

METHODOLOGY

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Development of a recombinase polymerase amplification combined with lateral flow dipstick assay for rapid and sensitive detection of bean common mosaic virus

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Abstract

Bean common mosaic virus (BCMV) is one of the most widespread and damaging viruses of cultivated legumes in the world. In addition to serious yield reduction and germplasm decline, BCMV infection also makes legumes more vulnerable to other pathogens. Early diagnosis of the virus is particularly important in limiting its spread. Recombinase polymerase amplification (RPA) is a novel isothermic amplification technology. The whole reaction can be done outside the laboratory environment after the nucleic acid sample is obtained. In this study, we established a rapid and sensitive RPA combined with the lateral flow dipstick (LFD) assay for detection of BCMV, based on the conserved BCMV coat protein (CP) gene sequence. Specific primers and a probe were designed, which amplify ~ 150 bp CP fragments from BCMV-infected samples under a constant temperature of 37 °C for 20 min. The end-labeled amplification products were detected by high-affinity LFD within 5 min. Sensitivity of this RPA-LFD assay was 1000 times greater than that of the conventional polymerase chain reaction (PCR) assay. Furthermore, when the primers/probe were used against related potyviruses including soybean mosaic virus (SMV), bean yellow mosaic virus (BYMV), and turnip mosaic virus (TuMV), the three potyviruses were not detected, indicating that the assay was BCMV species-specific. The RPA-LFD assay was also successfully applied for the detection of seed-borne BCMV in beans. The RPA-LFD assay has great potential application in the rapid diagnosis of BCMV in the field.

Keywords: Potyvirus, RPA, LFD, Field detection, Viral diagnosis, *Phaseolus angularis*, *Glycine max*

Background

Bean common mosaic virus (BCMV, genus *Potyvirus*) is a pathogen of common beans (*Phaseolus vulgaris*), as well as other cultivated and wild legumes (Morales 2006). Yield loss can reach 100% in severely BCMV-infected bean fields (Saqib et al. 2010; Li et al. 2014), and the virus is internationally distributed. Isolates of

BCMV have been identified and characterized from different countries, including USA, India, China, Iran, South Korea, Turkey, and Australia, based on analysis of complete or partial genome sequences (Flores-Estévez et al. 2000; Saqib et al. 2005; Damayanti et al. 2008; Cui et al. 2014; Seo et al. 2015; Jang et al. 2018). BCMV is divided into strains based on host symptoms, serological relationships, high performance liquid chromatography peptide mapping, and genome sequence (Drijfhout et al. 1978; McKern et al. 1992; Mink and Silbernagel 1992; Feng et al. 2014a, 2014b; Li et al. 2016). An RT-PCR-based method combined with restriction enzyme analysis was first used to distinguish between BCMV and its

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closest relative, bean common mosaic necrosis virus (BCMNV) (Saiz et al. 1994). Flores-Estévez et al. (2003) used RT-PCR with primers based on the viral coat protein (*CP*) gene sequence to differentiate BCMV from BCMNV. A disadvantage of conventional RT-PCR-based assays for BCMV is their unsuitability for rapid detection in the field.

