Detection of Rice Tungro Bacilliform Virus by Polymerase Chain Reaction for Assessing Mild Infection of Plants and Viruliferous Vector Leafhoppers

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ABSTRACT

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The polymerase chain reaction (PCR) was 10^3–10^4 times more sensitive for detecting rice tungro bacilliform virus (RTBV) DNA extracted from infected rice plants than was enzyme-linked immunosorbent assay (ELISA). The greater sensitivity of PCR enabled the detection of the virus in individual RTBV-exposed leafhoppers, *Nephotettix virescens*, a semipersistent vector of RTBV. Detection of RTBV in the vector by ELISA has been impossible. To evaluate resistance to RTBV, seedlings of rice cultivars Utri Merah, Utri Rajapan, or Balimau Putih, were inoculated with RTBV, and infection of the cultivars with the virus was indexed by PCR and ELISA. When inoculated seedlings with no clear symptoms were tested by ELISA, only some seedlings of the three cultivars produced positive reactions, and their ELISA values were generally low. When DNA extracted from the same leaf samples was amplified by PCR, RTBV DNA was detected in all plants that reacted in ELISA. In addition, among the plants that produced ELISA values lower than 0.05, a value twice the average of the uninfected controls and considered noninfected with RTBV, 19% of Utri Merah, 4.2% of Utri Rajapan, and 60% of Balimau Putih plants were positive according to PCR. These results indicate that ELISA failed to detect RTBV in many infected rice plants with tolerance to the virus. The results clearly demonstrate the effectiveness of PCR as a method for evaluating rice cultivars for tolerance or resistance to RTBV.

Additional keywords: diagnosis, evaluation of resistance and tolerance.

Tungro is the most serious virus disease of rice (*Oryza sativa* L.) and the major constraint to stable rice production in South and Southeast Asia. It is a composite disease caused by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (12). Both RTBV and RTSV are transmitted in a semipersistent manner by the rice green leafhopper, *Nephotettix virescens* (Dist.) and by other leafhopper species (4,8). RTBV contains a circular double-stranded (ds) DNA (7) with 8,002 base pairs (registered in DNA Data Bank of Japan, Nucleotide Sequence Data Base, no. D01149) (10), and RTSV contains single-stranded RNA. Sensitive serological methods have been developed to detect the viruses in plant extracts (16). However, none of the methods detected the viruses in vector leafhoppers previously exposed to the viruses (Y. Takahashi, *unpublished data*).

Cultivar resistance to tungro is an important breeding objective for rice improvement in South and Southeast Asia (6). Rice cultivars with resistance to RTBV and/or RTSV have been identified through indexing by enzyme-linked immunosorbent assay (ELISA) and by evaluating symptom severity (6). In some cultivars, RTBV-infected plants show very mild or no clear symptoms and have less grain-yield reduction (3). In some of the cultivars with tolerance to RTBV, virus concentration in infected tissues is low (3), and this makes indexing by ELISA less reliable. A more sensitive detection method is required to make indexing of inoculated plants reliable, especially when evaluating rice cultivars for resistance to RTBV.

The polymerase chain reaction (PCR) method has been developed for the in vitro amplification of nucleic acid sequences (14,18). The method is highly sensitive and capable of detecting even a single copy of a DNA molecule (18). Recent progress in nucleotide sequence analysis on RTBV (10) made possible the application of the PCR technique for detecting small amounts of RTBV in vector insects and in rice plants with tolerance to the virus.

MATERIALS AND METHODS

Virus, plant, and insect. The original tungro disease source was collected in the Philippines and has been maintained in Japan since 1977 on rice cultivar Taichung Native 1 (TN1), with successive transfers with the leafhopper *N. virescens*. Viruliferous leafhoppers were obtained by allowing *N. virescens* adults to feed for 2 days on RTBV plus RTSV infected TN1 plants. Seedlings of rice cultivars TN1, Utri Merah (International Rice Germplasm Center, no. 16680), Utri Rajapan (no. 16684), and Balimau Putih (no. 17204) at the three-leaf stage were inoculated by confining them for 6 h in test tubes with two or three viruliferous leafhoppers per seedling. Inoculated seedlings were grown in an air-conditioned greenhouse (27 ±3 C). One month after inoculation, the second youngest leaf was harvested from each inoculated plant, a 10-cm-long portion was cut from the leaf to test in ELISA, and the rest of the leaf was used in PCR. A total of 60 plants for Utri Merah, 31 for Utri Rajapan, and 25 for Balimau Putih were inoculated in two trials, and a total of 16 TN1 plants were inoculated as the control. Leaf samples from inoculated plants were stored at −20 C. Viruliferous insects also were stored at −20 C prior to use in PCR. Uninoculated leaf samples and leafhoppers unexposed to RTBV also were stored at −20 C and used as the negative controls in ELISA and PCR.

