.

Genetic analysis of Tolerance to Rice Tungro Bacilliform Virus in Rice (*Oryza sativa* L.) through Agroinoculation

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Abstract

Balimau Putih [an Indonesian variety tolerant to rice tungro bacilliform virus (RTBV)] was crossed with IR64 (RTBV, susceptible variety) to produce three filial generations F_1, F_2 and F_3. Agroinoculation was used to introduce RTBV to the test plants. RTBV tolerance was based on RTBV level in plants by analysis of coat protein using enzyme-linked immunosorbent assay. The level of RTBV in cv. Balimau Putih was significantly lower than that of IR64 and the susceptible check, Taichung Native 1. Mean RTBV levels of the F_1, F_2, and F_3 populations were comparable with one another and with the average of the parents. Results indicate that there was no dominance and an additive gene action may control the expression of tolerance to RTBV. Tolerance based on the level of RTBV coat protein was highly heritable (0.67) as estimated using the means of F_3 lines, suggesting that selection for tolerance to RTBV can be performed in the early selfing generations using the technique employed in this study. The RTBV level had a negative correlation with plant height, but positive relationship with disease index value

Introduction

Rice tungro is one of the most economically important viral diseases of rice in South and Southeast Asia. It is a complex disease caused by two viruses, ' rice tungro bacilliform virus' (RTBV) and 'rice tungro spherical virus' (RTSV) (Hibino et al.,1978). For their transmission, the viruses depend on green leafhoppers (GLH), mainly *Nephotettix vi rescens* (Distant). In nature, RTSV can be transmitted independently by the vector, but RTBV transmission always depends on the presence of RTSV (Cabauatan et al., 1988). Tungro symptoms, such as yellowing and reddening (or yellow to yellow-orange discoloration) and stunting, are mainly caused by RTBV, while RTSV causes mild symptoms but is essential for vector transmission (Hull, 1996). The symptoms are accentuated when both RTBV and RTSV are present in the plant. Consequently screening for RTBV resistance has been complicated due to the required presence of RTSV and vector. Conventional screening using the vector transmission technique is laborious and is known to be less efficient as some plants may escape virus infection (Shahjahan et al., 1991) and due to the difficulty to quantify and control the uniformity and the concentration of virus inoculum delivered to each plant. Agroinoculation technique that delivers RTBV alone into the rice plant via *Agrobacterium tumefaciens* is a breakthrough in tungro research (Dasgupta et al., 1991). Agroinoculation is fast and efficient technique for determining tungro virus resistance as it eliminates the use of insect vector. This technique has been used to introduce RTBV into rice plant and produced typical RTBV symptoms (Dasgupta et al., 1991, Sta Cruz et al., 1999).

At present, there are few RTBV-tolerant varieties. One that has shown consistent tolerance to RTBV is the Indonesian rice cultivar, Balimau Putih (Daquiog et al., 1986; Hasanuddin et al., 1988). Line IR68305-18-1, which was derived from this tolerant variety, has been tested on multi-location trials in the Philippines. In all these trials, the line has shown tolerance to RTBV comparable with Balimau Putih (Cabunagan et al., 1999). However, there is as yet limited information on the genetics of tolerance to RTBV in

Balimau Putih, particularly its inheritance. This study therefore, was conducted to understand the genetics of RTBV tolerance in order to contribute to integrated tungro management through fast and efficient identification of sources of RTBV resistance that could be combined with the available RTSV resistant varieties.

Materials and Methods

Analysis of RTBV DNA by Southern blot hybridization

Southern blot hybridization was done to monitor and compare RTBV DNA profiles at various times post agroinoculation in both susceptible and tolerant hosts. Seedlings from Balimau Putih and TN1 were infected with RTBV (B) using agroinoculation technique. Samples were collected from the youngest and fully expanded leaves at 5, 7, 14, 21 and 28 days post inoculation (dpi). Total DNA isolation, digestion, hybridization and detection were done following methods described by Cabauatan et al., (1998). Approximately 5 ug of total DNA was run using 1.0% agarose in 1X TBE by gel electrophoresis for 5 hr. Hybridization was performed using DNA probe consisting of a full length cloned RTBV DNA. Hybridized bands were detected via ECL chemiluminescent detection system (Amersham).

