marker Assisted Backcrossing (MAB) has been used to transfer single genes or QTLs into various backgrounds. Marker assisted backcrossing has been used in rice for diseases like bacterial leaf blight, blast, sheath blight disease and also in combining insect and bacterial resistance (Jiang *et al.*, 2004). Sundaram *et al.* (2008) successfully introgressed three BLB resistance genes *Xa21 xa13* and *xa5* into Samba Mahsuri, an elite indica rice variety which is high yielding and possesses exceptional grain and cooking quality. A major QTL on chromosome 9, *Sub1,* has provided the opportunity to apply maker assisted backcrossing to develop submergence tolerant varieties. Neeraja *et al.* (2007) successfully developed the submergence tolerant version of the widely grown cultivar, Swarna through marker assisted backcrossing.

Foreground selection

In the present study, BC_3F_1 seeds of the cross between ASD 16_3 X PB1 were sown in portrays in the green house along with the recurrent parent. The foreground analysis for selecting the plants that contain the RTBV resistant gene was done using functional primer Hyg forward and reverse. Out of 129 plants surveyed, 44 plants showed amplification

Table 1. Recurrent parent genome contribution in BC3F3 plants of ASD 16

	AS 16- 59- 4	AS 16- 88- 3	AS 16- 115-3	AS 16- 120- 4
No. of polymorphic markers surveyed	20	20	20	20
No. of markers amplfied	19	20	17	20
No. of markers exhibiting ASD 16 allele	19	20	17	20
% of recurrent genome recovery (G)	100	100	100	100

for the functional primer used (Fig 1). The PCR positive transgenic plants of ASD 16 were forwarded to BC_3F_2 generation. The identified PCR positive plants of ASD 16 in BC_3F_2 generation were harvested, dried and sown to raise the BC_3F_3 generation. Out of 38 BC_3F_3 plant progenies, in three progenies, all the 30 plants were surveyed in order to study the inheritance pattern of the particular gene. Four progenies *viz*., AS 16- 59- 4, AS 16- 88- 3, AS 16- 115- 3 and AS 16- 120- 4 were considered as homozygotes (Fig 2) for the RTBV resistant gene as all the 30 progeny of all the four plants were positive for the functional primer used. These 4 plants were subjected to background selection for finding out the recurrent parent genome contribution. The plants in which some of the progeny were negative for the primer used were not surveyed further.

Background analysis

Marker assisted background selection is the selection of backcross derived plants which possess maximum recurrent parent genome recovery. Phenotypic selection for 'good agronomic type' has always been practiced along with backcross selection (Allard, 1960), but genotypic selection - monitoring the parental origin of alleles using markers throughout the genome in backcrossing - was originally proposed by Young and Tanksley (1989) and later was termed as background selection (Hospital and Charcosset, 1997). The objective of the background selection is to accelerate the return to recipient parent genome outside the target gene. Basically, markers allow one to have a good idea of how much of the recurrent parent genome has been recovered in any particular BC progeny and to select for the best backcross progeny available in any generation. This ability to select for recurrent parent genotype outside of the target locus can greatly reduce the number of generations required to develop lines that possess the desired gene, but are otherwise nearly isogenic with respect to the recurrent parent.

In several studies on background analysis, various types of markers viz., sequence tagged

microsatellite marker (Shanti *et al*., 2010), RFLP (Chen *et al*., 2000), SSR (Sundaram *et al*., 2009 and Perumalsamy *et al.,* 2010) and AFLP (Joseph *et al*., 2004) has been used. Of the various types of markers available for background selection, the microsatellite markers are considered robust due to the availability of high density genome map especially in the case of rice (McCouch *et al*., 2002).

