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Rapid detection of sweepoviruses through lateral flow dipstick-based recombinase polymerase amplification

He Wang[1,2,3#], Xiukun Yang[1,2,3#], Decai Tuo[2,3], Yonghua Liu[1], Peng Zhou[2,3], Wentao Shen[2,3*], Guopeng Zhu[1*]

[1]College of Horticulture & Hainan Key Laboratory for Quality Regulation of Tropical Horticultural Crops, Hainan University, Haikou 570228, P. R. China; [2]Hainan Key Laboratory for Protection and Utilization of Tropical Bioresources & Institute for Tropical Agricultural Resources, Haikou 571101, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, P. R. China; [3]Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture and Rural Affairs & Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, P. R. China

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Summary. – Sweepoviruses represent a phylogenetic group of begomoviruses that cause significant sweet potato (Ipomoea batatas) production losses in various countries across the world. For rapid identification of sweepoviruses, we developed a technique based on isothermal recombinase polymerase amplification in conjunction with lateral flow dipsticks (RPA-LFD). The optimum reaction conditions for the RPA were 20 min incubation at 37°C. The RPA-LFD specifically detected distinct sweepovirus species, with no other viruses infecting sweet potato causing a cross-reaction. The detection limit of the RPA-LFD was 1.0×10^4 copies of the target DNA molecule per reaction, and it exhibited a 10-fold greater sensitivity than the conventional PCR. Furthermore, when coupled with an alkaline polyethylene glycol-based crude genomic DNA extraction, the entire procedure was completed in 30 min without the use of any special instruments other than a water bath. Therefore, the RPA-LFD technique is a potential sweepovirus diagnostic tool that can be used in the field with fewer available resources.

Keywords: detection; sweepoviruses; recombinase polymerase amplification; lateral flow dipstick

Sweet potato (*Ipomoea batatas*) has long been a staple food and economic crop in both industrialised and developing countries (Neela *et al*., 2019). However, the threat of virus epidemics is a major constraint, which leads to considerable sweet potato production losses worldwide (Loebenstein, 2012). So far, approximately 30 viruses from various families that are known to infect sweet potatoes have been identified (Clark *et al*., 2012; Loebenstein, 2015). Typically, sweepoviruses represent a phylogenetic group of begomoviruses (the family *Geminiviridae*) with high incidence and economic harm and are capable of infecting members of the family *Convolvulaceae*, including sweet potato (Briddon *et al*., 2010; Albuquerque *et al*., 2012; Liu *et al*., 2017). In total, 14 sweepovirus species were accepted by the International Committee on Taxonomy of Viruses in 2020 (https://ictv.global/taxonomy). Sweet potato leaf curl virus (SPLCV) found in China was the first species of sweepovirus identified (Chung *et al*., 1985). Sweepoviruses spread across plants by the insect vector *Bemisia tabaci* and through micropropagation, grafting, or seeds (Valverde *et al*., 2004; Kim *et al*., 2015). They often induce up-

*Corresponding author. E-mail: shenwentao@itbb.org.cn, guopengzhu@163.com; phone: +86-898-6890687. #These authors contributed equally to this work.

Abbreviations: AGE = agarose gel electrophoresis; LFD = lateral flow dipsticks; PEG= polyethylene glycol; RPA-LFD = recombinase polymerase amplification in conjunction with lateral flow dipsticks; ; SPBV = sweet potato badnavirus; SPCFV = sweet potato chlorotic fleck carlavirus; SPFMV = sweet potato feathery mottle virus; SPLC = sweet potato leaf curl; SPLCCV = SPLC China virus; SPLCGxV = SPLC Guangxi virus; SPLCGV = SPLC Georgia virus; SPLCRV = SPLC Canary virus; SPLCSdV = SPLC Shandong virus; SPLCHbV = SPLC Hubei virus; SPGVKV = sweet potato golden vein Korea virus; SPLCSiV-1/2 = SPLC Sichuan virus 1/2 ; SPLCV = sweet potato leaf curl virus; SPVC/G = sweet potato viruses C/G

ward leaf curling with mottle or vein yellowing in young sweet potato plants; however, symptoms are sometimes not seen in older plants (Miano *et al*.,2006). In addition, sweepovirus infections result in sweet potato production losses, quality reduction and cultivar decline (Wasswa *et al*., 2011; Clark *et al*., 2012).