DNA extraction from rice leaf tissues. The three methods described below were compared for extracting RTBV DNA from a portion (2 cm long) of frozen, infected TN1 leaves. Approximately 20 mg of leaf tissue was used for the test.

Method A followed the procedure of Kaye et al (11). Leaf tissue was crushed in 200 μl of extraction buffer 1 (10 mM Tris-HCl, pH 8.3; 10 mM sodium ethylene diamine tetraacetate (EDTA); 10 mM NaCl; 0.5% sodium dodecyl sulfate (SDS); 1 mg of proteinase K per milliliter). Extracted DNA was dissolved in 50 μl of water and used in PCR.

Method B followed the procedure of Soler et al (15). Leaf tissue was crushed in 200 μl of extraction buffer 2 (100 mM Tris-HCl, pH 8.3; 1 mM EDTA; 0.5% SDS; 1 mg of proteinase K per milliliter). Extracted DNA was dissolved in 50 μl of water and used in PCR.

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TABLE 1. Primer used in polymerase chain reaction

Primer	Localization[a]	Sequence[b]	Product size (bp)
P1	655-678 (sense)	5′-ATCAAGCTACATGAGCGCTGATTA-3′	269
P2	899-923 (antisense)	3′-CATCAAGTCGTCCTCGATATCATG-5′	
P3	6,519-6,542 (antisense)	5′-TTACAGAAGGATTGTGAACCCTTT-3′	633
P4	7,128-7,151 (antisense)	3′-CGAACCGGTCTTGTACTACCTCTG-5′	

[a] Based on the DNA sequence data of rice tungro bacillioform virus in DNA Data Base of Japan (no. D01149).
[b] Twenty-four-mer sequences.

Method C followed the procedure of Higuchi (9). The extract was used directly in PCR.

DNA extraction from vector leafhoppers. Individual viruliferous leafhoppers were crushed in 200 μl of extraction buffer 1 or 2, and DNA was extracted by method A or B. Extracted DNA was resuspended in 20 μl of water.

PCR amplification. Four primers were identified in the nucleotide sequence of RTBV DNA (DNA Data Bank of Japan) and were synthesized with an automatic DNA synthesizer (Model 394, Applied Biosystems, Inc., Foster City, CA) (Table 1).

Five to 20 μl of DNA extracts from rice leaves or individual leafhoppers was combined to produce a final volume of 100 μl, with the reaction mixture containing 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 0.01% gelatin; 200 μM of each deoxynucleotide; 1 μl each of four primers (A_{260nm} = 10); and 2.5 U of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer/Cetus, Norwalk, CT). The mixture was overlaid with mineral oil to prevent evaporation and was subjected to 30–50 cycles of amplification in a DNA thermal cycler (Perkin-Elmer/Cetus). A cycle included denaturation for 30 s at 94 C, primer annealing for 1 min at 55 C, and primer extension for 2 min at 72 C. To detect virus DNA in individual leafhoppers, 10 μl of the PCR products were further amplified in a second PCR. Furthermore, 10 μl of the second PCR products were amplified in a third PCR to confirm the results of the second PCR amplification. Five microliters of each PCR product was subjected to electrophoresis in a 1% agarose gel. The gels were stained with 0.5 μg of ethidium bromide per milliliter. Leaf samples that produced no specific DNA products were tested an additional one or two times to confirm they were not infected.

ELISA. The leaf sample was homogenized in 10 volumes of PBS-T (0.02 M sodium phosphate; 0.85% sodium chloride, pH 7.4; 0.5% Tween 20). RTBV-IgG and IgG-alkaline phosphatase conjugate were used as described previously (16). Each sample was tested in two wells. Average absorbance at 415 nm was measured 1 h after the addition of the substrate.

