

Article

Comparison of sensitivity and rapidness of PCR, recombinase polymerase amplification, and RNA-specific amplification for detection of *Rice yellow mottle virus*

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***Rice yellow mottle virus* (RYMV) is a plant pathogenic virus that often causes a serious damage to rice production in Africa. In this study, we developed detection systems of RYMV DNA or RNA using each of PCR, recombinase polymerase amplification (RPA), and an RNA-specific amplification. The sensitivities were in the range of several copies of the target DNA for PCR and RPA and dozens copies of the target RNA for RNA-specific amplification. The cycle numbers or reaction times required for amplification from 10⁹ copies of the target DNA or RNA were 15 cycles (27 min) for the PCR-based system and 5 min for RPA- or RNA-specific amplification-based systems. These results suggested that isothermal RPA and RNA-specific amplification-based detection systems of RYMV will be more suitable for quick detection of RYMV-infected rice plants than the PCR-based one.**

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Key words: recombinase polymerase amplification (RPA); *Rice yellow mottle virus*; RNA-specific amplification.

Introduction

Rice yellow mottle virus (RYMV) causes rice yellow mottle disease, which is severe disease of rice in Africa. It was first reported in 1966 in Kenya, and subsequently widely spread in almost all rice-producing regions in Africa. RYMV belongs to *Sobemovirus* genus [1, 2]. The genome of RYMV is a single-stranded, positive sense RNA

of 4,450 nucleotide (nt) [3]. It contains four open reading frames (ORF1 to ORF4) (Fig. 1). ORF1 encodes a 157 amino-acid protein whose function is unknown. ORF2 encodes a 999 amino-acid polyprotein comprised of the genome-linked protein, the viral protease, the helicase, and the reverse transcriptase, RNA-dependent RNA polymerase. ORF3 encodes a 126 amino-acid protein whose function is unknown. ORF4 encodes a 239 amino-acid coat protein.

Rapid and sensitive detection of RYMV in plants is required to control rice yellow mottle disease. For this purpose, visual assessment,

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Comparison of sensitivity and rapidness of PCR

serological assay, and enzyme linked immunosorbent assay were first developed. Then, PCR assay was developed [4, 5], which is now widely used. However, when the assay is performed out of a sophisticated laboratory, such as a farm, an isothermal nucleic acid amplification-based system is more preferable to a PCR-based system because thermal cycler is not necessary for an isothermal nucleic acid amplification.

Recombinase polymerase amplification (RPA) and RNA-specific amplification are isothermal reactions. RPA specifically amplifies a target DNA sequence at around 37-42°C with recombinase, single-stranded DNA-binding protein, and strand-displacing polymerase [6]. RNA-specific amplification specifically amplifies a target RNA sequence at around 40-43°C with reverse transcriptase and RNA polymerase [7-9]. Various reports have revealed that both isothermal reactions are useful to detect various pathogens.

In the present study, we established an RPA assay and an RNA-specific amplification assay to detect RYMV. We evaluated their sensitivities and specificities using *in vitro* synthesized standard nucleic acid. We also evaluated the effects of leaf extracts on the reaction efficiency.

Materials and Methods

Preparation of standard DNA The 240-nt single strand DNA fragment of the RYMV gene, corresponding to DNA sequence 3421-3660 deposited in GenBank (L20893.1), was purchased from Eurofins Genomics (Tokyo, Japan). The 240-bp DNA was amplified by PCR using primers RYMV-F1 and RYMV-R3 and *Taq* polymerase (Toyobo, Osaka, Japan) under 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and purified using MagExtractor (Toyobo). The concentration of purified DNA was determined spectrophotometrically at A_{260} and stored at -20°C

for subsequent use.

Preparation of standard RNA *In vitro* transcription was carried out with 0.024 µg/ml standard DNA above mentioned as a template using RiboMAX™ Large Scale RNA Production System (Promega, Madison, WI) at 37°C for 3 h. The resultant RNA was purified by NICK Columns (GE Healthcare, Buckinghamshire, UK) and then extracted by ethanol precipitation. The concentration of purified RNA was determined at A_{260} and stored at -80°C for subsequent use.

