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Ultra-sensitive detection of papaya ringspot virus using single-tube nested PCR

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Summary. – Aphid-transmitted papaya ringspot virus (PRSV) is the greatest disease threat to the commercial production of papaya worldwide. Specific ultrasensitive assays are important for the early detection of PRSV in the field. We have developed a single-tube nested PCR (STNP) assay to address this need. Two nested PCR primer sets were designed to target the P3 gene of PRSV. The annealing temperatures and concentrations of both primer pairs were optimized to reduce potential competition between primer sets in STNP. The assay is more sensitive than regular RT-PCR as determined by serial dilutions of cDNA and RNA templates and sample extracts from infected plants. STNP is capable of detecting PRSV in plants 7 days post-inoculation, whereas RT-PCR and ELISA are capable of detecting PRSV 14 to 21 days post-inoculation. This new assay can also detect PRSV from virus infected but asymptomatic plants. This system could assist epidemiological studies in the field and in quarantine protocols by enabling early detection of very low PRSV infection rates in the field and in imported plant samples.

Keywords: early detection; quarantine protocols

Introduction

Papaya (*Carica papaya* L.) is a widely grown fruit crop in tropical and subtropical areas that is valued for its nutritional, digestive, and medicinal properties (Manshardt, 1992; Hamim *et al.,* 2014). Several RNA and DNA plant viruses pose a serious threat to papaya production (Tripathi *et al.*, 2008; Maoka *et al.*, 1996; Wang *et al.,* 2013; Chang *et al.*, 2003; Singh-Pant *et al.*, 2012; Yue *et al.*,2009). Among these, *the* aphid transmitted papaya ringspot virus (the genus *Potyvirus*, the family *Potyviridae*) causes the most widespread, destructive and commercially important disease that results in serious economic and agronomic impacts on papaya production worldwide (Tripathi *et al.*, 2008; Lu *et al.,* 2008; Purciful *et al.*, 1984). PRSV is grouped into two types, PRSV-P and PRSV-W, that are serologically indistinguishable but that have different host specificities. PRSV-P infects papaya and cucurbits, while PRSV-W infects only cucurbits (Purciful *et al.*, 1984; Yeh *et al.*, 1984; Chen *et al.*, 2008). PRSV-P causes mosaic, chlorosis, mottling, vein clearing and distortion of the leaves, typical "ringspot" and streaking on fruits, and water-soaked streaks on stems and petioles (Tripathi *et al.*, 2008; Gonsalves *et al.*, 2008). The international trade of papayas threatens commercial papaya production especially with regard to the emergence of new PRSV isolates (Bau *et al.*, 2008; Shen *et al.*, 2014a,b; Tuo *et al.*, 2013). Therefore, the accurate and effective diagnosis of PRSV is crucial for monitoring early infections and minimizing economic losses and the introduction of new PRSV isolates arising from world-wide trade.

Over the past few decades, various diagnostic methods have been developed to detect PRSV including both serological and molecular based methods. ELISA is widely used to detect PRSV, but the sensitivity of this method is less than that of the molecular diagnostic methods – such as PCR, real-time (quantitative) PCR, loop-mediated isothermal amplification (LAMP) and molecular hybridizations (Ling *et al.,* 1991; Chiang *et al.,* 2001; Noa-Carrazana*et al.,* 2006; Cruz *et al.,* 2009; Shen *et al.,* 2014a,b; Usharani*et al.,* 2013). However, highly sensitive real-time PCR requires sophisticated and expensive instruments, chemicals and complicated protocols that limit its use by many laboratories in developing

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Abbreviations: dpi = day(s)-post-inoculation; LAMP = loop-mediated isothermal amplification; PRSV = papaya ringspot virus; STNP = single-tube nested PCR

countries (Shen *et al.,* 2014a,b). A comparatively new and rapid detection assay LAMP requires complex reagents and protocols and also considerable skill in order to use this method reliably. These shortcomings make these molecular assays prohibitive for many laboratories worldwide.