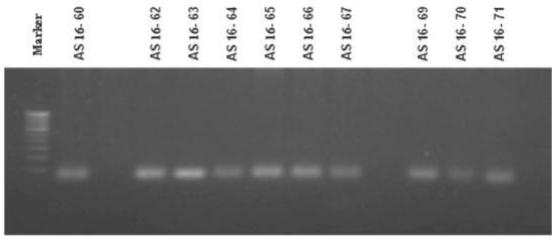

Fig. 1 PCR positive plants of ASD16 selected in BC_3F_1

Microsatellites or SSRs are a unique class of repetitive DNA sequences that are highly polymorphic and abundant throughout the genomes of eukaryotes (Rakoczy and Bolibok, 2004). They provide several advantages over the other types of markers when applied in a plant breeding program. Markedly, they are based on the polymerase chain reaction technique, co-dominant in nature, represent

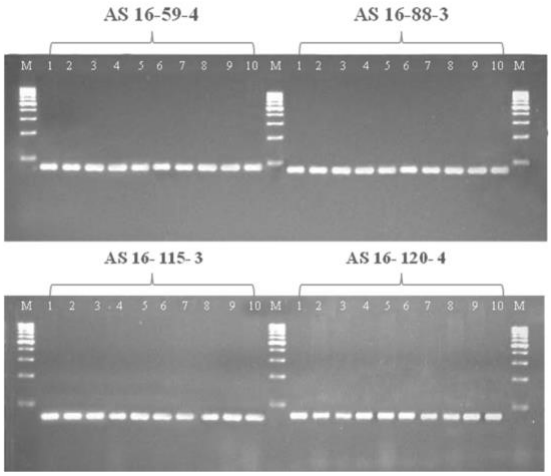

Fig.2 Homozygous plants identified through PCR

single loci, chromosome specific and can detect high levels of polymorphism (Roder *et al.,* 1998) which ultimately makes them ideal tool for tracing out the origin of genetic content in backcross progeny.

The effect of marker density on the recovery of the recurrent genome was first investigated by Hospital *et al.* (1992) and they concluded that addition of two to three markers per 100 cM as optimal for controlling the genetic background of the recurrent parent. Neeraja *et al.* (2007) has used a minimum of three polymorphic microsatellite markers in each chromosome. Background selection was performed using markers that are unlinked to target trait but polymorphic between both donor and recurrent parent. This leads to the recovery of recurrent genome to the maximum level.

In the current study, around 100 SSR markers that are distributed across the rice genome were screened for parental polymorphism. Out of 100 markers, 24 were identified as polymorphic between the donor parent (PB-1) and recurrent parent (ASD 16). These 24 markers which showed polymorphism between the recurrent parent and donor parent were utilized for screening the foreground selected RTBV resistant homozygotes. In plant no. AS 16- 59- 4, out of 24 markers used, 23 markers showed amplification and all these 23 markers exhibited ASD 16 allele. In AS 16- 88- 3 and AS 16- 120- 4, all the 24 markers amplified were similar to ASD 16 allele. In AS 16-115-3, all the 21 amplified markers exhibited recurrent parent allele. These results reveal that there is 100 % recurrent parent genome recovery in the identified BC_3F_3 homozygotes (Table 1).

Agronomic performance of the PCR positive plants

No significant alteration was observed in the agronomic characteristics of the identified transgenic lines compared to normal ASD 16. From the mean performance study of BC_3F_2 population 10 plants were selected based on their performance in comparison with their parent to raise the BC_3F_3 generation to identify the homozygous transgenic lines. The selected plants were AS 16- 42- 7, AS 16- 57- 3, AS 16- 57- 5, AS 16- 59-4, AS 16- 66- 4, AS 16-88- 3, AS 16- 88- 4, AS 16- 115- 3, AS 16- 117- 2 and AS 16- 120- 4. Among the selected plants, AS 16- 42- 7, AS 16- 59- 4, AS 16- 66-4, AS 16- 115- 3 showed more number of productive tillers than the parent. Almost all the selected plants have longer panicles which is one of the contributing factor for yield. As 16- 120 - 4 showed the longest panicle length of 30.5 cm. The plant yield of selected plants ranged from a minimum of 52.1 g in AS 16- 120- 4 to a maximum of 60 g in AS 16- 57- 3.The mean yield of the parent was 52.53 g which showed that almost all the selected plants yielded more than the parent. The spikelet fertility percentage of all the selected plants was more than that of the parent. The gain in yielding traits over the control parent might have been also due to some favourable introgressions other than transgene from PB-1. The resultant plants however did not exhibit any major trait variation from ASD16 making them ideal candidates for adoption.

As a final conclusion, in the present study, the RTBV resistant transgene were introgressed into ASD 16 with a maximum recurrent genome recovery of 100 % in the identified homozygous transgenic lines which are ready for phenotyping for RTD resistance.

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