RESEARCH NOTE

Detection of probable marker-free transgene-positive rice plants resistant to rice tungro disease from backcross progenies of transgenic Pusa Basmati 1

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Introduction

Rice tungro disease (RTD) is caused by simultaneous infection of rice tungro bacilliform virus (RTBV), a pararetro virus having a dsDNA, belonging to genus *Tungrovirus* (Hull *et al*. 2005); and rice tungro spherical virus (RTSV), a positive ssRNA virus of the genus *Waikavirus* (Murphy *et al*. 1995). Typical RTD symptoms include stunting, yellow-orange discolouration of plants, reduced tillering and incomplete panicle emergence with chaffy grains (Azzam and Chancellor 2002). The major vector of virus complex over much of Southeast Asia is the rice green leafhopper, *Nephotettix virescens* (Hibino and Cabauatan 1987).

The viruses (RTBV and RTSV) are found in most, if not all, rice growing countries of South Asia and Southeast Asia. RTD is reported to be responsible for 5–10% annual losses of rice yield in Asia (Dai and Beachy 2009) and about 2% in India (Muralidharan *et al*. 2003). Due to the economic significance of RTD, incorporation of resistance has been an important objective in rice improvement programme in Asia. Most attempts to develop RTD resistance through classical breeding have not been durable (Dahal *et al*. 1990; Azzam and Chancellor 2002; Khush *et al*. 2004). Biotechnological approaches have also been employed to develop transgenic plants for resistance against RTBV and RTSV (for review see Dai and Beachy 2009). The transgenic strategies for RTD resistance are promising, although pathogen derived resistance for RTD has reported as being only partially effective (Dai and Beachy 2009). Recently, some work on the development of transgenic resistance to RTD have been done targeting RTBV, as it is the causative agent of tungro symptoms (Dai *et al*. 2008; Tyagi *et al*. 2008; Ganesan *et al*. 2009). These reports showed considerable resistance against the disease in controlled laboratory conditions. However, the level of resistance and its durability in field condition is important.

The selectable markers used for plant transformation with *Agrobacterium* (e.g. antibiotic resistance) provide a convenient source of dominant markers for segregation analysis. The markers (in either positive or negative selection) are considered to be undesirable once a transgenic plant is developed. Several strategies have been developed and adopted to keep the marker gene in containment (for review see Puchta 2003).

The diversification of transgenic resistance by backcross (BC) breeding approaches can be useful in managing RTD through the incorporation of the transgene into several high-yielding-commercial rice varieties. Efforts have been made in a multi-institutional project funded by Department of Biotechnology, Government of India, to incorporate the transgene, conditioning resistance to RTBV through RNAi, from transgenic Pusa Basmati 1 into two commercial high-yielding rice cultivars IET4094 and IET4786 through transgene-based marker assisted BC breeding (Roy *et al.* 2011). Repeated backcrosses have been performed and the progenies were evaluated for the presence of transgene and their reaction to artificial virus inoculation. During the regular breeding programme to incorporate the transgene, we observed that in some of the backcrossed plants (BC_2F_1 and BC_2F_2) the selectable marker gene is not detectable through

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PCR based screening for the transgene. In this paper we report our observation on these transgenic backcrossed plants for their probable marker-free condition.

Materials and methods

Plant materials and growth condition

The transgenic Pusa Basmati 1 line (PB1/RTBV-O-Ds2) expressing the RNAi construct involving DNA encoding *ORF-IV* (open reading frame IV) of RTBV in sense and anti-sense orientation was used as the source of resistance (Tyagi *et al*. 2008). Two commercial high-yielding rice cultivars, IET4094 and IET4786, of West Bengal, India were used as recipient of the transgene. The transgene cassette present in donor parent PB1/RTBV-O-Ds2 consisted of a RTBV DNA fragment between residues 5700 and 7026 encoding *ORF IV* of an Indian isolate, RTBV-AP, cloned in between 35S promoter and nopaline synthase transcription termination signals separately in sense as well as anti-sense orientation with a selectable marker hygromycin phosphotransferase (*hpt*). The details of the transgene construct were reported earlier by Tyagi *et al.* (2008). Transgenic PB-1/RTBV-O-Ds2 line was developed through mediated transformation by *Agrobacterium tumefaciens*, which resulted in integration of the same transgene at two different sites (double-copy) in the genome (Tyagi *et al*. 2008).

Seed sowing, planting and crossing were performed under containment poly-greenhouse. Seeds of the parents and crossed progenies were first germinated in plastic trays and the 30-day-old seedlings were transplanted into the pots filled with equal volume of clay loam soil and supplemented with need-based chemical fertilizers (N, P and K). The temperature of the poly-greenhouse was kept between 28 and 31°C and the photoperiod was maintained at 14/10 h light/dark using supplementary lighting.