Another very sensitive molecular technique is two-step nested PCR that has been used in laboratories worldwide for sensitive detection of viruses including PRSV (Huo-gen *et al.,* 2000; Jeong*et al.,* 2014). It does not require costly equipment or chemicals like real-time PCR, or the use of complex of protocols like LAMP (Jeong*et al.,* 2014; Dey *et al.*, 2012). However, nested PCR requires the manipulation of previously amplified material and involves the transfer of DNA from one PCR tube to another for the second round PCR raising the risk of cross-contamination (Jeong *et al.,* 2014; Dey *et al.,* 2012; Lin *et al.,* 2010). This potential problem can be overcome by the use of a single-tube nested polymerase chain reaction (STNP) assay capable of reliably detecting very low titer viral targets (Dey *et al.,* 2012).

In the present study, we have developed an ultra-sensitive STNP assay to detect PRSV and compared its sensitivity to regular PCR and ELISA. This new method could be of use by the papaya industry and plant quarantine offices of governments to reliably and sensitively detect very low titers of PRSV.

Materials and Methods

Sample collection, RNA extraction and cDNA synthesis. PRSV-infected papaya leaves were collected from the island of Oahu in Hawaii, USA (Fig. 1) and used to mechanically inoculate PRSV onto 4-week-old healthy papaya cv. Sunset in greenhouse studies (Tennant *et al.,* 1994). Total cellular RNAs were extracted from systemically infected leaves using the RNeasy® plant mini kit (Qiagen, Inc., USA) according to the manufacturer's protocol. RNAs were eluted into 80 µl of RNase -free water and stored at -80°C until used. The yield and quality of RNA samples was monitored by electrophoresis in 1% agarose gels and on a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). cDNAs were synthesized in reverse transcription (RT) reactions of total RNAs as described previously (Dey *et al*., 2012) with minor modifications. The reaction mixture consisted of 2 µl RNA, 1 µl random hexamer primers (50 µg/ml) and 6.5 µl RNase-free H_2O. Then reaction was incubated for 10 min at 72°C and quickly chilled on ice. A cocktail of 5 µl dNTP mixture (2.5 mmol/l each), 4 µl 5x MMLV reaction buffer, 1 µl MMLV reverse transcriptase (200 U/µl) and 0.5 µl RNase inhibitor (40 U/µl) was added. The RT reaction was incubated for 10 min at 25°C followed by 50 min at 42°C. The products were chilled on ice and stored at -20°C until RT-PCR and STNP reactions were performed.

Primer design. Two nested primer sets (outer and inner) were designed targeting the P3 gene of PRSV using Primer 3® software (http://frodo.wi. mit.edu/) (Shen *et al.,* 2014a,b; Dey *et al.,* 2012; Lin *et al*., 2010). In designing the inner primers, a GC content of 40–50%, a melting temperature (Tm) of 53–57°C, primer lengths from 18–22 bp, and predicted amplicon sizes from 120–200 bp were considered. In designing, the outer primers, the GC content was similar to that of inner primers, but the melting temperatures were required to be at least 10°C higher than the melting temperatures of the inner primers. Lengths for the outer primers were similar to that of the inner primers but primers were chosen to produce amplicon sizes from 300–500 bp (Fig. 2) (Dey *et al.,* 2012; Lin *et al.,* 2010). All of the designed primers were checked for their uniqueness to PRSV against the available microbial and papaya sequences in GenBank for their uniqueness to PRSV (Lin *et al.*, 2010).

Sensitivity of STNP. Sensitivity of the STNP assay was compared to conventional RT-PCR using the inner primer set. cDNAs generated from 130 ng of cellularRNAs were serially-diluted in nuclease free water from 0–10^7-fold and then used as templates. We also evaluated STNP sensitivity using similar RNA dilutions of cellular RNAs extracted from PRSV infected papaya leaves in both assays. Serially diluted RNAs (100 ng–100 fg) were converted into cDNAs and the STNP and RT-PCR assays were performed as described above. The sensitivity of STNP was further compared to results from RT-PCR and ELISA using serial dilutions of extracts from infected papaya leaf tissues (Table 1). ELISA was performed according to the manufacturer's (Agdia, USA) instructions and absorbance of 405 nm was measured with a Bio-Rad Model 680 microplate reader (BioRad, USA). Absorbance ratios (I/H ratios) of the infected (I) and healthy tissues (H) were calculated from mean values of absorbance for each extract dilution. A positive/negative threshold was set for each plate at two times the absorbance of healthy control (I/H = 2) for each dilution (Sreenivasulu *et al.*, 2010).