For genomic profiling of RTBV with various types of inocula, total DNA extracts from plants infected with either RTBV + RTSV (BS) or B alone (infection with B alone was done using agroinoculation whereas infection with BS was done by agroinoculating B first then insect inoculating with S). The total DNA from infected and uninoculated control plants were digested with 30 units *Eco*RV restriction enzyme at 37 ^{o} C for overnight. Southern blot hybridization analysis of the digested DNA followed similar procedure described above.

Sources of test plants

The F_1 and F_2 progenies from cv. Balimau Putih (RTBV tolerant variety) and IR64 (RTBV susceptible variety) were generated using the rapid generation advance facility at the International Rice Research Institute (IRRI), while the F_3 seeds were kindly provided by Tokio Imbe, formerly of the Plant Breeding, Genetics and Biochemistry Division in IRRI. TN1, which is susceptible to RTBV, RTSV and GLH, was used as a susceptible control. The experimental design for all experiments was completely randomized design. Each filial generation was evaluated in separate experiments.

Thirty F_1 seeds in single experiment, 250 F_2 seeds in two separate experiments, and 80 F_3 lines in 20 temporally separate experiments, were tested along with the parents and TN1. Each set of experiment for F_3 lines consisted of four lines (40 plants per line) taken at random. All seeds were pre-germinated and grown in pots with each pot containing five plants. Eight pots per entry were used in each set of experiment. Seedlings in six of the eight pots were agroinoculated with RTBV, while the remaining two pots were left un-inoculated to serve as the healthy control.

Agroinoculation of test plants

The agroinoculation technique was employed for routine production of RTBV- infected plants. All the experiments were conducted at IRRI Containment Level 4 (CL4). The inoculum was prepared from the RTBV infectious clone pRTRB1162 in *Agrobacterium* strain GV3850 (Dasgupta et al., 1991; Sta Cruz et al., 1999). About 100-200 µl of the bacterial inoculum carrying RTBV was injected at the crown of each two-week-old seedling using a 1-ml syringe.

Evaluation of RTBV coat protein level

From each inoculated plant and the healthy control, leaf samples were collected at 14 days post inoculation. All samples were tested for the presence of RTBV coat protein using enzyme linked immunosorbent assay (ELISA) (Bajet et al., 1985). The cutoff point for positive reaction in the ELISA test was computed using the formula: $X+ f * S.D.$ (Frey et al., 1998), where X is the average absorbance value of 10 healthy plants, f is the value of standard deviation multiplier for the 10 healthy plants, and S.D. is the standard deviation of the healthy plants. The f is multiplier of the S.D. and $f= t \sqrt{(1+ (1/n))}$ (Frey et al., 1998), where t is value for one tailed t-distribution and n is number of healthy plants used as a control in the experiment. Average ELISA value and their standard error are used in all the statistical analysis.

Data from two batches of F_2 generation were analyzed as a single trial after confirming the homogeneity of the data across batches for the parents using Bartlett's test (Snedecor and Cochran, 1989). Similarly, the F_3 population was analyzed as a single trial based on Bartlett's test for homogeneity in parents among the 20 batches.

Correlation of plant height and level of RTBV coat protein

Fourteen days after inoculation and just before leaf sampling for ELISA, the height of individual plants was measured to the longest tip and the symptoms were visually scored based on the standard evaluation system for rice (SES) (INGER, 1996). Average height reduction and percentage of infected plants for each entry was estimated as $((H_h - H_i)/ H_h) *100$ where H_h is mean height of healthy plants, and H_i is height of infected plant.

After conducting ELISA on every leaf sample, the ELISA value as indicator of the level of RTBV coat protein accumulation was tested for its correlation with the plant height data.

Disease Index

Disease index (DI) value for the genotype, which represents both disease incidence and symptom

severity, was computed as follows from the symptom severity score data as an indicator of virus resistance based on SES (INGER, 1996).

$$DI = \frac{n(3) + n(5) + n(7) + n(9)}{tn}$$

where: n(3), n(5), n(7), and n(9) = number of plants showing a reaction on scale of 3, 5, 7, 9 and tn = total number of plants.

The resulting DI values are classified according to SES (INGER, 1996):where 0-3 is resistant or tolerant, 4-6 is moderate and 7-9 is susceptible.