Backcrossing

To transfer the transgene, backcross breeding method was followed. Two sets of crosses viz. IET4094 × PB-1/RTBV-O-Ds2 (set-I) and IET4786 × PB-1/RTBV-O-Ds2 (set-II) were made using transgenic plants as pollen parent. The F_1 seeds of both crosses were raised and seedlings were screened for the presence of transgene by polymerase chain reaction (PCR) analysis using gene-specific primers. Screening was conducted for both the *ORF-IV* and *hpt* coding regions within the construct. The transgene positive F_1 plants were used as pollen parents in the backcrossing with the female recurrent parents (IET4094 and IET4786). The BC_1F_1 plants were screened for the presence of transgenes at the seedling stage and positive plants were used as pollen parents in next backcross. Subsequent backcrosses were performed similarly.

DNA and RNA isolations

DNA was isolated from the test plants following the SDS-based micro-Prep method (Banerjee *et al*. 2009). Total RNA was isolated from 100 mg of leaf tissue using TRIZOL reagent according to the manufacturer's protocol (Bangalore Genei, Bangalore, India). cDNA was prepared from the total RNA according to the procedures for the first-strand cDNA synthesis (Fermentas Life Sciences, Bangalore, India).

PCR analysis of transgene

The DNA from the transgenic donor (PB1/RTBV-O-Ds2) plants and nontransgenic recurrent parents were used as positive and negative controls, respectively. PCR was performed in a thermocycler to detect both the gene of interest (*ORF-IV*) and *hpt* coding region within the construct employing following specific primers for RTBV *ORF-IV* (underlying the junction of sense *ORF-IV* and anti-sense *ORF-IV*) and *hpt*. O-Ds2 F: 5′-CAAGGCAGAAGGTGAAAGGC-3′ (positions 5968–5987 of RTBV-AP DNA), O-Ds2R: 5′-AAGAAGTATTTGCTCCTGGGG-3′, (positions 6832–6852 in RTBV-AP DNA), HPTF: 5′-GCCTGAACTC AACG-3′ and HPTR: 5′-CTCATCGAGAGCCTGCGCG-3′. The reaction mixture containing template (50 ng of genomic DNA), primers, buffer, dNTPs and *Taq* DNA polymerase, was subjected to initial denaturation (94°C) for 5 min; followed by 35 cycles of denaturation (94°C) for 30 s, annealing (52°C for *ORF-IV* and 64°C for *hpt*) for 30 s, elongation (72°C) for 1 min and final extension at 72°C for 7 min. The amplified PCR products were resolved in 1% TBE-agarose gel and the view was captured in a gel documentation system.

Expression analysis of hpt marker gene: RT-PCR and functional assay

RT-PCR analysis was carried out by using cDNA (∼5 μg), prepared from the total RNA isolated from leaf tissues. The primers for *hpt* and PCR conditions were same as mentioned in previous section. The amplified PCR products were resolved in 1% TBE-agarose gel.

The leaf assay for hygromycin sensitivity of the transgenic BC_2F_2 progenies was performed by the following procedure. Approximately 2–3 cm long leaf tissues were excised from 1-month-old transgenic and parental seedlings (control) and dipped in hygromycin B solution (200 μg mL^{-1}). The leaves were kept in Petri dishes under a 16/8 h light/dark cycle with a temperature of 25–27°C under laboratory condition. For the assessment of sensitivity of seedlings to hygromycin, rice seeds were surface sterilized with 1% mercuric chloride and was rinsed at least five times. After drying, the seeds were germinated in Petri dishes for five days, followed by transfer to another set of Petri dishes containing blotting papers soaked in hygromycin B solution (200 μg mL^{-1}) in sterile

Table 1. Plants carrying the *ORF-IV* and selectable marker gene (*hpt*) in different generations.

Generation	Plants assayed	Transgene-positive plants (*ORF IV* and *hpt*)	*hpt*-negative plants
F_1			
Set-I (IET4094 × PB1/RTBV-O-Ds2)	80	68	0
Set-II (IET4786× PB1/RTBV-O-Ds2)	100	84	0
BC_1F_1			
Set-I	120	55	0
Set-II	120	70	8
BC_2F_1			
Set-I	100	62	2
Set-II	100	54	5

distilled water. The Petri dishes were kept under same laboratory conditions as mentioned above. The sensitivity of rice leaves and seedlings were evaluated after seven days.

