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Reverse transcription-recombinase polymerase amplification combined with lateral flow strip for detection of rice black-streaked dwarf virus in plants

Chong Zhao[a,b,c,1], Feng Sun[b,c,1], Xuejuan Li[a,b,c], Ying Lan[b,c], Linlin Du[b,c], Tong Zhou[b,c], Yijun Zhou[a,b,c,*]

[a] College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China
[b] Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
[c] Key Laboratory of Food Safety Evaluation, MOA, Nanjing, 210014, China

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ABSTRACT

Rice black-streaked dwarf virus (RBSDV) infects rice plants, a major crop, and is transmitted via the small brown planthopper (SBPH: *Laodelphax striatellus* Fallén), causing significant economic loss in China. To rapidly diagnose RBSDV, a reverse transcription-recombinase polymerase amplification (RT-RPA) method was developed using P10 virus-specific primers and probes. Detection of terminally labeled amplification products was achieved with the lateral flow strip method. Our results demonstrate that RT-RPA and RT-PCR assays offer similar sensitivity and specificity in RBSDV detection using cDNA as template. The optimum RT-RPA reaction temperature and time was 37 °C and 20 min, respectively. By screening twenty-one field suspected rice plants, the RT-RPA assay was confirmed to be simple, rapid and reliable. Thus, the RBSDV RT-RPA assay developed here will be a successful tool for quick diagnosis of RBSDV-infected rice plants.

Rice black-streaked dwarf virus (RBSDV), a member of the genus Fijivirus in the family Reoviridae, primarily infects rice and maize plants and causes rice black-streaked dwarf and maize rough dwarf diseases, which lead to severe rice and maize yield losses in China (Bai et al., 2001; Fang et al., 2001; Zhang et al., 2001; Wang et al., 2003). The virus is transmitted via the small brown planthopper (SBPH: *Laodelphax striatellus* Fallén) in a persistent, propagative manner (Wei and Li, 2016). Plants infected with RBSDV typically show severe disease symptoms such as stunting, darkening of leaves and white waxy galls along the veins on the leaf blades and sheaths (Sun et al., 2013). To successfully control the spread of the RBSDV disease, there is an urgent need to produce methods to rapidly diagnose RBSDV in these economically important crops and their insect vectors.

RBSDV is composed of 10 segments of double-stranded RNA encapsulated in a double-layered, icosahedral particle (Wang et al., 2003). Based on the viral RNA and protein components, several laboratory detection methods are available, including northern blot analysis (Isogai et al., 1998), enzyme-linked immunosorbent assay (ELISA) (Wang et al., 2006), dot immunobinding assay (DIBA) (Wu et al., 2013a,b), real-time reverse transcription polymerase chain reaction (RT-PCR) (Cho et al., 2013; Wu et al., 2013a,b; Zhang et al., 2013) and loop-mediated isothermal amplification (LAMP) (Le et al., 2010). Currently, the most commonly used method for detection of this virus in plants is RT-PCR. However, this method is time consuming and requires special equipment such as thermal cyclers and gel imagers, thus limiting application of this technology for rapid high-throughput diagnostic detection.

More recently, recombinase polymerase amplification (RPA) (Piepenburg et al., 2006) has been explored for molecular detection of diverse pathogens such as bacteria, fungi, parasites and viruses. The RPA process employs three enzymes, including recombinases, single-stranded DNA-binding proteins (SSBs) and strand-displacing polymerases and can operate at low and constant temperatures (Piepenburg et al., 2006). This sequence-specific isothermal nucleic acid amplification offers quick and sensitive detection without thermal cycling and has been reported for diagnosis of plant viruses such as *little cherry virus 2* (Mekuria et al., 2014), *plum pox virus* (Zhang et al., 2014) and *tomato yellow leaf curl virus* (Londoño et al., 2016). In this study, we explored and evaluated RPA combined with the lateral flow strip method for rapid molecular detection of RBSDV in plants.

Sources of plant samples: All RBSDV-infected rice plants showing severe stunting or dwarf symptoms used in this study were collected from an experimental field. The plants samples were frozen in liquid nitrogen and stored at −70 °C, and virus isolate was validated by

* Corresponding author at: College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China.
E-mail address: 2690065830@qq.com (Y. Zhou).
[1] These authors contributed equally to this work.