Recombinase polymerase amplification (RPA) (Piepenburg et al. 2006) has been explored for molecular detection of bacteria, fungi, and viruses. The RPA process employs three enzymes: a recombinase, a single stranded DNA-binding protein (SSB) and a strand-displacing polymerase (Piepenburg et al. 2006) to implement the amplification of a specific genome fragment. Unlike PCR-based methods, which require stringent temperature cycling, RPA reactions can be operated at a constant temperature of 37 °C, thereby avoiding the requirement of a thermal cycler. The reactions can be completed in 5–20 min depending on the starting copy number of the targeted template and the size of the amplicon. The RPA assay has been reported in diagnosis of several plant viruses, including those with DNA genomes such as wheat dwarf virus (Glais and Jacquot 2015), banana bunchy top virus (Kapoor et al. 2017), citrus yellow mosaic virus (Kumar et al. 2018), bean golden yellow mosaic virus, tomato mottle virus, tomato yellow leaf curl virus (Londoño et al. 2016; Wang and Yang 2019), milk vetch dwarf virus (Cao et al. 2020), and piper yellow mottle virus (Mohandas and Bhat 2020); and with RNA-based genomes such as little cherry virus-2 (Mekuria et al. 2014), plum pox virus (Zhang et al. 2014; Babujee et al. 2019), rose rosette virus (Babu et al. 2017), rice black-streaked dwarf virus (Zhao et al. 2019), cucumber mosaic virus (Srivastava et al. 2019), maize chlorotic mottle virus (Jiao et al. 2019a), apple stem pitting virus (Kim et al. 2019), cucumber green mottle mosaic virus (Jiao et al. 2019b; Zeng et al. 2019), cucurbit yellow stunting disorder virus (Kalischuk et al. 2020), and peach latent mosaic viroid (Lee et al. 2020). RPA has also been successfully applied for detection of the potyviruses plum pox virus (Zhang et al. 2014), yam mosaic virus and yam mild mosaic virus (Silva et al. 2015), potato virus Y (Glais and Jacquot 2015; Babujee et al. 2019), and chilli veinal mottle virus (Jiao et al. 2020). The type of tissue used as viral sources in RPA assays include leaves, budwood, grape mealybugs, stems, petals, pollen/anthers, whole flowers and roots, but seeds haven't been reported for the assay. In this study, we designed specific primers based on *CP* gene sequences of BCMV isolates to develop an RPA assay. RPA products can be analyzed by agarose gel electrophoresis or by using a lateral flow dipstick (LFD) assay that avoids the time and equipment required for electrophoresis (MileniaBiotec, Giessen, Germany). End-labeled RPA amplification products can be detected by LFD in 5

min. The main advantage of the new RPA-LFD assay described here is that the whole reaction process can be carried out in a non-laboratory environment after the nucleic acid sample is obtained.

The objectives of this study were to (i) develop primers and probe for specific detection of BCMV by RPA-LFD; (ii) evaluate the specificity of the assay against related potyviruses; (iii) compare the sensitivity of the new developed RPA-LFD assay with conventional PCR; (iv) evaluate the assay with different BCMV-infected legume species; (v) evaluate the assay in detecting seed-borne BCMV.

Results

Establishment of RPA-LFD assay for the detection of BCMV

To evaluate the simplicity and rapidity of RPA-LFD assay for the detection of BCMV in legume plants, BCMV-infected soybean samples exhibiting severe mosaic symptoms were collected from different experimental farms in Jiangsu Province, China. The cDNA synthesized from the total RNA of diseased soybean samples was used as template for PCR and RPA-LFD assays, with cDNA samples from the uninfected (healthy) soybean plants as negative controls. PCR results demonstrated that a 150-bp product corresponding to the partial BCMV *CP* gene was amplified from diseased soybean plants, but not from healthy soybean plants (Fig. 1a). Similarly, the RPA-LFD assay successfully detected BCMV in the diseased plants using specific primers and a probe against the partial BCMV *CP* gene, and did not detect BCMV in uninfected plants (Fig. 1b), indicating that RPA-LFD can be used for the detection of BCMV in soybean plants.

RPA-LFD assay is more sensitive than conventional PCR in the detection of BCMV

To evaluate the sensitivity of the RPA-LFD assay, the detection limit of the RPA-LFD assay was evaluated by testing 10-fold serial dilutions (starting from 4 ng/μL) of cDNA samples derived from BCMV-infected soybean, and by comparing results with those of conventional PCR. RPA-LFD successfully detected BCMV even when cDNA samples was diluted 10⁵-fold (Fig. 2a). In contrast, the PCR assay detected BCMV in cDNA samples diluted up to 10²-fold (Fig. 2b). Thus, the RPA-LFD assay was 1000 times more sensitive than the PCR assay.