Results and discussion

Backcrossing and progeny analysis

A large number of seeds were produced from two sets of crosses i.e., set-I: IET4094 × PB1/RTBV-O-Ds2 and set-II: IET4786 × PB1/RTBV-O-Ds2. Out of 80 F_1 plants tested of set-I, 68 plants; and among 100 F_1 plants tested of set-II, 84 plants were found to be transgene positive (table 1). Five healthy transgene-positive F_1 plants were used as pollen parent for backcrossing (BC) with two recurrent parents (IET4094 and IET4786). A total of 120 BC_1F_1 seeds from each of the two sets of crosses were raised. Out of 120 plants, 55 and 70 plants were transgene positive for set-I and set-II, respectively. Interestingly, out of 70 transgene-positive BC_1F_1 plants from set-II, eight plants were found to be negative for the *hpt* marker gene in PCR analysis but showed the presence of amplicon for *ORF-IV*. In BC_2F_1 generation also, 2 and 5 plants from set-I and set-II, respectively were

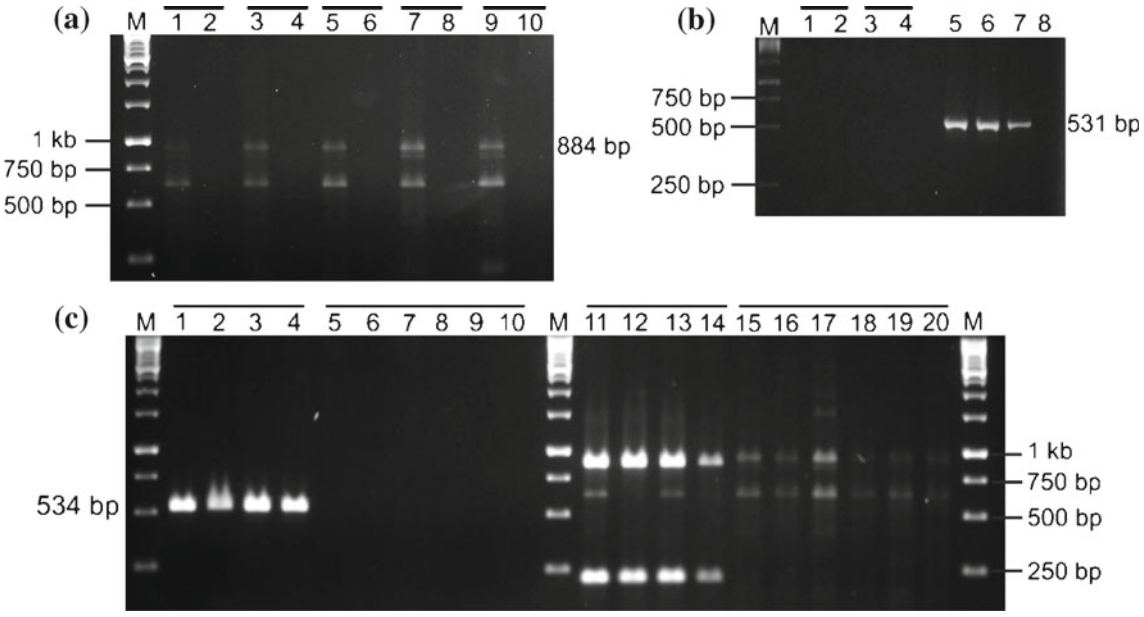

Figure 1. PCR analysis on *hpt*-negative backcross progenies. (a) BC_2F_1 progenies showing the presence of amplicon specific for *ORF-IV* (883 bp) but not that of *hpt*. lanes 1–6 BC_2F_1 progenies from set-I, lanes 7–10 BC_2F_1 progenies from set-II. Each bar over two adjacent lanes represents a single plant, first lane for *ORF-IV* and second for *hpt*. (b) Expression analysis of *hpt* marker gene by RT-PCR. M, molecular weight marker; lanes 1, 2 and 3, 4 *hpt*-negative BC_2F_1 plants from set-I and set-II, respectively; lanes 5–6 *hpt*-negative BC_2F_1 plants from set-I and set-II; lane 7 transgenic donor parent (PB1/RTBV-O-Ds2); lane 8 is blank. (c) PCR amplification using primers for *hpt* (lanes 1–10) and *ORF-IV* (lanes 11–20) in BC_2F_2 progenies. Lanes 1–4 and 11–14 represent four *hpt*-positive BC_2F_2 progenies whereas lanes 5–10 and 15–20 represent six *hpt*-negative BC_2F_2 progenies. The amplification product using *hpt* primers is 531 bp and that of *ORF-IV* is 883 bp, indicated at the sides.

found to be negative for amplicon specific to *hpt* marker gene. In each generation, the plants were screened for the transgene by PCR, which amplified an 883-bp fragment specific to *ORF-IV* and a 531-bp fragment for *hpt* coding region (figure 1).