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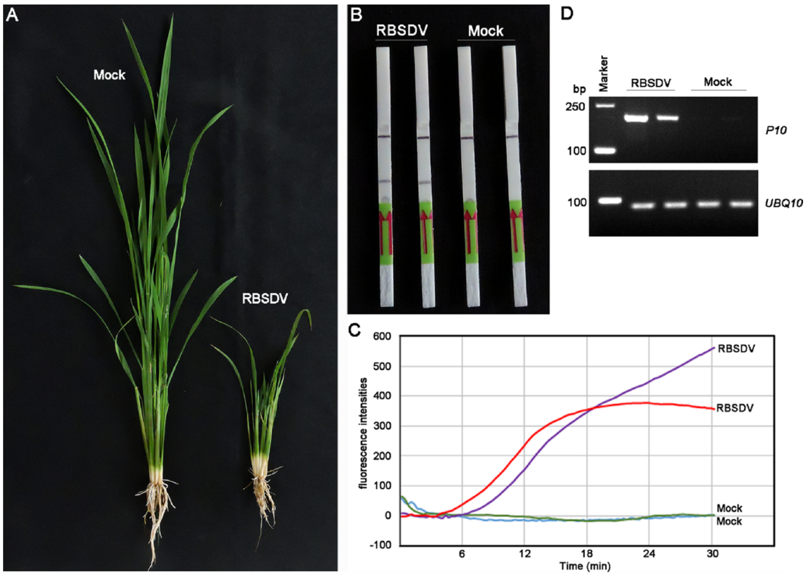

Fig. 1. Developed RT-RPA assay for RBSDV detection from RBSDV-infected rice plants. (A) Rice plants infected by RBSDV showed severe dwarf symptoms. (B) RBSDV was detected by RT-RPA combined lateral flow strip assay using *P10* specific primers and probe from RBSDV-infected rice plants. (C) RBSDV was detected by RT-RPA combined fluorogenic probe assay using *P10* specific primers and probe from RBSDV-infected rice plants. (D) RBSDV was detected by RT-PCR assay using *P10* specific primers from RBSDV-infected rice plants. Rice *UBQ10* gene transcript level was used as an internal standard.

reverse transcription-PCR (RT-PCR) using specific primers (P10-F: 5′−CCACGTTGCATCTTCCCAAA -3′, P10-R: 5′- CTTCGCTCAGGTCGTTG TTC -3′) (Zhou et al., 2018).

RNA extraction and cDNA synthesis: Total RNA was extracted from rice leaves using the RNAiso Plus Kit (TAKARA, Dalian, China), according to the manufacturer's instructions. The purity and yield of RNA extracts were measured by NanoDrop 2000C microvolume UV–vis spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA). First-strand cDNA was synthesized from 1 μg of total RNA in a 20 μl volume using the PrimeScript™ RT Master Mix Kit (TAKARA, Dalian, China), according to the manufacturer's instructions.

RT-PCR: RT-PCR reactions were performed using 10 μl 2 × Taq Master Mix (Dye Plus) (Vazyme Biotech, Nanjing, China), 0.2 μM forward and reverse primers and 1 μl cDNA in a 20 μl total volume. All samples were subjected to denaturation for 5 min at 95 °C,followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 10 min. RT-PCR products were separated on 2% agarose gels, and images were obtained using the Bio-Rad Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA) after staining with ethidium bromide. RBSDV P10 (P10-F: 5′−CCACG TTGCATCTTCCCAAA -3′, P10-R: 5′- CTTCGCTCAGGTCGTTGTTC -3′) (Zhou et al., 2018) and southern rice black-streaked dwarf virus (SRBSDV) P10 (SP10-F: 5′- TCAAAGACTTTCAGATACGATAATT -3′, SP10-R: 5′- TGAATATGTTCTTTAAGTTGAGTAA -3′) were target genes, and rice ubiquitin 10 (UBQ10) (UBQ10-F:5′- TGGTCAGTAATCAGCCA GTTTGG-3′, UBQ10-R:5′- GCACCACAAATACTTGACGAACAG -3′) was used as an internal standard (Fang et al., 2016).