Specificity of the RPA-LFD assay

Symptoms of BCMV infection can resemble those of other potyviruses that occur in legume crops. Therefore, isolates of soybean mosaic virus (SMV), bean yellow mosaic virus (BYMV) and turnip mosaic virus (TuMV), all belonging to the genus *Potyvirus*, were selected to test specificity of the RPA-LFD assay. Isolates of SMV,

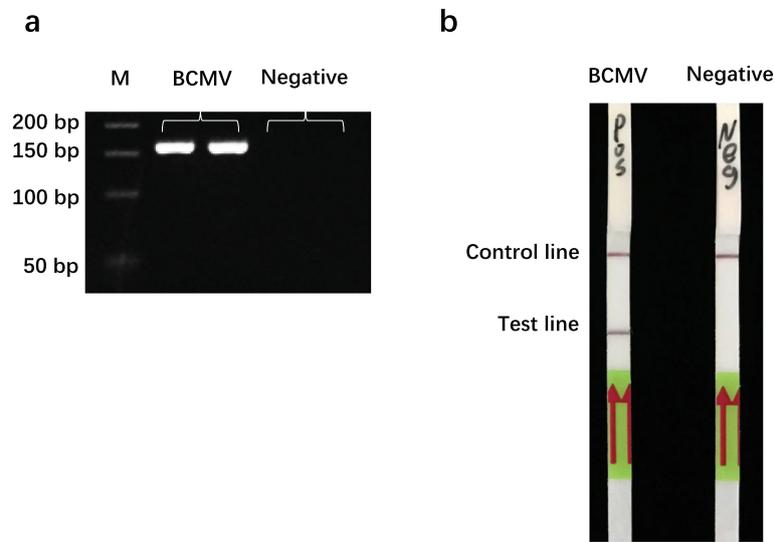


Fig. 1 Development of an RPA-LFD assay for the detection of BCMV from soybean plant tissues. **a** Detection of BCMV from BCMV-infected soybean plants by RT-PCR assay using BCMV-CP primers, with non-infected (healthy) plants as negative control. **b** Detection of BCMV from BCMV-infected soybean plants by RPA-LFD using BCMV-specific primers and probe, with non-infected healthy plants as negative control

TuMV and BYMV all consistently tested negative in both the RPA-LFD-based and PCR-based assays for BCMV (Fig. 3a, b).

Evaluation of the RPA-LFD assay using soybean and adzuki bean plants

To verify the effectiveness of the RPA-LFD method for detecting BCMV in legume plants, leaves of nine soybean (*Glycine max*) plants were manually-inoculated with sap from a BCMV-infected soybean plant. Untreated soybean plants served as negative controls. Two weeks later, all plants were sampled for RT, followed by PCR and RPA-LFD detection of BCMV. As expected, both RPA-LFD and PCR assay results showed that all diseased plants were positive for BCMV while all control plants were negative (Fig. 4a, b).

To further test the effectiveness of RPA-LFD in detecting BCMV in legume plants in the field, we collected 21 adzuki bean (*Phaseolus angularis*) samples from several

regions of Jiangsu Province, China, including 16 suspected virus-infected and five symptomless ones. These were tested by PCR and RPA-LFD for BCMV. There were 16 positive plants among the 21 tested samples, as detected by both RPA-LFD and PCR assays (Fig. 4c, d). Detailed results of the detection analyses are provided in Table 1.

Detection of seed-borne BCMV using RPA-LFD

BCMV is seed-borne in many legumes. We tested the effectiveness of the RPA-LFD assay for detection of seed-borne BCMV in adzuki beans. Total RNA was extracted from ten seeds derived from infected parental plants, and this was used as a template for RT, followed by RPA-LFD and PCR assays. Both assays detected BCMV in seeds (Fig. 5a, b). However, bands from the PCR assay were unclear when observed on an imager, because adzuki bean seeds contain large quantities of polysaccharides, proteins, secondary metabolites such as phenols and a fat content of up to 40%. Polysaccharides and