RESULTS

Development of the PCR. Five, 10, or 20 μl of DNA preparations extracted by each method from RTBV-infected TN1 plants were amplified with four primers (P1, P2, P3, and P4). Agarose gel electrophoresis revealed two DNA fragments of expected sizes (269 and 633 bp) for DNA preparations extracted by method A or B (data not shown). Method C failed to amplify the fragments. Either method A or B was used in the following experiments. When only primers P2 and P3 were used in the reaction, a single DNA fragment of the predicted size (2,407 bp) was obtained (data not shown). When DNA extracted from uninoculated TN1 leaf tissues by each method was processed similarly, no visible band was observed after the electrophoresis.

To determine the detection efficiency of PCR, a dilution series (10^{-1}–10^{-6}) was prepared from DNA extracts from a portion of a RTBV-infected TN1 leaf. The dilution end point for positive PCR was 10^{-5} for DNA extracted by method A and 10^{-6} for DNA extracted by method B (Fig. 1). Based on the original weight of the leaf used for DNA extraction by methods A and B, the dilution end points were calculated as 0.8×10^{-6} and 0.8×10^{-7}, respectively. When the remaining portion of the leaf was homogenized and tested in ELISA, the dilution end point was 1.6×10^{-3}.

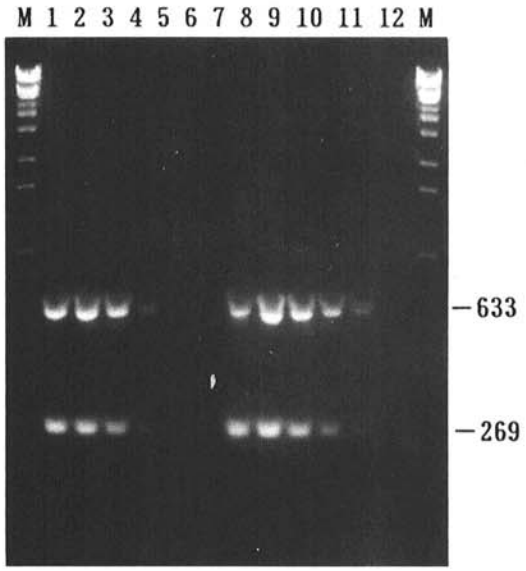

Fig. 1. Sensitivity of polymerase chain reaction (PCR) for detection of rice tungro bacilliform virus (RTBV) DNA in RTBV-infected rice (cv. Taichung Native 1) leaves. Lanes 1–6: DNA was extracted from leaf tissues by method A (described in text). The extract was diluted with water from 10^{-1} to 10^{-6} and subjected to PCR. Lane 7–12: DNA was extracted from leaf tissues by method B (described in text). The extract was diluted from 10^{-1} to 10^{-6} and subjected to PCR. Lane M: *Eco*T141-digested λ-phage as the DNA marker. Numbers to the right indicate number of base pairs.

Detection of RTBV DNA in viruliferous leafhoppers. When methods A and B were used to extract DNA from viruliferous leafhoppers, only method A produced specific DNA products in PCR.

DNA preparations from 10 individual viruliferous leafhoppers did not form bands in agarose gels after the first PCR (Fig. 2a). In six out of 10 such DNA preparations, however, the second PCR products formed two DNA bands of the predicted sizes (Fig. 2b). The third PCR products produced more intense bands than did those of the second PCR (data not shown). To confirm the specificity of the bands, the second PCR products were digested with restriction enzymes. The 269-bp PCR product had a *Spe*I site and was expected to be cleaved into a 236-bp segment by the enzyme. The 633-bp product had two *Acc*I sites and cleaved into a smaller 539-bp segment. Two DNA fragments amplified by PCR on DNA extracts from viruliferous leafhoppers were shortened to the expected sizes after digestion with *Spe*I or *Acc*I as were PCR products from cloned RTBV DNA (Fig. 3). All 90 individual viruliferous insects produced negative reactions in ELISA. None of 10 individual leafhoppers unexposed to virus produced DNA fragments after the second or third PCR.