The development of precise and quick diagnostic techniques for sweepoviruses will facilitate rapid screening for generating virus-free sweet potato planting materials, which will aid in the prevention of virus transmission across plants (Wondimu *et al*., 2012). Furthermore, early sweepoviruses field identification is critical for disease treatment (Loebenstein, 2015). The available techniques for the identification of sweepoviruses are PCR, real-time PCR, rolling-circle amplification, and next-generation sequencing technologies (Li *et al*., 2004; Kokkinos *et al*., 2006; Barkley *et al*., 2011; Trenado *et al*., 2011; Gu *et al*., 2014). Nonetheless, these detection methods take a long time and require expensive laboratory equipment and skilled personnel.

Recombinase polymerase amplification (RPA), an isothermal DNA amplification technique with excellent speed and sensitivity, takes 20–30 min and requires a temperature range of 37°C–42°C (Piepenburg *et al*., 2006). Agarose gel electrophoresis (AGE), fluorescent RT-PCR equipment, or lateral flow dipsticks (LFDs) coupled with anti-biotin antibodies and gold-labeled anti-carboxyfluorescein (FAM) are used to identify RPA products (Li *et al*., 2018). RPA in conjunction with LFD (RPA-LFD) enables quick acquisition of results in the visual read-out format and does not require any specific testing apparatus. RPA-LFD can identify pathogen infections quickly and sensitively, which makes this technique appropriate for use in the field and under resource-limited situations (James *et al*., 2015). RPA-LFD is used to rapidly identify plant viruses such as rice black-streaked dwarf virus, little cherry virus 2, tomato spotted wilt virus, bean common mosaic virus, and citrus tristeza virus (Mekuria *et al*., 2014; Zhao *et al*., 2019; Ghosh *et al*., 2020; Lee *et al*., 2021; Qin *et al*., 2021). The current study developed a fast detection method for sweepoviruses by combining RPA-LFD with a crude genomic DNA extraction based on alkaline polyethylene glycol (PEG) (Hwang *et al*., 2013). The specificity and sensitivity of our developed RPA-LFD-based method for sweepoviruses identification were then compared with those of traditional PCR (Li *et al*., 2004).