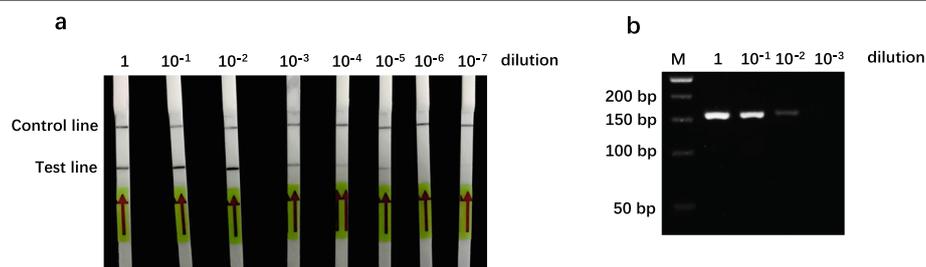
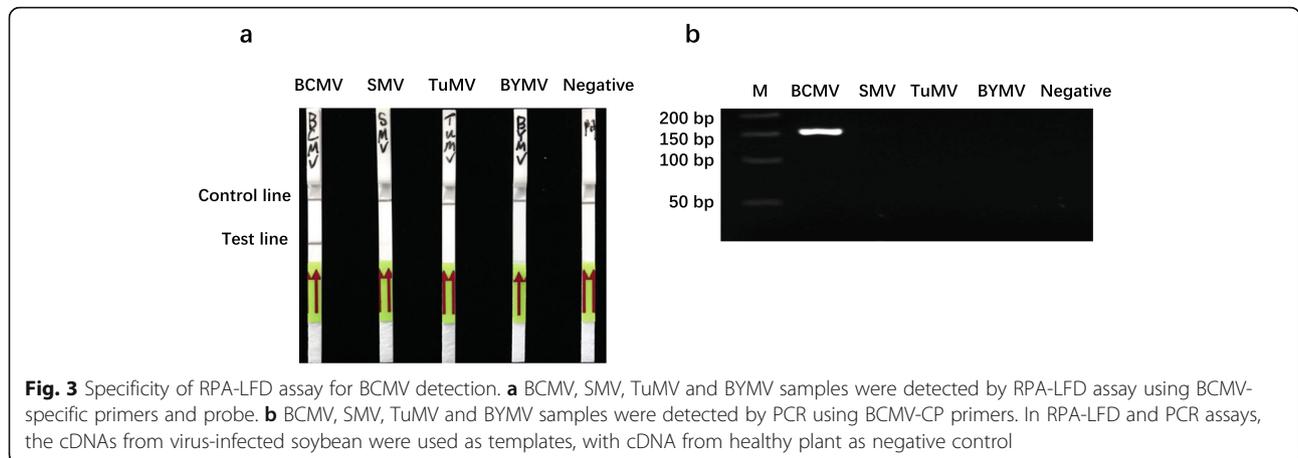


Fig. 2 Comparison of the sensitivity of RPA-LFD and PCR assay for the detection of BCMV. **a** Detection of BCMV from 10-fold serial dilutions of cDNA samples from BCMV-infected soybean by RPA-LFD. **b** Detection of BCMV from 10-fold serial dilutions of the same cDNA samples as in **a** by PCR assay



these other metabolites can inhibit the activity of many enzymes and therefore may interfere with PCR-based methods for BCMV detection. By contrast, using the same samples, we found the sensitivity of RPA-LFD to detect BCMV was 1000 times higher than that of the PCR method. The results of detection are read with a test strip, which is clear and intuitive. Therefore, when detecting BCMV in adzuki bean seeds, the RPA-LFD method was more sensitive and effective than the PCR.

Detection of crude plant extract using RPA-LFD

To further test the effectiveness of the RPA-LFD in detecting BCMV from the crude extract of plant samples, eight BCMV-infected soybean samples and four healthy

samples were used. In all samples, 0.1 g of fresh leaves were ground with 1 mL of buffer solution, then were centrifuged and the supernatant was used immediately to perform the RPA-LFD experiment. RT-PCR assay was performed on the corresponding samples using purified RNA extracts. The results showed that the RPA-LFD method can detect BCMV directly from crude soybean extracts and that the test results were consistent with results of RT-PCR (Fig. 6a, b). The whole RPA-LFD process can be completed within 30 min. The RPA-LFD is also sensitive to detect BCMV from crude extract of soybean leaves. It is suitable for non-professional experimenters to rapidly and simply detect BCMV infected-plants in the field.