When two to 10 viruliferous leafhoppers were combined and subjected to PCR, DNA fragments were not amplified even after two cycles of PCR. When 10 μl of nonviruliferous leafhopper extracts in water at a 1/200 dilution was added to reaction mixture containing DNA extracted from RTBV-infected rice leaves, the amplification of virus DNA was blocked (data not shown). When the head and breast portions were dissected from each viruliferous leafhopper and subjected to PCR, the two expected DNA frag-

ments were amplified by the second PCR.

Detection of RTBV by PCR and ELISA in infected rice plants with tolerance to RTBV. The results obtained on each cultivar in the two trials were comparable. All inoculated TN1 plants showed the typical tungro symptoms and produced high ELISA values for RTBV. None of the inoculated Utri Merah, Utri Rajapan, or Balimau Putih plants showed clear symptoms. Leaf samples from these inoculated plants were tested by ELISA for RTBV. Fifteen of 60 Utri Merah, four of 31 Utri Rajapan, and two of 25 Balimau Putih plants produced ELISA values higher than 0.10, the threshold level for positive detection adopted by Takahashi et al (16) (Fig. 4). When the threshold level was set at 0.05, a value twice the average of the uninfected controls was adopted in the earlier study (6), 34 of 60 Utri Merah, seven of 31 Utri Rajapan, and 10 of 25 Balimau Putih plants were scored as RTBV infected. All the inoculated TN1 plants tested produced ELISA values higher than 1.0, whereas none of the inoculated plants of Utri Merah, Utri Rajapan, and Balimau Putih produced ELISA values higher than 0.5 (Fig. 4). When DNA extracted by method A from the tissues of the same leaves used in ELISA were subjected to PCR, 39 Utri Merah, five Utri Rajapan, and 18 Balimau Putih plants showed the specific PCR products (Fig. 4). Among plants that produced ELISA values lower than 0.10, 54% of Utri Merah, 3.7% of Utri Rajapan, and 70% of Balimau Putih plants produced positive reactions with PCR. Among plants that produced ELISA values lower than 0.05, 19% of Utri Merah, 4.2% of Utri Rajapan, and 60% of Balimau Putih plants produced positive reactions with PCR. None of the plants that produced ELISA values lower than 0.03 produced positive PCRs. All plants that produced ELISA values higher than 0.06 also had positive

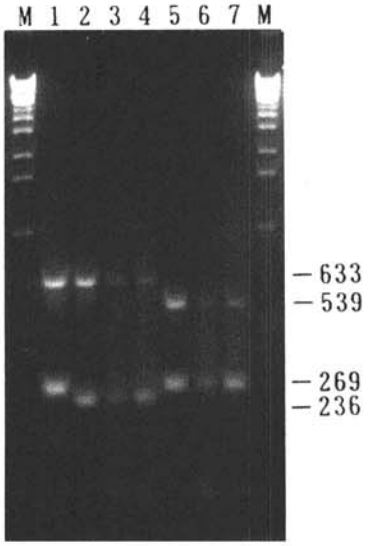

Fig. 3. Specific digestion of polymerase chain reaction (PCR) products with *Spe*I and *Acc*I. Five microliters of each enzyme-digested PCR product was electrophoresed on a 1% agarose gel. Lane 1: PCR products of rice tungro bacillioform virus-DNA cloned plasmid without restriction enzyme digestion. Lane 2: digestion with *Spe*I of PCR products of cloned plasmid. Lanes 3 and 4: digestion with *Spe*I of PCR products of DNA extracted from individual leafhopper adults. Lane 5: digestion with *Acc*I of PCR products of cloned plasmid. Lanes 6 and 7: digestion with *Acc*I of PCR products of DNA extracted from individual leafhopper adults. Lane M: *Eco*T141-digested λ-phage as the DNA marker. Numbers to the right indicate the number of base pairs.