Detection of RYMV DNA by PCR Reaction mixture (15 µl) was prepared by mixing 9.2 µl of water, 1.5 µl of 10 × PCR buffer [100 mM Tris-HCl buffer (pH 8.3), 500 mM KCl, 15 mM MgCl₂], 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1.2 µl of dNTPs (2 mM each), 1 µl of DNA, and 0.2 µl of 1 U/µl *Taq* polymerase. The cycling parameters were 95°C for 30 s, followed by 5-35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final extension at 72°C, 2 min. The amplified products were separated on 2.0% agarose gels and stained with ethidium bromide (1 µg/ml).

Detection of RYMV DNA by RPA Reaction was carried out with TwistAmp Liquid Basic (TwistDx, Cambridge, MA). Briefly, the reaction was started by mixing 7.5 µl of 2 × reaction buffer, 2.46 µl of dNTPs (2 mM each), 0.3 µl dH₂O, 1.5 µl of Basic E-Mix, 0.72 µl of 10 µM forward primer, 0.72 µl of 10 µM reverse primer, 0.75 µl of 20 × Core Reaction Mix, 0.75 µl of 280 mM Mg(CH₃COO)₂, and 0.3 µl of DNA in a PCR tube, and incubated at 41°C for 1-40 min. The amplified products were separated on 2.0% agarose gels and stained with ethidium bromide.

Detection of RYMV RNA by RNA-specific amplification Reaction mixture was prepared by mixing 2.5 µl of RNA and 10 µl of substrate primer solution [175 mM Tris-HCl (pH 8.6), 195 mM KCl, 26 mM MgCl₂, 1.5 mM dithiothreitol, 0.38 mM each dNTP, 2.8 mM each NTP, 5.4 mM

inositol triphosphate, 0.32 U/ μ l RNase inhibitor, 1.5 μ M forward primer, 1.5 μ M promoter-bearing reverse primer, and 19.5% dimethylsulfoxide] in a PCR tube and incubated at 65°C for 5 min and then at 41°C for 5 min. The reaction was started by adding 2.5 μ l of enzyme solution [0.72 mg/ml bovine serum albumin, 12% sorbitol, 1.6 U/ μ l AMV RT (Life sciences Inc, Petersburg, FL), 28.4 U/ μ l T7 RNA polymerase (Toyobo)] and continued at 41°C for 1-40 min. The amplified products were separated on 2.0% agarose gel and stained with ethidium bromide.

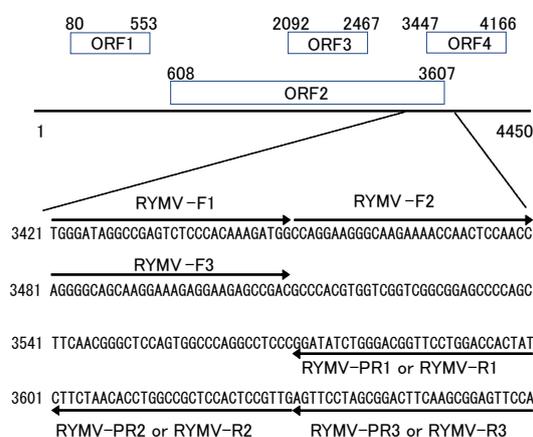


Fig. 1. Structure of RYMV and a target sequence for amplification. The sequences to which primers bind are indicated by arrows. Primer sequences (5'-3'): RYMV-F1: TGGGATAGGCCGAGTCTCCACAAAGATGG; RYMV-F2: CCAGGAAGGGCAAGAAAA CCAACTCCAACC; RYMV-F3: AGGGGCAGCAAGGAAAGAGGAAGAGCCGAC; RYMV-R1: ATAGTGGTCCAGGAACCGTCCCAGATATCC; RYMV-R2: CAACGGA GTGGAGCGGCCAGGTGTTAGAAG; RYMV-R3: TGGAACCTCCGCTTGAAGTC CGTAGGAAGT; RYMV-PR1, RYMV-PR2, and RYMV-PR3: those of RYMV-PR1, RYMV-PR2, and RYMV-PR3, respectively, to which the T7 promoter sequence (AATCTAATACGACTCACTATAGGGAGA) is added at the 5'terminus.