RT-quantitative real-time PCR (RT-qPCR): RT-qPCR reactions were performed using 10 μl 2 × TB-green Premix Ex Taq (TAKARA, Dalian, China), 0.5 μl 10 mM forward primer (P10-RPA-F: CGAACAACGACCT AAGTGAAGAATTTGTAG), 0.5 μl 10 mM reverse primer (P10-RPA-R: [5′BIOTIN]CTATACCAATATAACTATCATCGAGAATGA) and 1 μl cDNA in a 20 μl total volume. All samples were subjected to denaturation for 3 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s. Amplicon dissociation curves, i.e., melting curves, were recorded after cycle 40 by heating from 60 °C to 95 °C at a ramp speed of 1.9 °C/min. Data was collected and analysed using Bio-Rad iQ5 Real-Time PCR system (Bio-Rad, Hercules, CA, USA).

RT-RPA assays: RT-RPA primers were designed to target the RBSDV P10 gene (NCBI reference sequence: NC_003733.1) following the manufacture's specific parameters (TwistDx, UK). RT-RPA was carried out using the materials and protocols in the TwistAmp nfo Kit and TwistAmp Liquid exo/exo RT Kit (TwistDx, UK). RT-RPA-nfo reactions were performed in 50 μl reaction volumes containing 1 μl cDNA of RBSDV-infected rice plant tissue, 30 μl rehydration buffer, 2 μl 10 mM forward primer (P10-RPA-F: CGAACAACGACCTAA GTGAAGAATTTGTAG), 2 μl 10 mM reverse primer (P10-RPA-R: [5′BIOTIN]CTATACCAATATAACTA TCATCGAGAATGA), 0.5 μl 10 mM probe (P10-nfo-probe: [5′FAM] ttatttgctacctccaaacttgatgctgaa [THF]aaatagaacgtgtaca[3′BLOCK]) and 2.5 μl 280 mM magnesium acetate. All RPA samples were incubated at 37 °C for 20 min. For analysis by lateral flow, RT-RPA reaction products were diluted 10-fold with assay buffer and 100 μl per sample was loaded on the sample application areas of Milenia Genline HybriDetect test strips (Milenia, Germany).

RT-RPA-exo reactions were performed in 50 μl reaction volumes containing 1 μl cDNA of RBSDV-infected rice plant tissue, 25 μl 2 × reaction buffer, 9 μl dNTP (2.5 mM each), 5 μl 10 × probe E-mix, 2 μl 10 mM forward primer (P10-RPA-F), 2 μl 10 mM reverse primer (P10-RPA-R), 0.6 μl 10 mM probe (P10-exo-probe: ACAAATAGAACGTGTAC AAATTGAGACCC[FAM-dT]*A*[THF]*C*[BHQ1-dT]GAAGGAAACAT TAC[C3-spacer]), 2.5 μl 20 × core reaction mix, 1 μl 50 × exo and 2.5 μl 280 mM magnesium acetate. The reaction mix was then incubated at 37 °C and fluorescence intensity was recorded with Bio-Rad iQ5 Real-Time PCR system (Bio-Rad, Hercules, CA, USA).

To evaluate the RT-RPA assay for simple and rapid molecular detection of RBSDV in plants, diseased rice plants showing severe dwarf symptoms (Fig. 1A) were collected from an experimental field in Jiangsu Academy of Agricultural Sciences. cDNA synthesized from purified plant total RNAs were used as templates in RT-PCR and RPA assays. The RT-PCR results showed that amplification of the RBSDV P10 gene using specific primers resulted in a product of 197 bp in samples from diseased rice plants and no amplification in samples from healthy plants (Fig. 1D). The ubiquitin 10 (UBQ10) reference gene was used as an internal control in the RT-PCR assay to ensure that negative results could be attributed to lack of virus RNA (Fang et al., 2015). Using specific primers and probes targeted to the P10 viral gene, the RT-RPA assay successfully detected RBSDV from diseased rice plants but not from healthy plants with lateral flow strip at end-point assay (Fig. 1B). With FAM-labeled fluorogenic probe, RBSDV RT-RPA test results were detected in a real-time assay (Fig. 1C). These results showed that RT-RPA was suitable for detection of RBSDV in plants.