In a previous study, we assessed the RTD resistance in BC_2F_1 generation by inoculating test plants with the rice tungro viruses through GLH-mediated inoculation by taking TN1, an RTD-susceptible rice cultivar, as a susceptible control. The development of symptoms in the recurrent parents were typical of RTD, whereas those in the PB1/RTBVO-Ds2 and BC_2F_1 plants were very mild. Observations on plant height and leaf yellowing in the inoculated plants indicated that inoculation with rice tungro viruses resulted in the reduction in height of parental plants as well as of the BC_2F_1 plants. The reduction in plant height and leaf yellowing were most prominent in the case of nontransgenic parental lines and TN1 (Roy *et al*. 2011). The significant amelioration of tungro symptoms in the BC_2F_1 progenies strongly indicates that RTBV resistance has been transferred from the transgenic donor plant.

RT-PCR assay of probable marker-free BC_2 progenies

We have performed repeated PCRs on the genomic DNA to check the consistency of our results. The PCR analysis on

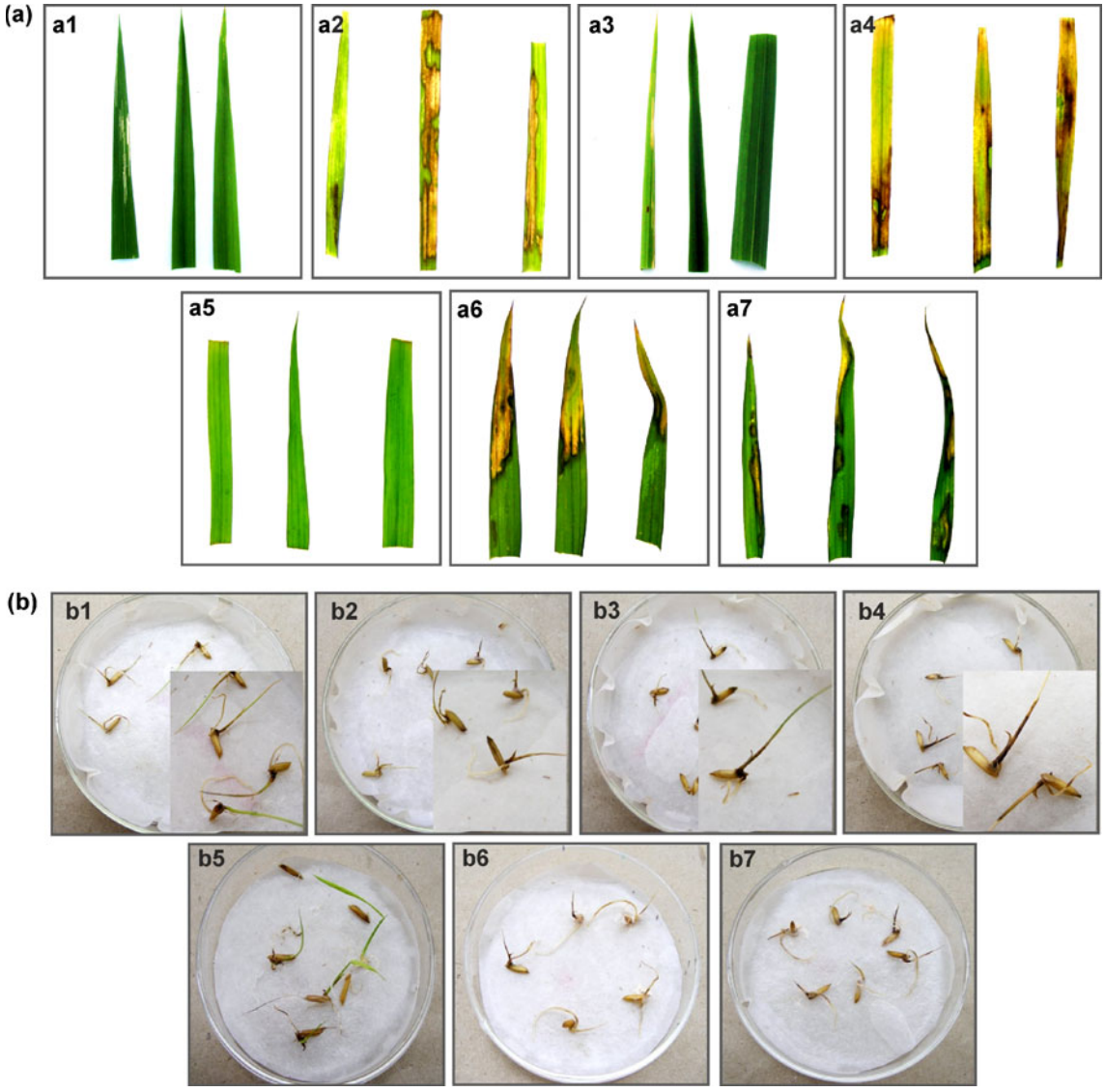

Figure 2. (a) Test for hygromycin sensitivity of the leaves and (b) seedlings in BC_2F_2 generation. a1–a2 And b1–b2 *hpt*-positive and *hpt*-negative progenies of IET4094, respectively. a3–a4 And b3–b4 *hpt*-positive and *hpt*-negative progenies of IET4786, respectively. a5–b5, a6–b6 And a7–b7 donor parent, IET4094 and IET4786, respectively.

these plants resulted in the amplification of specific amplicon (883 bp) for *ORF-IV* but nil for *hpt* (figure 1a). These BC_2F_1 progenies were marked and evaluated further for the expression of the gene at mRNA level by RT-PCR. The RT-PCR analysis indicated the absence of expected 531-bp amplicon specific to marker gene in these *hpt* negative plants (figure 1b, lanes 1–4), whereas, the *hpt* specific amplicon (531 bp) was observed in the marker-positive BC_2F_1 plants (figure 1b, lanes 5 & 6) and PB1/RTBV-O-Ds2 (figure 1b, lane 7). The selected BC_2F_1 plants were allowed for one cycle of selfing and the resultant BC_2F_2 progenies were again checked for the absence of marker gene. In all the BC_2F_2 progenies that obtained from the *hpt*-negative BC_2F_1 plants, the PCR analysis of leaf DNA resulted in amplification of 883 bp product specific to *ORF-IV* region (figure 1c). Though, in all these cases a faster-migrating band was also noted in addition to 883-bp amplicon for *ORF-IV*, this might be due to double copy of the transgene in the donor parent. Specific amplicons for *hpt* and *ORF-IV* were found in all the progenies of transgene-positive BC_2F_1 lines (figure 1c, lanes 1–4 and 11–14).