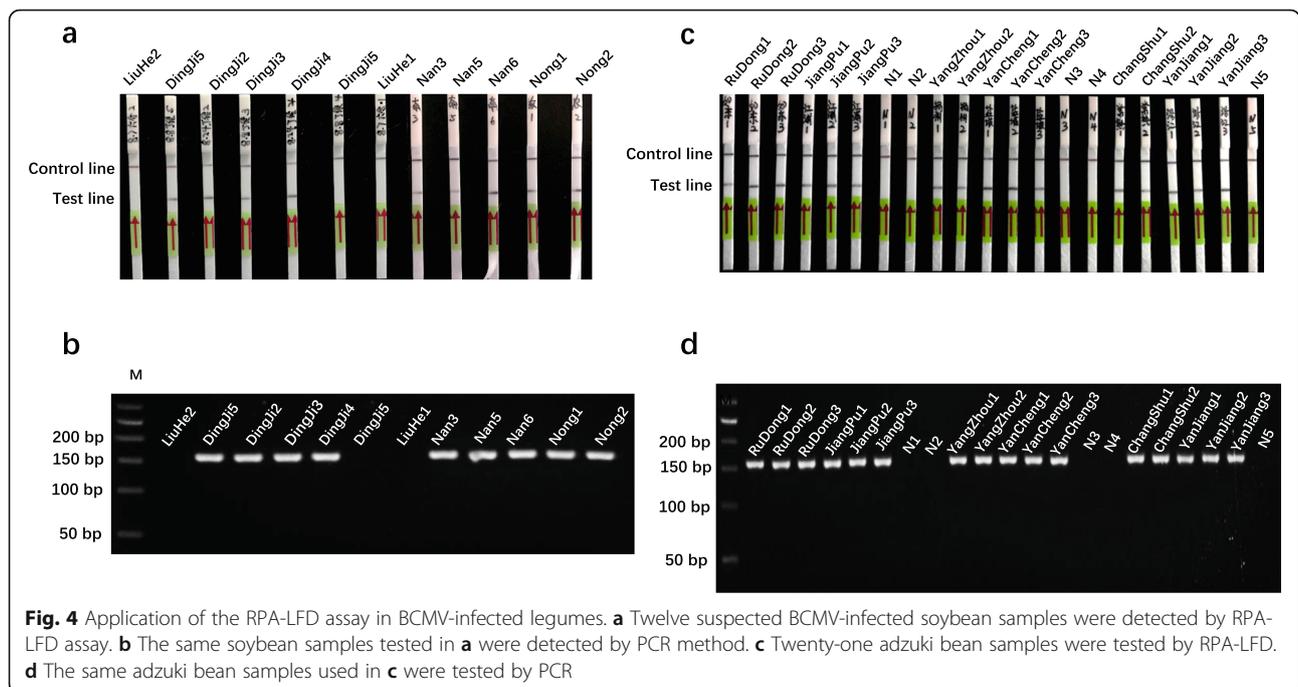


Table 1 Detection results by RPA-LFD for BCMV in soybean and adzuki bean samples from different regions of Jiangsu Province, China