First, we aligned complete coat protein genes for 14 sweepovirus species or strains [namely SPLCV, sweet potato leaf curl (SPLC) Georgia virus, SPLC South Carolina virus SPLC Canary virus (SPLCRV), SPLC Sao Paulo virus, SPLC China virus (SPLCCV), SPLC Shandong virus (SPLC-SdV), SPLC Henan virus, SPLC Guangxi virus (SPLCGxV), SPLC Hubei virus (SPLCHbV), sweet potato golden vein Korea virus (SPGVKV), sweet potato mosaic virus, SPLC Sichuan virus 1 (SPLCSiV-1) and 2 (SPLCSiV-2)], which were downloaded from GenBank (Acc. Nos. JF768740 KC253233, KX611145, KX033431, FJ529203, MK931309, MK951978, KX033440, NC_024693, MK951968, KJ476509, KC907406, MK951975, MH577011, NC_025681, MF359266, KU323597.1, KX033426, NC_038465, KX033437, OK067241, HQ333144, NC_015317, KT992056, NC_015324, MW574043 and MW574041, respectively). On the basis of the principle of RPA (Piepenburg *et al*., 2006), we selected a highly conserved region (329–541 nt, Acc. No. OK067241) in the CP gene as the target to design the sweepoviruses-specific RPA primer set and probe by using Primer Premier 6 (http://www.premierbiosoft.com/index.html). The forward primer, SPV-F (5'-AACTTCGAGACAGCTATCGT GCCCTACACTG-3'), was unlabeled, whereas the reverse primer, SPV-R (5'-biotin-CCCTTGTAAAAT CCGAGACA CAGACAAACG-3'), was biotin-labeled at the 5' end. The probe, SPV-P (5'-FAM-TGCTGTCCCAATTGCTGCCCGAAGC TATGT-THF-CCGGTTTCAAG AGGC3-C3 spacer-3'), contained one 5'- FAM antigenic label, one internal tetrahydrofuran (THF) residue and one 3'-C3-spacer (Piepenburg *et al*., 2006). These RPA primer pair and probe were prepared by GenScript (Nanjing, China). Furthermore, the full-length genome sequence of SPLCSiV-2 isolate Hainan (Acc. No. OK067241) was cloned into the pTA/Blunt-Zero Cloning vector (Vazyme, China) to create a recombinant plasmid, pSPLCSiV, as the standard DNA template for the development of the sweepoviruses RPA-LFD method. The standard DNA copy number (number/microlitre) was calculated using an online calculator (https://horizondiscovery.com/en/ordering-and-calculation-tools/dna-copy-number-calculation). The sweepovirus RPA reaction was carried out using the ERA kit (GenDx Biotech, China), which is a modified commercial RPA product (Xia *et al*., 2020). First, 48 µl of the ERA reaction Premix solution was prepared by sequentially adding 20 µl of ERA buffer, 0.6 µl of the 10 µM SPV-P probe, 2.1 µl of the 10 µM SPV-F/R primer, DNA template, and nuclease-free water into a lyophilised pellet. Then, the reactions were initiated by mixing 2.0 µl of ERA activator and followed by 20–30 min of incubation in a 37°C–42°C water bath. Subsequently, the FastPure Gel DNA Extraction Mini Kit (Vazyme) was used to purify reaction products, which were then tested using 2.0% agarose gel. Alternatively, we diluted RPA products at 1:200 with nuclease-free water, and then, LFDs (GenDx Biotech) were dipped into the diluted solution for 5 min at room temperature until the control line could be observed. The positive results were indicated by the visible test line, whereas negative reactions only showed a control line.

Six temperature gradients (20°C, 25°C, 30°C, 37°C, 40°C, and 45°C) in 30-min reactions were investigated to deter-