Next, RT-RPA assay sensitivity for detection of RBSDV using cDNA as a template was assessed. Using a dilution range of 10^4-1 fold of cDNA as template, RT-RPA, RT-PCR and RT-qPCR were performed. As shown in Fig. 2A and B, the RT-RPA assay successfully detected RBSDV in a 10 dilution of the cDNA, and RT-PCR targeting the same regions yielded

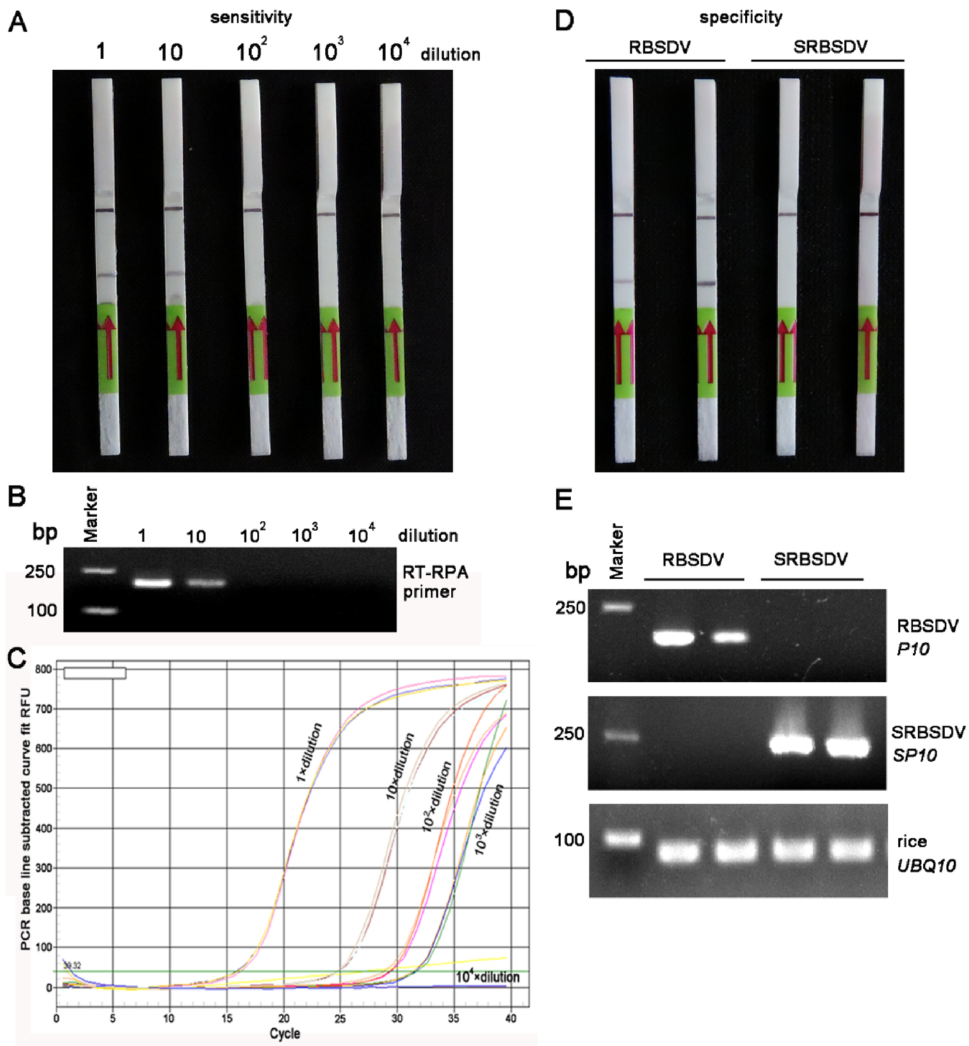

Fig. 2. Sensitivity and specificity of the RT-RPA and RT-PCR assays for detection of RBSDV. (A) Detection of RBSDV in the 10-fold serial dilutions of cDNA from an RBSDV-infected rice plant using RT-RPA asssy. (B) RT-PCR assay was performed with RT-RPA primer pair P10-RPA-F and P10-RPA-R using dilutions of cDNA. (C) RT-qPCR assay was performed with primer pair P10-RPA-F and P10-RPA-R using dilutions of cDNA. (D)Southern rice black-streaked dwarf virus (SRBSDV) samples were tested for RBSDV by RT-RPA assay but yielded negative results. (E) RBSDV and SRBSDV were detected by RT-PCR assay using virus *P10* specific primers from rice plants. Rice *UBQ10* gene transcript level was used as an internal standard. The RBSDV RT-RPA assay was specific for RBSDV and did not react with SRBSDV.