Results and Discussion

Establishment of PCR, RPA, and RNA-specific amplification assays for detection of RYMV

Figure 1 shows the structure of RYMV. We selected a part of ORF2 (nt: 3421-3660) as a target sequence, due to that the divergences of ORF2 and ORF4 are lower than other ORFs [10]. In our experience, the followings are important to design primers in RPA and RNA-specific amplification: (i) GC content does not elevate 60%; (ii) the length of primer region that binds to a target DNA or RNA is about 30 nt; and (iii) the size of amplified DNA is 100-300 bp, and that of amplified RNA is 100-300 nt. However, unlike the case with PCR, no method for rational design of primers has yet been developed for RPA and RNA-specific amplification, necessitating trial and error.

Table 1. PCR and RPA primer combination and expected size of amplicon.

No.	Primer combination		Expected size of amplified DNA (bp)
	Forward	Reverse	
1	RYMV-F1	RYMV-R1	180
2	RYMV-F1	RYMV-R2	210
3	RYMV-F1	RYMV-R3	240
4	RYMV-F2	RYMV-R1	150
5	RYMV-F2	RYMV-R2	180
6	RYMV-F2	RYMV-R3	210
7	RYMV-F3	RYMV-R1	120
8	RYMV-F3	RYMV-R2	150
9	RYMV-F3	RYMV-R3	180

Table 2. RNA-specific amplification primer combination and expected size of amplicon.

No.	Primer combination		Expected size of amplified RNA (nt)
	Forward	Reverse	
1	RYMV-F1	RYMV-PR1	186
2	RYMV-F1	RYMV-PR2	216
3	RYMV-F1	RYMV-PR3	246
4	RYMV-F2	RYMV-PR1	156
5	RYMV-F2	RYMV-PR2	186
6	RYMV-F2	RYMV-PR3	216
7	RYMV-F3	RYMV-PR1	126
8	RYMV-F3	RYMV-PR2	156
9	RYMV-F3	RYMV-PR3	186

Comparison of sensitivity and rapidness of PCR

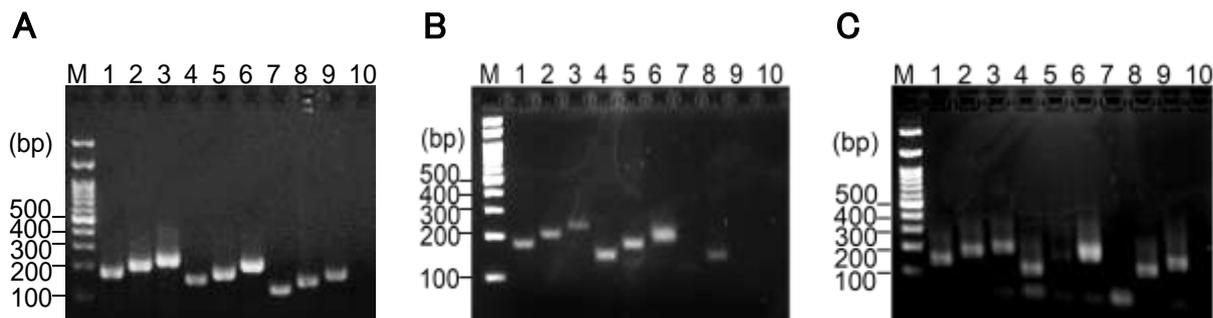


Fig. 2. Comparison of primer combination. PCR (A), RPA (B), and RNA-specific amplification (C) were carried out from 10^9 copies of standard DNA (A, B) or 10^9 copies of standard RNA (C). The cycle number of PCR was 35 (A), and the reaction time of RPA (B) and RNA-specific amplification (C) were 20 min. Lanes: M, DNA ladder DM2300; 1-9, reaction solution with primer combinations No. 1-9, respectively; and 10 corresponds to the reaction without primers. Amplified products were applied to 2.0% agarose gel followed by staining with ethidium bromide (1 $\mu\text{g/ml}$).