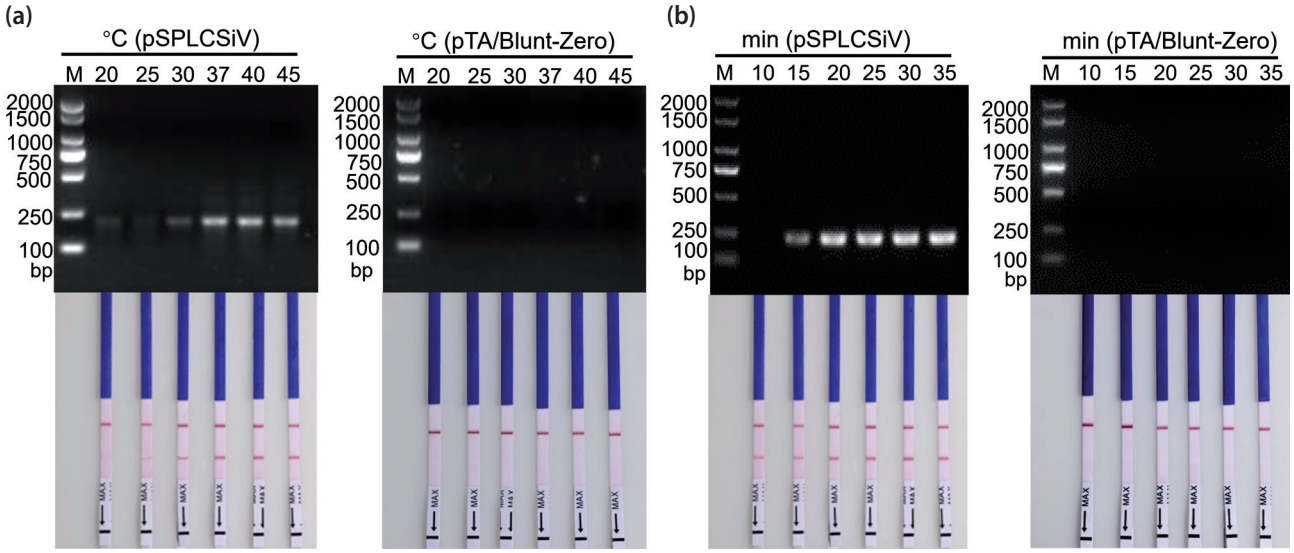

Fig. 1

Optimisation of the RPA-LFD reaction conditions

(a) RPA-LFD reaction temperature optimisation. RPA-LFD assays were performed at 20, 25, 30, 37, 40, and 45°C for 30 min by using standard positive plasmids pSPLCSiV (1×10^5 copies/reaction) as templates. AGE (top panel) and LFD (bottom panel) were conducted to detect RPA amplification products. **(b)** RPA-LFD reaction time optimisation. RPA-LFD assays were conducted at 37°C for 10, 15, 25, 30, 25, and 35 min by using the standard plasmids pSPLCSiV (1×10^5 copies/reaction). The RPA amplification products were detected by AGE (top panel) and lateral flow strip (bottom panel). Lane M: DNA marker. The pTA/Blunt-Zero Cloning vector (1×10^5 copies) served as a negative control.

mine the optim um reaction temperature for RPA-LFD by utilising the templates of standard plasmid pSPLCSiV and pTA/Blunt-Zero Cloning vector (negative control) at 1×10^5 copies/reaction. At 37°C–45°C, AGE of purified RPA reaction products revealed the specific amplification of evident target bands of 214 bp from the pSPLCSiV template (Fig. 1a). Furthermore, by utilising the LFD, we detected unambiguous positive test lines over a wide temperature range of 30°C–45°C (Fig. 1a). These findings indicated that the SPV-F/R primer set and SPV-P probe may be utilised for RPA-LFD assays. Because the RPA amplification efficiency at 37°C was greater than at 30°C, we considered 37°C as the optimal RPA reaction temperature (Fig. 1a). Afterwards, the RPA reactions were run at 37°C for 5, 15, 20, 25, 30, and 35 min to calculate the optimal amplification time. Both AGE and RPA-LFD demonstrated that a 20-min RPA reaction had a greater amplification efficiency than a 15-min reaction and was sufficient for unambiguous detection by LFD (Fig. 1b). After 20 min, no substantial difference was discovered (Fig. 1b). As a result, the optimum reaction conditions for RPA-LFD were at 37°C for 20 min incubation.

We tested RPA-LFD by using some major sweet potato-infecting viruses across China (Gu *et al*., 2014; Liu *et al*., 2017), which included eight sweepoviruses (SPLCSiV-2, SPLCV, SPLCRV, SPLCCV, SPLCGxV, SPLCHbV, SPLCSdV, and SPGVKV), two badnaviruses [sweet potato badnavirus A (SPBVA)/B (SPBVB); the family *Caulimoviridae*], three potyviruses [sweet potato feathery mottle virus (SPFMV), sweet potato viruses C (SPVC)/G (SPVG); the family *Potyviridae*], and sweet potato chlorotic fleck carlavirus (SPCFV; the family *Flexiviridae*). These virus-infected samples were collected and provided by The Chinese Academy of Tropical Agricultural Sciences. Total RNA and DNA were extracted from 100-mg samples by using the RNAprep Pure Plant Plus Kit and Super Plant Genomic DNA Kit (Tiangen, China), respectively. Afterwards, we used the extracted RNA as a template to create cDNA by using PrimeScript 1st-strand cDNA Synthesis Mix (TaKaRa, Japan). By using RPA-LFD, 1.0 ng of total DNA from sweepovirus-infected samples and cDNA from RNA virus-infected samples were separately identified. The positive signal on the LFD test line was found only in the samples infected with eight sweepoviruses (SPLCSiV-2, SPLCV, SPLCRV, SPLCCV, SPLCGxV, SPLCHbV, SPLCSdV, and SPGVKV), and no test bands were detected in the samples infected with other sweet potato viruses (Fig. 2). AGE of the RPA reaction products yielded results that were compatible with those of RPA-LFD (Fig. 2). These results confirmed that our developed RPA-LFD was specific for detecting sweepoviruses. To test RPA-LFD sensitivity, standard plasmids pSPLCSiV at 1.0×10^{10} to 10^1 copies/