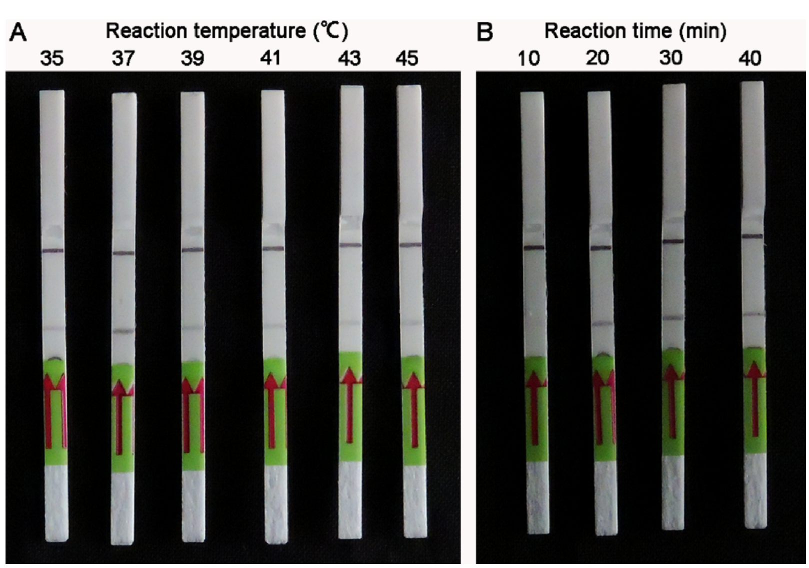

Fig. 3. Optimum of RT-RPA reaction temperatures and times. (A) Efficacy of RT-RPA assay at temperatures from 35 °C to 45 °C. RT-RPA assay works effectively in a wide range of constant reaction temperatures. (B) RT-RPA reactions were performed from 10 min to 45 min. The test line was visible on the test strip after 20 min of RT-RPA reaction.

similar sensitivity. RT-qPCR assay successfully detected RBSDV in a 10^3 dilution of the cDNA and showed the highest sensitivity (Fig. 2C). The symptoms of RBSDV can be associated with the southern rice black-streaked dwarf virus (SRBSDV) (Zhou et al., 2013). As demonstrated in Fig. 2D and E, two SRBSDV samples were tested for RBSDV by RT-RPA and RT-PCR assays but yielded negative results. The results indicate that RT-RPA for RBSDV is specific for RBSDV and does not react with SRBSDV.

To optimize reaction temperature in the RBSDV-RT-RPA technique, a set of independent reactions were performed on a gradient from 30 °C