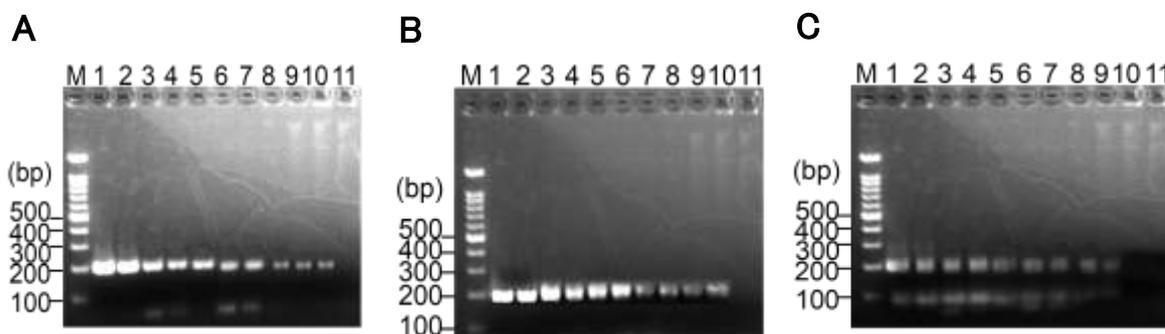


Fig. 3. Effects of initial copies on the amplification. PCR (A), RPA (B), and RNA-specific amplification (C) were carried out with primer combination No. 6. The cycle number of PCR was 35 (A), and the reaction time of RPA (B) and RNA-specific amplification (C) were 20 min. Lanes: M, DNA ladder DM2300; 1-9, reaction solutions from 10^9 (lane 1), 10^8 (lane 2), 10^7 (lane 3), 10^6 (lane 4), 10^5 (lane 5), 10^4 (lane 6), 10^3 (lane 7), 10^2 (lane 8), 10 (lane 9), 1 (lane 10), and 0 (lane 11) copies of standard DNA (A, B) or standard RNA (C). Amplified products were applied to 2.0% agarose gel followed by staining with ethidium bromide.

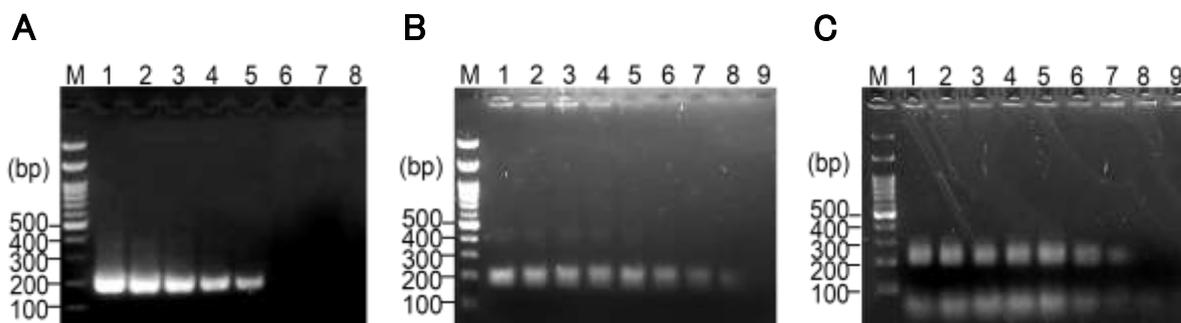


Fig. 4. Effects of cycle number or reaction time on the amplification. PCR (A), RPA (B), and RNA-specific amplification (C) were carried out with primer combination No. 6 from 10^9 copies of standard DNA (A, B) or standard RNA (C). Lanes: M, DNA ladder DM2300; 1-9 in (A), reaction solutions at 35 (lane 1), 30 (lane 2), 25 (lane 3), 20 (lane 4), 15 (lane 5), 10 (lane 6), 5 (lane 7), and 1 (lane 8) cycles; 1-9 in (B and C), reaction solutions at 40 (lane 1), 35 (lane 2), 30 (lane 3), 25 (lane 4), 20 (lane 5), 15 (lane 6), 10 (lane 7), 5 (lane 8), and 1 (lane 9) min. Amplified products were applied to 2% agarose gel followed by staining with ethidium bromide.

We designed three forward primers (RYMV-F1, RYMV-F2, and RYMV-F3), three reverse primers (RYMV-R1, RYMV-R2, and RYMV-R3), and three promoter-bearing reverse primers (RYMV-PR1, RYMV-PR2, and RYMV-PR3) (Fig. 1). The expected sizes of the amplified DNA and RNA of each combination are shown in Tables 1 and 2, respectively.