Fig. 1

PRSV-infected papaya samples collected from Island of Oahu, Hawaii

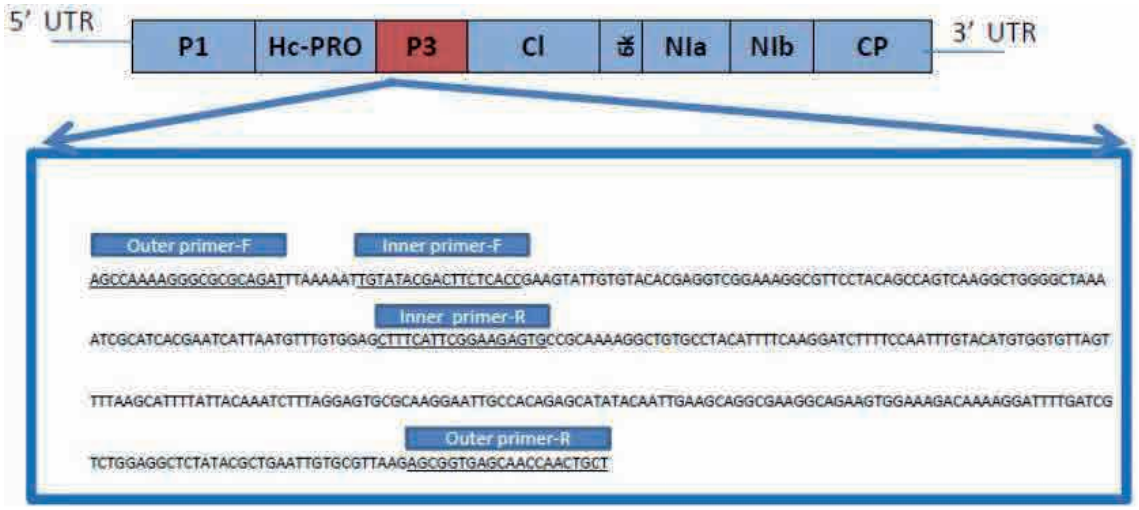

Fig. 2

Primers used in the single tube nested PCR (STNP) assay for the detection for PRSV

F, sense primer; R, antisense primer.

Inoculation of papaya plants to monitor PRSV infection. We mechanically inoculated 4-week-old papaya plants of cv. Sunset with PRSV inoculum prepared from PRSV-infected papaya plants. To prepare the virus inoculum, 6-week post inoculation leaves of papaya plants showing PRSV symptoms were ground in 10 volumes (vol/wt) of inoculation buffer (0.01 mol/l potassium phosphate, pH 7.4) (Tennant *et al.,* 1994). The inoculum was then applied to the leaves of six papaya plants that had been dusted with carborundum. Leaves with inoculum were gently rubbed with glass rods and then rinsed in dH_2O. Inoculated plants were kept in the greenhouse for symptom development. Young leaves were collected from the inoculated plants at 7-day intervals, beginning 7 days post inoculation (dpi) and continuing until 42 dpi. STNP, RT-PCR and ELISA were used to detect PRSV from the inoculated samples at each time point. This experiment was repeated twice.

Detection of PRSV from symptomatic and asymptomatic samples. A total of 52 papaya leaf samples were collected for diagnosis of PRSV from the island of Oahu. Of these 52 samples, 26 displayed typical PRSV symptoms including mosaic, ring spots, and leaf distortions of leaves, and water soaked streaks on petioles. The remaining samples were asymptomatic. An STNP assay was performed as described above and its detection limits compared to those generated by RT-PCR and ELISA.