Leaf sample	Original host	Geographical origin ^a	BCMV-specific RPA-LFD assay	BCMV-specific PCR assay	Symptoms
LiuHe 2	Soybean	JAAS	–	–	Symptomless
DingJi 5	Soybean	JAAS	+	+	Mosaic and shrinking
DingJi 2	Soybean	JAAS	+	+	Mosaic
DingJi 3	Soybean	JAAS	+	+	Mosaic and shrinking
DingJi 4	Soybean	JAAS	+	+	Mosaic and shrinking
DingJi 5	Soybean	JAAS	–	–	Symptomless
LiuHe 1	Soybean	JAAS	–	–	Symptomless
Nan3	Soybean	JAAS	+	+	Mosaic and shrinking
Nan5	Soybean	JAAS	+	+	Mosaic and shrinking
Nan6	Soybean	JAAS	+	+	Mosaic
Nong1	Soybean	JAAS	+	+	Mosaic
Nong2	Soybean	JAAS	+	+	Mosaic
RuDong1	Adzuki bean	Rudong, Jiangsu, China	+	+	Mosaic
RuDong2	Adzuki bean	Rudong, Jiangsu, China	+	+	Mosaic
RuDong3	Adzuki bean	Rudong, Jiangsu, China	+	+	Mosaic
JiangPu1	Adzuki bean	Nanjing, Jiangsu, China	+	+	Mosaic
JiangPu2	Adzuki bean	Nanjing, Jiangsu, China	+	+	Mosaic
JiangPu3	Adzuki bean	Nanjing, Jiangsu, China	+	+	Mosaic
N1	Adzuki bean	JAAS	–	–	Symptomless
N2	Adzuki bean	JAAS	–	–	Symptomless
YangZhou1	Adzuki bean	Yangzhou, Jiangsu, China	+	+	Mosaic
YangZhou2	Adzuki bean	Yangzhou, Jiangsu, China	+	+	Mosaic
YanCheng1	Adzuki bean	Yancheng, Jiangsu, China	+	+	Mosaic
YanCheng2	Adzuki bean	Yancheng, Jiangsu, China	+	+	Mosaic
YanCheng3	Adzuki bean	Yancheng, Jiangsu, China	+	+	Mosaic
N3	Adzuki bean	JAAS	–	–	Symptomless
N4	Adzuki bean	JAAS	–	–	Symptomless
ChangShu1	Adzuki bean	Changshu, Jiangsu, China	+	+	Mosaic
ChangShu2	Adzuki bean	Changshu, Jiangsu, China	+	+	Mosaic
YanJiang1	Adzuki bean	Rugao, Jiangsu, China	+	+	Mosaic
YanJiang2	Adzuki bean	Rugao, Jiangsu, China	+	+	Mosaic
YanJiang3	Adzuki bean	Rugao, Jiangsu, China	+	+	Mosaic
N5	Adzuki bean	JAAS	–	–	Symptomless

^a JAAS: Jiangsu Academy of Agricultural Sciences

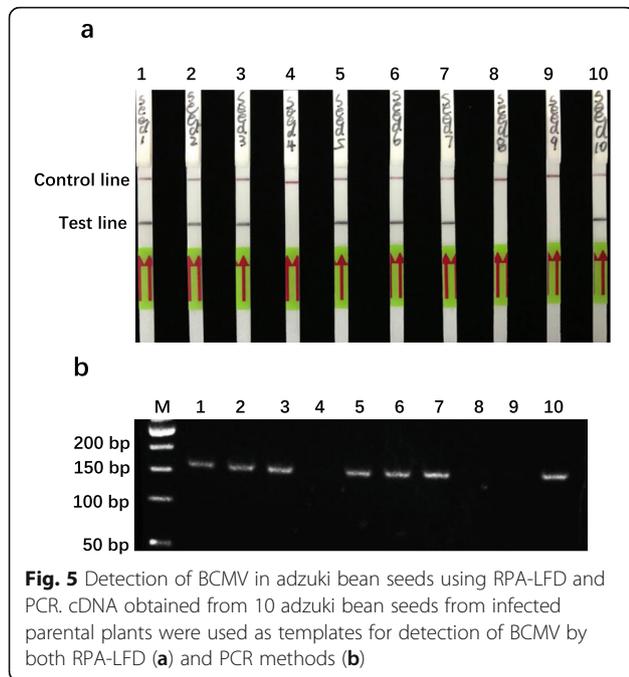
Discussion

We developed an RPA-LFD assay to detect BCMV from legume plants and compared outcomes with a conventional PCR assay. The RPA-LFD assay was 1000 times more sensitive than the PCR, and had no cross-reactivity with three other potyviruses. Reaction time of the RPA-LFD assay was less than 20 min, compared with 150 min for the PCR assay using samples of the same target concentration. Detection of BCMV in adzuki bean leaves and seeds proved applicability of the RPA-LFD assay

beyond soybean. RPA-LFD was able to detect BCMV from crude leaf extracts, without tedious extraction and purification of total RNA. This method is suitable for rapid on-site diagnosis.