Heritability analysis

The analysis of variance for the F_3 population is given in Table 3. The heritability (H) of RTBV tolerance was estimated as: $H = \delta^2_g / (\delta^2_g + \delta^2_{ge}/N + \delta^2_e/ NS)$ (Fehr, 1998, Halluar and Miranda 1981), in this study it is presented as: $H = \delta^2_L / (\delta^2_L + \delta^2_{PwL}/N + \delta^2_{Pwp} / NS)$ Where δ^2_g (δ^2_L) is genetic variance among lines, δ^2_{ge} (δ^2_{PwL}) is genetic by environment variance which is variance among pots within line, and δ^2_e (δ^2_{Pwp}) is environmental variance which is variance among plants within pot. In this experiment N is the number of plants in a pot, while S is the number of pots per entry.

Results

Analysis of RTBV DNA by Southern blot hybridization

RTBV DNA profile was consistent, between hosts during the initial infection cycle (up to 28 days) and there was no genomic DNA detected from un-inoculated "healthy" plants by Southern blot hybridization (Fig. 1).

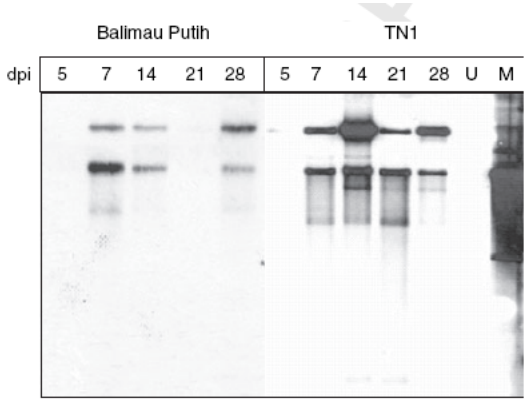

Fig. 1 Comparison of rice tungro bacilliform virus (RTBV) DNA profiles in the tungro-susceptible variety, Taichung Native 1, and the RTBV-tolerant variety, Balimau Putih at 5, 7, 14, 21, and 28 days postagroinoculation and as analysed by Southern blot hybridization. Approximately 5 ug of total DNA were loaded per lane and the linear-ized full length RTBV-Ic plasmid was used as a probe. U, total DNA from an uninoculated plant; M, marker (linearized full-length RTBV-Ic clone combined with an identical genome that was EcoRV restricted)

To verify the stability and consistency of this genomic profile with various types of inocula, total DNA extracts from plants infected with either RTBV + RTSV (BS) or RTBV alone (B) were digested with *Eco*RV and compared. Results show that RTBV-EcoRV- pattern was maintained in both BS- infected plants and B-infected plants in both the susceptible and tolerant hosts and there were no genomic DNA detected from the uninoculated "healthy" plants (Fig. 2).

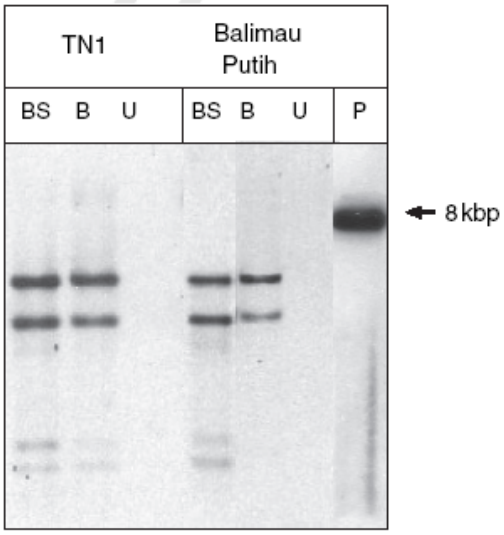

Fig. 2 Patterns of restricted rice tungro bacilliform virus (RTBV) DNA from total DNA extracts that originated from plants infected with either RTBV + rice tungro spherical virus (BS), RTBV (B) or un-inoculated (U) of Taichung Native 1 and Balimau Putih varieties at 14 days post-agroinoculation. Total DNA (5–10 ug) were digested with EcoRV restriction enzyme, run in 1% agarose gel, transferred into a nylon membrane, and probed with a linearized full-length RTBV DNA probe. P, RTBV linearized genome devoid of the plasmid (8 kbp)

Level of RTBV coat protein in F_1 progenies and the parents

The frequency distribution curves for the level of RTBV coat protein of the parents that are obtained from ELISA data are shown in Fig. 3. Balimau Putih had a significantly lower level of RTBV coat protein (1.064 ± 0.299) than IR64 (2.4±0.125) and the susceptible check TN1 (2.5± 0.454). The level of RTBV for F_1 progenies was continuous with average value 1.640± 0.229 (Figure 4), indicating the quantitative nature of the trait. The average RTBV level of the F_1 progenies was significantly lower than that of IR64 and significantly higher than that of Balimau Putih, however, it was not significantly different from the average of the two parents (1.731).