Functional assay

The leaf assay for sensitivity to hygromycin showed contrasting responses in *hpt*-positive and *hpt*-negative plants. The leaf tips of transgenic donor parent (PB1/RTBV-O-Ds2) and *hpt*-positive BC_2F_2 plants remained green or very less affected (no bleaching or necrosis) when exposed to hygromycin solution, showing the resistance capacity of the plants to hygromycin (figure 2a). Whereas, the leaves of *hpt*-negative transgenic BC_2F_2 progenies showed necrosis and brown patches similar to that of nontransgenic parents (figure 2a). The seedling mortality assay also produced similar results. The seedlings of nontransgenic control plants and *hpt*-negative BC_2F_2 plants became bleached and died at the seventh days after treatment unlike the transgenic parental and the *hpt*-positive BC_2F_2 seedlings that remained green and produced leaves (figure 2b). Similar trends were recorded in progenies for both recurrent parents.

The previous report of Tyagi *et al*. (2008) indicated that the transgene was integrated in two different position of PB1/RTBV-O-Ds2. In this double-copy insertion, if the marker gene (*hpt*) was failed to be inserted in one copy out of the two, the chance of getting marker-free plants in advanced backcross progenies is more. The segregation patterns of *ORF-IV* and *hpt* genes in the backcross generations showed that in some progenies the marker gene might have been lost during crossbreeding.

In this present study, RT-PCR approach takes advantage of the detection of mRNA transcript of the specific genes. It is essential for a gene to form the mRNA copy and then protein specifying its expression. So, for the detection of expression of any gene its transcription can be assayed by PCR amplification by using cDNA prepared from the mRNA copies of the gene (Erlich *et al*. 1991). The RT-PCR amplification indicated the transcription *hpt* gene in *hpt*-positive plants but no amplification was observed in case of *hpt*-negative plants (figure 1b). The amplicon for *ORF-IV* in BC_2F_1 plants and PB1/RTBV-O-Ds2 was not observed in RT-PCR analysis (data not shown). This might be resulted due to cleavage of *ORF-IV* transcripts by dicer protein of the host as an outcome of RNAi mechanism. An in-depth investigation on these probable marker free transgenic progenies could confirm the true nature of these plants, which would be extremely valuable before any future release of these lines for cultivation, keeping in mind the current regulations on the release of genetically modified crops in various countries.

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