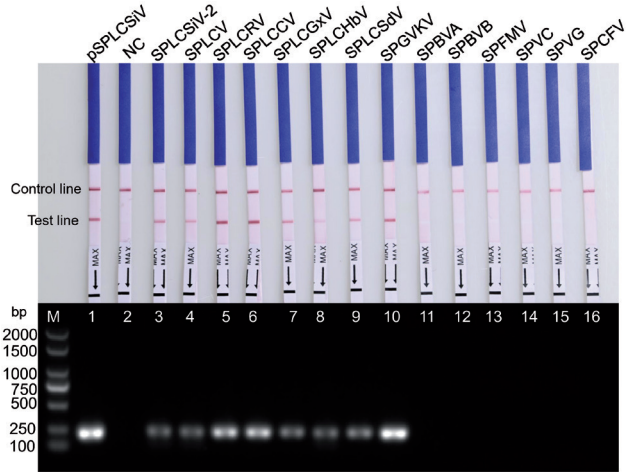

Fig. 2

RPA-LFD assay specificity for sweepoviruses

LFD (top panel) and 2.0% AGE (bottom panel) were conducted to analyse the amplified RPA products. Lane M: DL2000 marker. The plasmids pSPLCSiV and virus-free sweet potato sample served as the positive (Lane 1) and negative control (NC, Lane 2), respectively. Lane 3–16: SPLCSiV-2-, SPLCV-, SPLCRV-, SPLCCV-, SPLCGxV-, SPLCHbV-, SPLCSdV-, SPGVKV-, SPBVA-, SPBVB-, SPFMV-, SPVC-, SPVG-, or SPCFV-infected sweet potato samples.

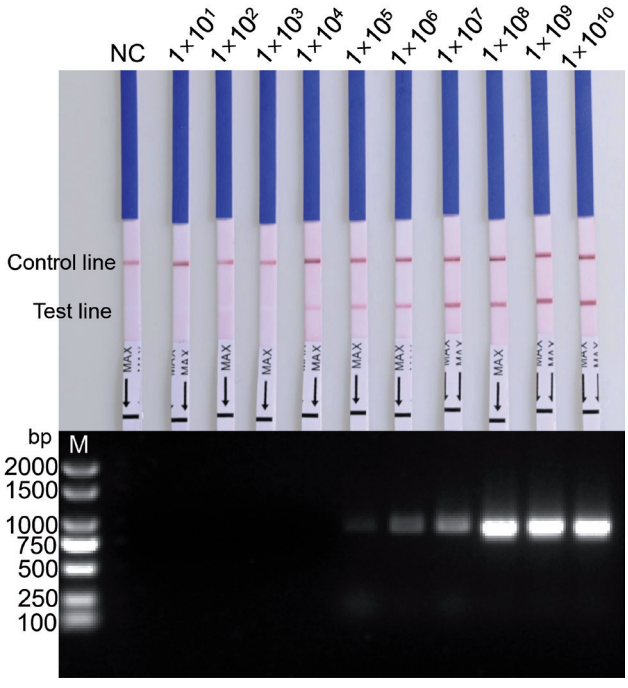

Fig. 3

Sensitivity comparison between RPA-LFD and the conventional PCR assays with standard control plasmids pSPLCSiV at 1×10^{10} to 1×10^{1} copies/reaction

LFD (top panel) was conducted to analyse the amplified RPA products. Then, 2.0% AGE (bottom panel) was conducted to detect the conventional PCR reaction products. Nuclease-free water served as the negative control (NC). Lane M: DL2000 marker.