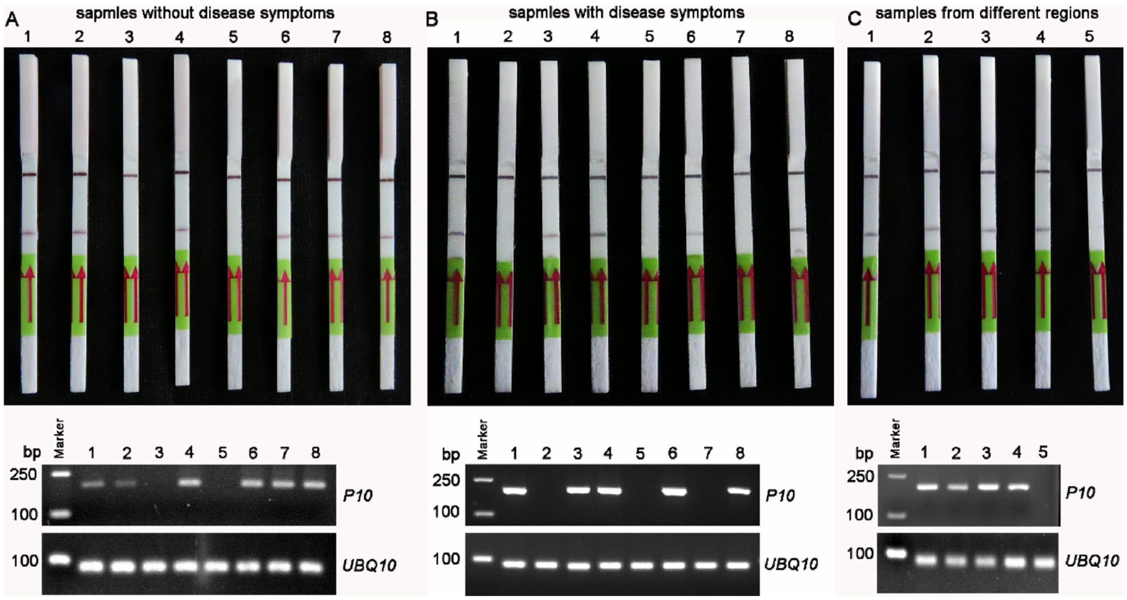

Fig. 4. Comparison of RT-RPA and RT-PCR assays for detecting RBSDV in field rice samples. Eight field samples at early infection stage without obvious symptoms (A), eight samples at late infection stage with dwarf symptoms (B), and five samples from different regions in China (C) were tested by RT-RPA and RT-PCR assays using purified RNA extracts. Both RT-RPA and RT-PCR assays showed identical results: of the 21 suspected field samples, 15 yielded a positive result, while the other 6 samples were negative. (C) Samples 1 and 5 were collected at Nanjing, Jiangsu, Samples 2 and 3 were collected at Jianhu, Jiangsu, and Sample 4 was collected at Kaifeng, Henan.

to 45 °C. It was found that RT-RPA reactions were reproducible in the temperature range tested, and the best amplifications were observed at 37 °C (Fig. 3A). Similarly, for optimizing reaction time, RT-RPA reactions were conducted at 37 °C for 10–40 min. Our results demonstrate that RBSDV can be detected by RT-RPA amplification in 20 min, and continued incubation up to 40 min resulted in comparable signal (Fig. 3B). Thus, optimized RT-RPA reactions were performed at 37 °C for 20 min for RBSDV detection.

To check the efficacies of developed RT-RPA assays, eight field samples at early infection stage without obvious symptoms, eight samples at late infection stage with dwarf symptoms and five samples from different regions in China were collected from the field. RT-RPA and RT-PCR analyses of each sample for RBSDV detection were compared. The results from both methods were identical: 15 samples were positive by both methods (Fig. 4). Thus, the accuracy of the RT-RPA method was similar to that of the RT-PCR method for detecting RBSDV in rice plants.

RBSDV disease control strategies such as disease forecast and identification of resistant varieties mostly depends on early detection of the virus in plants, even before disease symptom appearance and spread of the virus in the field. In this study, the application of the RT-RPA method for the detection of RBSDV in rice plants was analyzed and compared to the currently used RT-PCR assay. The RT-RPA assay was developed to target the P10 region of the virus genome which encodes the viral outer coat protein (Liu et al., 2007) and has been a target region for RBSDV detection by RT-PCR (Zhou et al., 2018). Our results demonstrate that the RT-RPA assay shows similar sensitivity and accuracy for RBSDV detection in rice plants compared with the RT-PCR assay (Figs. 2 and 4). However, the time and equipment required to perform RT-RPA is considerably reduced compared to RT-PCR. The RT-RPA reaction developed in this study could be accomplished in 20 min (Fig. 3B), while the RT-PCR reaction generally needs approximately 2–3 h. In addition to the shorter time, RT-RPA assays can be performed at a constant temperature of 37 °C using simple equipment such as water or dry baths (Fig. 3A), while RT-PCR requires a thermocycler and use of the mutagen ethidium bromide for visualization of PCR amplification products. Thus, the RT-RPA assay would be a rapid, sensitive and reliable method for RBSDV detection for both field and laboratory tests. To our knowledge, this is the first report to show application of RT-RPA technology for the detection of RBSDV.

In previous studies, the loop-mediated isothermal DNA amplification (LAMP) assay has been developed as a simple alternative to PCR-based amplification for detection of nine rice viruses, including RBSDV (Le et al., 2010). For RBSDV detection, the RT-LAMP assay employs two pairs of primers that recognize six regions of a target sequence, and the optimal amplification condition was 60 min at 61 °C (Le et al., 2010). The RT-RPA assay developed in this paper can be completed in 20 min at 37 °C and demonstrates a shorter required reaction time and a lower reaction temperature than the LAMP technique, avoiding the need for sophisticated thermocyclers. In addition, RT-RPA products can be readily detected by using lateral flow strips, while LAMP products must be detected by agarose gel electrophoreses or use of fluorescent detection reagent. Obviously, RT-RPA assay makes it easier for visualization of amplification products compared with LAMP. In conclusion, the RBSDV RT-RPA assay developed here could be used successfully for quick diagnosis of RBSDV-infected rice plants.

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