Plant height and disease index in F_1 progenies

As shown on Table 1, level of RTBV coat protein and plant height in all entries of this experiment was negatively correlated for both parents and F_1 progenies. The average percent height reduction of the F_1 progenies was moderate compared with those of IR64 and TN1, however, it was higher than that of Balimau

Putih. The height reduction was highly significant for all entries.

Based on disease index (DI) value, Balimau Putih and IR64 were classified as moderately tolerant and susceptible varieties, respectively, while the F_1 generation fell under intermediate category (Table 1).

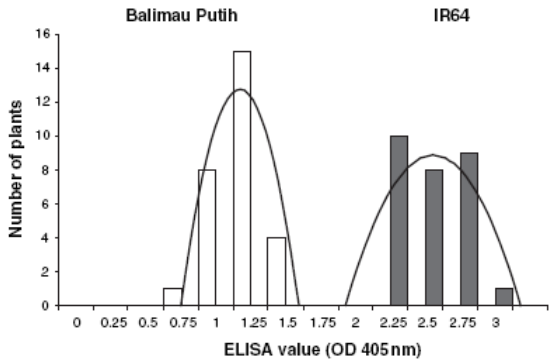

Fig. 3 Frequency distribution of rice tungro bacilliform virus coat protein level based on enzyme-linked immunosorbent assay value with fitted normal curve for parents: Balimau Putih and IR64

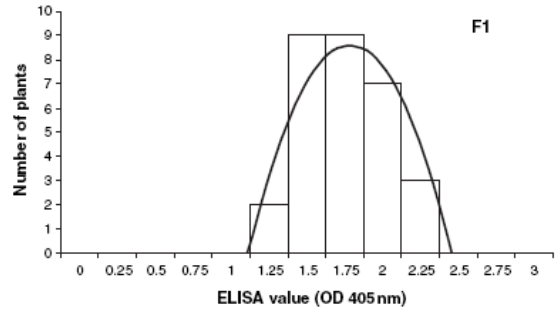

Fig. 4 Frequency distribution of rice tungro bacilliform virus coat protein level based on enzyme-linked immunosorbent assay value with fitted normal curve for F_1 progenies

Table 1
Correlation, average per cent height reduction and disease index values in the F_1 progenies

Test material	Correlation[a]	Average height reduction (%)	Disease index
Balimau Putih	−0.65*	8.3*	3.7
F_1	−0.74*	11.2*	4.3
IR64	−0.84*	21.9*	6.7
TN1	−0.79*	25.9*	6.9

[a]Correlation between the level of rice tungro bacilliform virus protein and height of the infected plants.
*Highly significant at 1%.

Level of RTBV coat protein in F_2 progenies

The F_2 population showed an approximately normal distribution for the level of RTBV coat protein (Fig. 5), which is of a typical polygenic trait. The mean level RTBV coat protein for F_2 progenies (1.793±0.119) was not significantly different from that of the mean value of the parents (1.802) and the F_1 progenies (1.64 ± 0.229). However, it was significantly different from the mean level of Balimau Putih (1.28± 0.123), IR64 (2.264± 0.115) and TN1 (2.44±0.364).

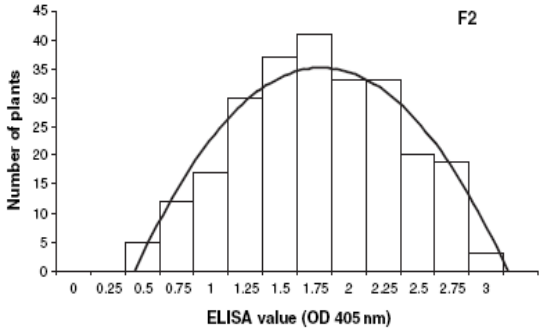

Fig. 5. Frequency distribution of rice tungro bacilliform virus coat protein level based on enzyme-linked immunosorbent assay value with fitted normal curve for F_2 progenies

Plant height and disease index in F_2 progenies

Table 2 shows that level of RTBV coat protein and height were negatively correlated. In the RTBV-infected plants height reduction was significant for F_2 plants and the parents. However, mean plant height reduction in the F_2 population was lower than Balimau Putih but higher than IR64. When assessed with DI value F2 progenies had an intermediate level of tolerance to RTBV.