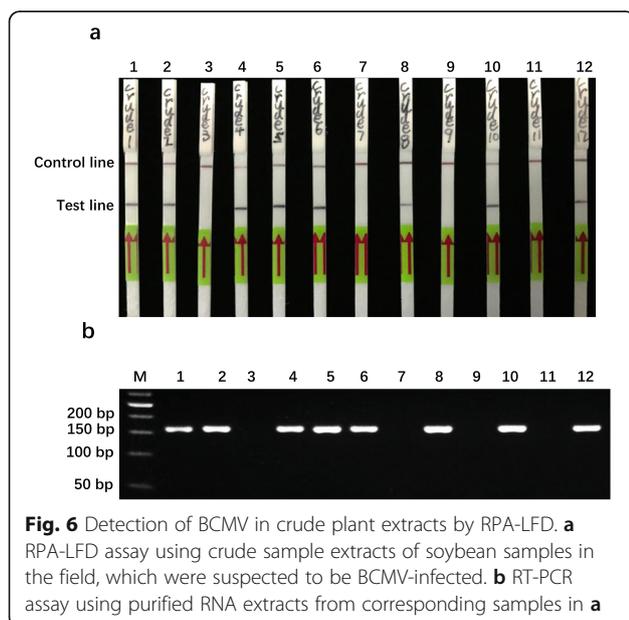
Compared with other plant virus assays, the RPA-LFD method has the following advantages:

- (1) Primer design is simple. Compared with the loop-mediated isothermal amplification method (LAMP), a constant temperature amplification technology



which requires a complex primer design, RPA-LFD detection requires only a pair of primers and one probe to complete amplification;

- (2) It does not require thermal cycling instruments. The RPA-LFD isothermal amplification reaction can be achieved under isothermal conditions from 37 °C–42 °C, requiring only a water heater and thermostat;
- (3) The reaction time is only 15–30 min;



- (4) RPA combined with a lateral flow dipstick (RPA-LFD) avoids a gel electrophoresis step;
- (5) It has high sensitivity and can directly detect BCMV in seeds. Although RT-PCR could detect BCMV in seeds, sensitivity was low. Polysaccharides, proteins and secondary metabolites in legume seeds may interfere with RNA extraction and inhibit subsequent PCR-based assays. As a result, no clear BCMV-CP gene bands were observed using gel imaging system, which may lead to false negatives. In contrast, RPA-LFD is more sensitive than PCR, and the results of detection are more clearly visualized without misjudgment;
- (6) It was able to detect BCMV in soybean and adzuki bean.

Although RPA has clear advantages over PCR in diagnosis of plant viruses, a limitation is the cost. Cost of reagents per sample is much higher than the cost of a PCR. The authors believe that as RPA technology becomes more widely adopted, the cost per unit will eventually be closer to that of PCR reagents.

Conclusions

The RPA-LFD method for detecting BCMV established in this study is simple, specific and highly sensitive. It can detect BCMV in dry seeds and in different legume crops including soybean and adzuki bean. We believe that with the continuous development of RPA technology, it will play a wider role in the rapid diagnosis of BCMV and other viruses of concern in agriculture and elsewhere.

Methods

Sources of plant samples

All BCMV-infected legume plants including soybean (*Glycine max*) and adzuki bean (*Phaseolus angularis*) showing severe mosaic symptom were collected from different regions of Jiangsu Province, China. The positive control for the test is the confirmed BCMV source from the laboratory and the negative control is set as healthy soybean leaves. All samples were stored at –80 °C after PCR detection and sequence determination. The virus samples of SMV and BYMV were stored in our laboratory and TuMV full length cDNA infectious clone vector was kindly provided by Prof Aiming Wang (Agriculture and Agri-Food, Canada).

RNA extraction, cDNA synthesis

Total RNA of soybean and adzuki bean leaves and adzuki bean seeds was extracted using a Plant Total RNA extraction kit from Tiangen Biotechnology Co., Ltd.. The purity and yield of RNA extracts were measured by NanoDrop 2000C microvolume UV-Vis

spectrophotometer. 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. The cDNA was stored at -20 °C.