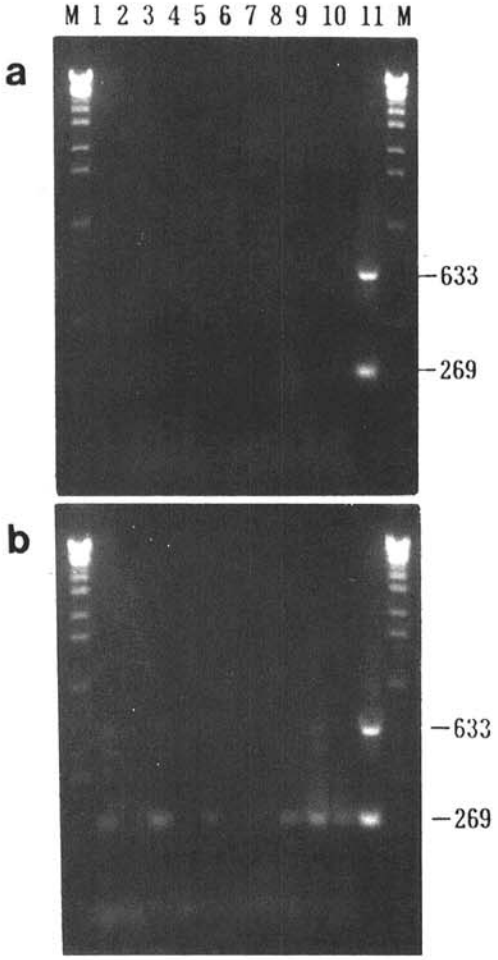

Fig. 2. A, Rice tungro bacilliform virus (RTBV) DNA extracted by method A (described in text) from individual leafhopper adults previously exposed to tungro-infected plants (lanes 1–10) and RTBV-DNA cloned plasmid (10) (lane 11) were subjected to polymerase chain reaction (PCR); **B,** 10 μl of each PCR product was amplified again in a second PCR. Five microliters of the first or second PCR products was electrophoresed on a 1% agarose gel. Lane M: *Eco*T141-digested λ-phage as the DNA marker. Numbers to the right indicate the number of base pairs.

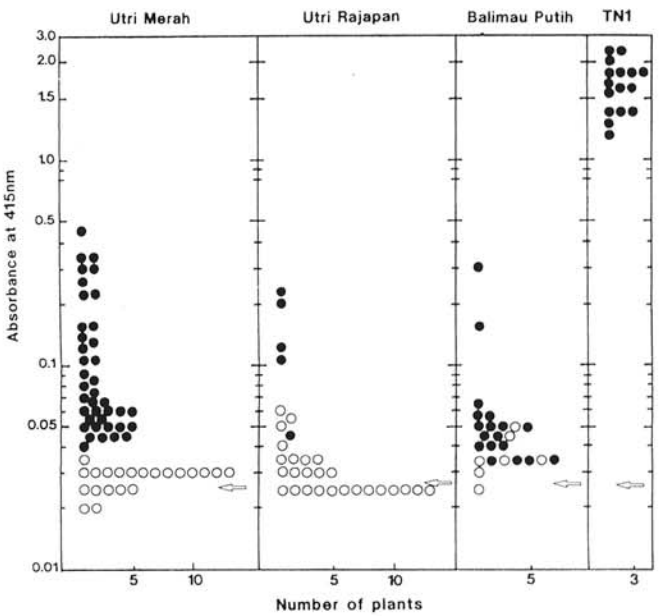

Fig. 4. Detection of rice tungro bacilliform virus by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) in tungro-inoculated rice plants of cvs. Utri Merah, Utri Rajapan, Balimau Putih, or Taichung Native 1 (TN1). The tissues of the second youngest leaf of each plant were subjected to ELISA and PCR. Absorbance at 415 nm in ELISA was measured 1 h after addition of the substrate and plotted (one open or closed circle indicates one plant). Arrows indicate average absorbance of uninoculated plants. Closed and open circles indicate the individual plants that produced positive and negative reactions in PCR, respectively.

results in PCR. None of the uninoculated leaf samples of the four cultivars were positive according to PCR or ELISA.

DISCUSSION

RTBV dsDNA is circular and has two discontinuities (gaps), one in each strand (7). To detect RTBV DNA in plants and vector leafhoppers by PCR, the two target regions selected in the nucleotide sequence (10) did not include the gaps. When P2 and P3 were used as primers, a single DNA fragment of 2,407 bp, which was the predicted size, was amplified. One of the gaps is located between the sequences corresponding to the P2 and P3 primers, indicating that the polymerization can proceed through the gap on the template RTBV DNA during amplification. A nested PCR approach (13) also could be carried out to further check specificity using P2 and P3 as an outer primer set and P1 and P2, or P3 and P4, as an inner primer set. Use of more than two sets of primers, called multiplex PCR (15), has advantages over the use of one set to detect virus DNA, because DNA of field virus isolates may have mutations in their sequence and the region corresponding to one of the primers may be lost or changed completely. The PCR method that uses two sets of primers should be suitable for detecting DNA of RTBV field isolates.