Level of RTBV coat protein in F_3 lines

The frequency distribution of level of RTBV coat protein in the F_3 population showed a slight skewness toward the susceptible parent IR64 (Fig. 6). Nevertheless, the shape of the curve approximated a normal distribution for a typical quantitative trait. The mean level of RTBV for F_3 lines (1.849 ± 0.105) was significantly different from the mean level of Balimau Putih (1.176± 0.125), and IR64 (2.347± 0.145) and the mean value of the parents (1.765) (Fig. 4). However, the F_3 value was only 5% higher than that of the average of the two parents; as a result the mean level of RTBV coat protein for F_3 lines did not significantly differ from that of the F_1 and F_2 populations. Consequently, the characteristic intermediate reaction of the F_1 population relative to the parents remained the same throughout F_2 to F_3 generations which strongly suggest an additive type of gene action.

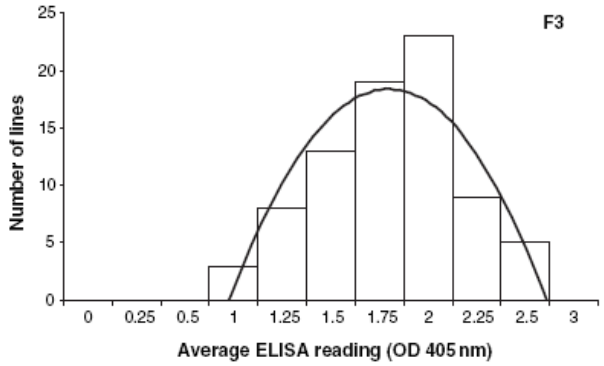

Fig. 6. Frequency distribution of rice tungro bacilliform virus coat protein level based on enzyme-linked immunosorbent assay value with fitted normal curve in F_3 lines.

Table 2
Correlation, average per cent height reduction, and disease index values in the F_2 generation

Test material	Correlation[a]	Average height reduction (%)	Disease index
Balimau Putih	−0.55*	13.6*	4.4
F_2	−0.88*	18.4*	5.3
IR64	−0.89*	20.1*	7.0
TN1	−0.86*	24.8*	7.1

[a]Correlation between the level of rice tungro bacilliform virus protein and height of the infected plants.
*Highly significant at 1%.

Plant height and disease index in F_3 lines

As in the previous two experiments, the correlation between plant height and level of RTBV coat protein for all entries was negative (Table 3). The average percent height reduction in the F_3 population was higher than Balimau Putih but lower than IR64 or TN1. However, the reduction was statistically significant for all entries in the experiment. The DI for the F_3 population was higher than those of the F_1 and F_2 populations but still it fell within the intermediate group (Table 3).

Table 3
Correlation, average per cent height reduction, and disease index values for all entries in the F_3 generation

Test materials	Correlation[a]	Average height reduction (%)	Disease index
Balimau Putih	−0.77	9.7*	4.0
F_3	−0.98	16.8*	5.7
IR64	−0.61	24.2*	7.2
TN1	−0.55	20.7*	7.3

[a]Correlation between the level of rice tungro bacilliform virus protein and height of the infected plants.
*Highly significant at 1%.

Heritability

Table 4 shows the ANOVA for the level of RTBV in F_3 population and their parents. The variability of the F_3 population was partitioned into variation among and variation within F_3 lines and highly significant differences were observed among the F_3 lines.

The pooled variation within the F_3 lines did not differ significantly from the pooled mean square of the parents. Out of the 80 F_3 lines there were three lines, which had an average level of RTBV coat protein comparable with that of Balimau Putih; 63 lines with average value between IR64. The heritability estimate based on entry mean values analysis for F3 lines (Table 4) was computed to be 0.67.