Crude sample extraction

Approximately 100 mg of fresh soybean leaf tissue was homogenized in a centrifuge tube with 1 mL 1 × PBS buffer (pH 7.4) containing 2% Tween-20. The supernatant of crude extracts were performed reverse transcription immediately after centrifugation at 4 °C.

Detection of BCMV using RPA-LFD

RPA primers were designed to target the partial BCMV CP gene (An alignment of CPs from different strains of NCBI reference sequence) following the manufacturer's specific parameters (TwistDx, Cambridge, UK). RPA was carried out using the materials and protocol (TwistDx, Cambridge, UK) from the TwistAmp® nfo kit. The RPA reaction was performed in a 50-µL reaction volume containing 1 µL of BCMV-infected soybean plant cDNA, 29.5 µL rehydration buffer, 2.1 µL of 10 mM forward primer (RPA-BCMV-F:5'-TTAGATCATCTGTTGGAT TACAAGCCAGAACA-3'), 2.1 µL of 10 mM reverse primer (RPA-BCMV-R:5'-CACACACCATAAAGCCA TTCATCACAATTGA -3'), 0.6 µL of 10 mM probe (BCMV-nfo-probe: [5'FAM] ACAAAGATGCAGTT TGAAATGTGGTACAAT [THF] CTGTGAAGGA AGAGTA [3'BLOCK]) and 2.5 µL of 280 mM magnesium acetate. All RPA samples were incubated at 37 °C for 20 min. For lateral flow analysis, with reference to the kit operation (Milenia Genline HybriDetect test strip, Germany), 5 µL of RPA-amplified products were mixed with 100 µL of assay buffer (HybriDetect assay Buffer) in a new reaction tube. Then the LFD strip was immersed into the mixture and incubated for 5 min at room temperature. A band with one visible line in the control area was considered as negative, and a band with two visible lines in both the control area and test area was considered as positive.

Detection of BCMV using PCR

The PCR reactions were performed using 10 µL 2 × Tap Master Mix (Dye Plus) (Vazyme Biothe, Nanjing, China), 0.2 µM each of forward primer (BCMV-CP-F:5'-TTAG ATCATCTGTTGGATTGG-3') and reverse primer (BCMV-CP-R:5'-CACACACCATAAAGCCATTC-3') and 1 µL cDNA in a 20 µL reaction volume. All samples were denatured at 95 °C for 5 min. Then 30 cycles were performed (95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s), and the last step was 72 °C for 10 min. The obtained PCR product was separated on a 3% agarose gel. After staining with ethidium bromide, images were observed

using a Bio-Rad Molecular Imager gel DOCXR system (Bio-Rad, Hercules, CA, USA).

Specificity and sensitivity assay

The specificity of the designed primers/ probe was tested using RPA-LFD, against other potyviruses infecting legume or no host-pathogen. A positive control is the cDNA from the BCMV-infected soybean samples and the negative control is the cDNA from healthy soybean plant. For the sensitivity assay, a series of 10-fold dilutions (to 10⁵) of cDNA from 4 ng were prepared in diethyl pyrocarbonate (DEPC)-treated water for RPA-LFD and PCR analysis using the RPA primers.

Abbreviations

BCMV: Bean common mosaic virus; BCMV: Bean common mosaic virus; BYMV: Bean yellow mosaic virus; cDNA: Complementary DNA; CP: Coat protein; LAMP: Loop-mediated isothermal amplification method; LFD: Lateral flow dipsticks; PBS: Phosphate buffer saline; PCR: Polymerase chain reaction; RPA: Recombinase polymerase amplification; RPA-LFD: Recombinase polymerase amplification combined with the lateral flow dipstick; RT-PCR: Reverse transcription-polymerase chain reaction; SMV: Soybean mosaic virus; TuMV: Turnip mosaic virus

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Authors' contributions

XYC conceived the project. XYC and XHC supervised the work. JCQ performed most of the experiments with assistance from ZY, XC, DYS and HTC. All authors analyzed the data. JCQ and XYC wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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