PCR successfully detected small amounts of RTBV DNA in infected rice plants or viruliferous vector leafhoppers. Theoretically, PCR could detect a single copy of a DNA fragment (18). However, RTBV DNA is coated with capsid proteins and is not immediately available for PCR. Generally, success in amplifying target DNA depends on the extraction method. Among the three extraction methods tested, methods A and B were suitable for detection of RTBV DNA from infected rice plants by PCR, but only method A was suitable for detection of RTBV DNA in extracts of individual vector leafhoppers.

The dilution end point for RTBV detection in infected rice plants (TN1) was 10^{-3} for ELISA and was 10^{-6}–10^{-7} for PCR. Therefore, the PCR technique used in these experiments, is 10^{3}–10^{4} more sensitive than ELISA. In our earlier attempts, ELISA failed to detect RTBV in viruliferous leafhoppers. The leafhopper transmits RTBV in a semipersistent manner, and viruliferous leafhoppers are supposed to contain small numbers of virus particles in the mouth or fore alimentary canal (5). The success of PCR in detecting RTBV in plants at such a sensitive level encouraged us to use it to assess viruliferous leafhoppers. In PCR, however, DNA extracted from individual viruliferous leafhoppers failed to amplify sufficient DNA fragments to form visible bands in agarose gels. A second amplification of the first PCR products was required to form visible bands. The fact that leafhopper extracts inhibited amplification of RTBV DNA from rice leaf extracts may indicate that the difficulty in PCR detection of RTBV-DNA in leafhoppers is not only due to extremely low RTBV concentration in leafhopper extracts but also to the presence in leafhopper extracts of a substance or substances that inhibit PCR of RTBV DNA. Extracts of gladioli corms were reported to contain a substance or substances that interfere with PCR (17). Detection by PCR of RTBV at high efficiency in individual leafhoppers can be achieved by removing impurities from DNA preparations. However, complicated steps for DNA preparation are not practical for routine detection of the virus. Method A is considered suitable for extracting DNA from plant and leafhopper tissues for PCR, although further improvement of the method may be desired.

ELISA is one of the most sensitive and reliable serological techniques and has been used to evaluate rice cultivars with resistance to tungro (6). In some cultivars with tolerance to RTBV, however, indexing of inoculated plants by ELISA is often difficult because of low ELISA values, and percent-infection rates obtained often vary among trials (1–3,6). Through the use of PCR in these experiments, RTBV was detected in Utri Merah, Utri Rajapan, and Balimau Putih plants that produced ELISA values as low as 0.03 or 0.04. The average reading of control plants by ELISA was 0.025. Accordingly, infection rates of these cultivars based on ELISA would change depending on the threshold level set for positive detection. If a threshold level of 0.10, four times the average of the uninfected controls, was adopted (16), it would successfully exclude false positives but fail to count many mildly infected plants as infected. If a threshold level of 0.05 was adopted (6), it would reduce false negatives but could bring a few uninfected plants into the positive category. The variation in percent-infection rates of cultivars with tolerance to RTBV among trials was due to low virus concentration in plants and its variation among the trials. Setting the threshold level high at 0.10 is desired in testing cultivars sensitive to RTBV. On the other hand, setting the level lower at 0.05 is desired in testing cultivars with tolerance to RTBV. PCR disclosed the difficulty of setting the threshold level for detecting virus in mildly infected plants. These results clearly demonstrate the effectiveness of PCR in evaluating rice cultivars with tolerance to RTBV. Further evaluation of more rice cultivars with tolerance or resistance to RTBV by PCR is desired.

To our knowledge, this is the first reported instance of applying PCR to evaluate cultivars for resistance to a virus. PCR requires DNA extraction, amplification, and agarose gel electrophoresis, so the number of samples that can be handled in one experiment is limited. PCR can be a useful supplement when evaluating rice cultivars after the primary screening for resistance to RTBV, which can be done by ELISA.

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