Table 4
Analysis of variance (ANOVA) for parents and F_3 population

Sources of variation	Degrees of freedom (df)	Mean squares (MS)	Expected MS	Observed df	Observed MS
Among treatments	$T-1$				
Among parents	$P-1$			1	354.283*
Among F_3 lines	$L-1$	MSA_L	$\delta^2_{Pwp} + N\delta^2_{pwL} + NS\delta^2_L$	79	1.056*
Parents vs. F_3 lines	1			1	7.617*
Among pots/treatments	$T(S-1)$				
Among parents	$P(S-1)$			238	
Among F_3 lines	$L(S-1)$	MSB_L	$\delta^2_{Pwp} + N\delta^2_{pwL}$	400	0.334
Among plants pots/lines	$TS(N-1)$				
Within parents	$PS(N-1)$	MSC_P		804	
Within F_3 lines	$LS(N-1)$	MSC_L	δ^2_{Pwp}	1845	0.260
Total	$TSN-1$			3368	

T, treatments; P, parents; L, lines; S, number of pots within each treatment; N, number of plants within a pot; MSAL, lines mean of squares; MSBL, among pots within lines mean of squares; MSC, among plants within pot within treatment mean of squares; δ^2 L, variance among lines; δ^2 pwL, variance among pots within lines; δ^2Pwp, variance among plants.

Discussion

During the conduct of this study, the presence of RTBV-like sequences integrated into the rice genome was reported (Nagano et al., 2000). Such finding and the recent article of Kunii et al. (2004) raise the possibility for a potential interaction between RTBV-like sequences that may be present in IR 64 and/or Balimau Putih and the RTBV inoculum that was introduced by agroinoculation. However, our preliminary experiments do not confirm such a possibility. Prior to conducting this study, we monitored and compared RTBV DNA profiles at various times post agroinoculation in both susceptible and tolerant hosts. These results suggest that the interaction did not occur or if it occurred, it was not detected by the Southern blot hybridization method that we used. Moreover, our previous work (Sta Cruz et al., 2003) showed that the trend of RTBV accumulation in agroinoculated Balimau Putih or TN1 was consistent with that detected in insect inoculated plants suggesting that there were no detectable interaction.

The RTBV level based on coat protein analysis of tolerant parent Balimau Putih was always significantly lower than that of susceptible parent IR64 in the three experiments. In general, the average RTBV levels of the F_1, F_2, and F_3 populations were comparable with one another and intermediate to the parents. This indicates that no gene dominance, but rather an additive gene action may control the expression of tolerance to RTBV. The results from this study suggest that multiple genes govern RTBV tolerance. This polygenic type of RTBV resistance was also observed on other varieties (Shahjahan et al., 1990).

Comparing the RTBV level of the 80 F_3 lines with that of the parents, around 96% of the F_3 lines had significantly higher RTBV level than that of Balimau Putih and 80% had significantly lower RTBV level than that of IR64. It is likely that the number of F_3 lines evaluated was not enough to include all ranges of phenotypes observed in F_2. However, around 4% of the F_3 lines had comparable RTBV levels with Balimau Putih. Furthermore the tolerant trait was found to be highly heritable (0.67) indicating that it is not difficult to obtain improved lines with tolerance to RTBV.

All experiments had consistently shown a highly significant negative correlation between RTBV level and plant height. Height reduction in IR64 and TN1 was nearly twice than that in Balimau Putih. The mean height reduction of each filial generation studied was close to the average height reduction of the parents. In general high level of RTBV coat protein accumulation correlated with significant height reduction in all experiments, which is a typical symptom of RTBV. The intermediate value of disease index for the three filial generations conformed with the observed intermediate value of RTBV coat protein accumulation and moderate height reduction.

Therefore, this study further confirms that tolerance to RTBV in Balimau Putih was quantitatively inherited. Since the trait was highly heritable, like plant height and growth duration, selection for RTBV tolerance can be done at early

selfing generations. Thus plant breeders who develop new lines with resistance to RTBV can improve their selection efficiency by employing the agroinoculation technique and screen for tolerant plants to RTBV in the early selfing generations.

This is the first time the agroinoculation was used as a tool to study the genetics of inheritance to tungro resistance. In this study agroinoculation was shown to be very useful in selecting virus resistant or tolerant cultivars in as early as F_3 population. This quick and efficient screening technique when combined with monitoring of the RTBV DNA profile, facilitates identification of more sources of RTBV resistance that could be combined with the currently available RTSV resistant genes in an integrated tungro resistance breeding programs to keep rice breeders one step ahead of the ever evolving viral pathogen. It will be more interesting to see similar approach for efficient screening of RTSV resistant varieties.

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