We evaluated the effects of primer combination on the amplification efficiency of these three assays. PCR, RPA, and RNA-specific amplification were carried out with the primer combinations 1-9 with the initial copy number of 10^9 copies (Fig. 2). The bands corresponding to amplified products appeared for the primer combinations No. 1-9 in PCR, No. 1-6 and 8 in RPA, and No. 1-4 and 6-9 in RNA-specific amplification, but did not for No. 7 and 9 in RPA and No. 5 in RNA-specific amplification. Primer combination No. 6 gave the best performances, and thus this combinations was used for subsequent analyses.

Comparison of the sensitivity and rapidness of the PCR, RPA, and RNA-specific amplification assays

We compared the sensitivity of the three assays. When the cycle number was set at 35 for PCR and the reaction time was set at 20 min, the intensities of the bands corresponding to the amplified products decreased with decreasing initial copy numbers, but the weak bands were observed even in the reaction from one copy for PCR and RPA and that from 10 copies for RNA-specific amplification (Fig. 3). In RNA-specific amplification, bands of smaller size corresponding to non-specific primer-derived amplified products also appeared (Fig. 3C). Considering the error of dilution, these results indicated that the sensitivities were in the range of several copies of the target DNA for PCR and RPA and dozens copies of the target RNA for

RNA-specific amplification.

We next compared the rapidness of the three assays. When the initial copy numbers of the target DNA or RNA were set at 10^9 , the band appeared at 15 cycles for PCR and 5 min for RPA and RNA-specific amplification (Fig. 4). Considering that 15 cycles takes 27 min in PCR, these results suggested that RPA and RNA-specific amplification are more rapid than PCR.

Effects of plant extracts on the RPA, and RNA-specific amplification assays

Inhibitors to RPA and/or RNA-specific amplification may be present in plant samples. We thus addressed this issue by examining if plant extracts inhibited RPA and RNA-specific amplification. However, it is forbidden to import plant organs, such as leaves or stems, of the rice cultivated in Kenya to Japan. We thus used extracts of *Morus australis* leaf [K.K., T.T., and K.Y. manuscript in preparation] and *Ficus carica* leaf [11] instead. As shown in Fig. 5, neither *M. australis* leaf nor *F. carica* leaf extract inhibited the reaction. These results suggested that plant extracts will not much affect the performance of the RPA- or RNA-specific amplification-based detections systems for RYMV.

One of the merits of RPA and RNA-specific amplification over PCR is that they are isothermal reactions, and thus has the potential to eliminate the use of specialized equipment to provide the complex temperature control. It should be noted that RPA reaction occurs even at the human body temperature (37°C). Recently, a completely instrument-free, RPA-based system to detect *Leishmania* species was reported [12]. Thus, RPA might be the most ideal nucleic acid amplification method for use in point-of-care diagnosis. In accordance with this trend, various technologies have been combined with RPA such as lateral flow assay [13], enzyme-linked oligonucleotide assay [14], and electrochemical assay [15].

Comparison of sensitivity and rapidness of PCR

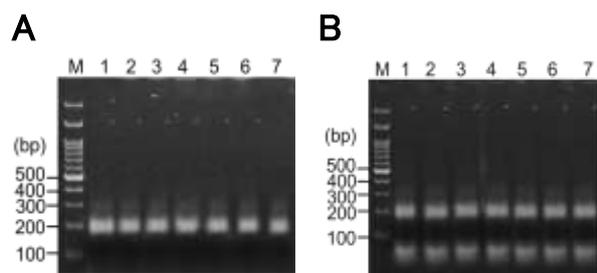


Fig. 5. Effects of leaf extracts on the amplification. RPA (A) and RNA-specific amplification (B) were carried out from 10^9 copies of standard DNA (A) or RNA (B) for 20 min. Lanes: M, DNA ladder DM2300; 1-7, reaction solutions without (1) or with *M. australis* (2-4) or *F. carica* (5-7) leaf extract. The protein concentrations of the extracts in the reaction solutions were 5 (lane 2), 0.5 (lane 3), 0.05 (lane 4), 45 (lane 5), 4.5 (lane 6), and 0.45 $\mu\text{g/ml}$ (lane 7). Amplified products were applied to 2.0% agarose gel followed by staining with ethidium bromide.

In conclusion, we developed specific and rapid RPA and RNA-specific amplification systems for detection of RYMV. These assays can be expected to be suitable for routine use. We are currently preparing a large-scale field survey in Kenya as a joint research with Plant Health Inspectorate Service (KEPHIS).

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