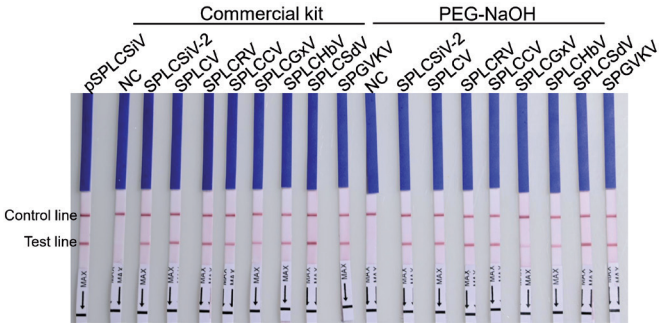

Fig. 4

Comparison between the commercial kit and alkaline (NaOH) PEG-based plant

DNA extraction approach in the RPA-LFD detection of sweepoviruses (SPLCSiV-2, SPLCV, SPLCRV, SPLCCV, SPLCGxV, SPLCHbV, SPLCSdV and SPGVKV). The virus-free sweet potato sample served as the negative control (NC).

reaction were used as RPA templates. We compared the sensitivity of the conventional PCR technique for sweepoviruses detection by using primers SPG1/SPG2 with that of the RPA-LFD test (Li *et al.*, 2004). As a consequence, SPLCSiV RPA-LFD detected the template with 1.0×10^{4} copies/reaction, and its sensitivity was 10 times greater than that of the conventional PCR (1.0×10^{5} copies/reaction) (Fig. 3). Because the RPA-LFD is quite specific and sensitive, it is likely to be used as a fast sweepoviruses detection technique in field applications when combined with an alkaline PEG-based plant genomic extraction approach (Hwang *et al.*, 2013). Briefly, 50 mg sweet potato leaf sample infected with a sweepovirus (namely SPLCRV, SPLCCV, SPLCGxV, SPLCHbV, SPLCSdV, SPLCSiV-2, or SPGVKV) was put into 1.5-ml microcentrifuge tube with 200 µl fresh extraction buffer (20 mM NaOH and 6% PEG 200). The plant tissues were then crushed and ground for 2 min using a pipette tip. We then used the resulting crude lysate as an RPA-LFD template. At the same time, the same volume of sweepovirus-infected tissue was used to extract plant genomic DNAs using the commercial kit (Tiangen). Using the RPA-LFD assays, 2 µl genomic DNAs and crude plant lysates were amplified independently. Each sweepovirus may be identified in a crude plant extract, which is consistent with the genomic DNAs from the commercial kit as templates (Fig. 4).

In total, 23 asymptomatic and 30 symptomatic sweet potato leaf samples with upward leaf curling were obtained in May 2021 from different planting areas in Hainan, China. We extracted plant genomic DNAs from all samples by using the aforementioned PEG-NaOH-based technique, and 2 µl of extracted lysate was utilised to identify sweepoviruses by using RPA-LFD and the

Table 1 Sweepoviruses detection in field samples by using RPA-LFD and conventional PCR

No. of samples	Disease symptoms	RPA-LFD positive[c]	PCR positive[c]
23	No[a]	9	5
30	Yes[b]	30	30

[a]Symptomless sample; [b]Samples with upward curling and vein yellowing of sweet potato leaves; [c]Among the 9 RPA-LFD-detected positive samples, 4 samples tested negative through conventional PCR.

traditional PCR method (Li *et al*., 2004). The findings of both RPA-LFD and PCR assays revealed that 30 of the 30 symptomatic samples were positive for sweepoviruses. By contrast, of the 23 asymptomatic samples, 5 samples were found to be positive for sweepoviruses using PCR and RPA-LFD assays, whereas another 4 RPA-LFD-positive samples tested negative for these viruses in the PCR assay (Table 1). These findings indicated that RPA-LFD exhibited more sensitivity than PCR in identifying low quantities of sweepoviruses.

In conclusion, the specific and sensitive RPA-LFD technique was successfully developed for the diagnosis of sweepoviruses. The combination of RPA-LFD and PEG-NaOH-based crude genomic DNA extraction approach allowed the identification of sweepoviruses in 30 min, without necessitating the use of any specific equipment other than a water bath. Thus, the simple and quick RPA-LFD technique may be useful for detection of sweepoviruses in the field and under resource-limited situations.

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