Results

Sensitivity of single-tube nested-PCR (STNP)

The STNP assay was designed based on similar assays reported earlier (Dey *et al.*, 2012; Lin *et al.*, 2010). For the inner

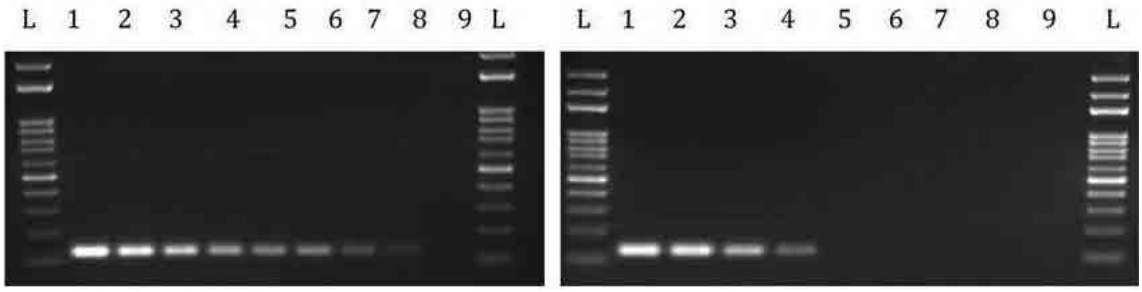

Fig. 3

Sensitivity of STNP (a) and RT-PCR (b) using serially diluted cDNAs prepared from PRSV-infected plant RNA

Lane numbers (1–8) correspond to cDNA dilutions from 0–10^7-fold. Lane 9 is cDNA from healthy control plants. L is the 100 bp ladder. Expected bands are 128 bp.

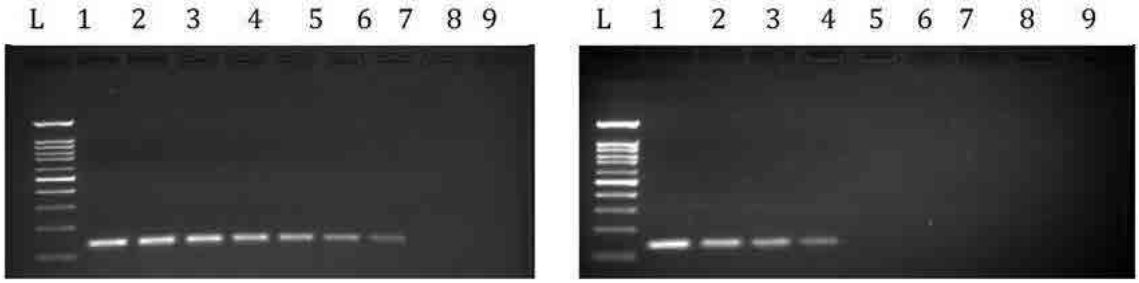

Fig. 4

Sensitivity of STNP (a) and RT-PCR (b) using total RNAs serially diluted from 100 ng – 10 fg.
cDNAs were reverse transcribed from diluted total RNAs. Lanes 1–8 correspond to 100 ng–10 fg of total RNA from papaya leaves. Lane 9 is RNA from healthy control plant. L is 100 bp ladder. Expected band are 128 bp.

and outer primers, annealing temperatures were optimized at 55°C and 65°C, respectively. The optimal amounts of the outer and the inner primers were 0.1 pmol and 10 pmol, respectively.

The detection limit of the STNP assay was determined in replicated experiments using serially-diluted cDNAs as templates generated from RNAs isolated from PRSV-infected plants. The STNP assay was able to detect cDNA templates diluted up to 10^7-fold, whereas the detection limit of conventional PCR was 10^3-fold, as indicated by the presence of the predicted 128 bp amplicon in gel electrophoresis (Fig. 3).

The sensitivities of the newly developed PRSV specific STNP and RT-PCR assays were also compared using total RNAs serially diluted from 100 ng–10 fg RNA isolated from papaya leaves infected with PRSV. cDNAs were reverse transcribed from diluted total RNAs and used as templates in STNP and conventional PCR. STNP was able to detect the template in concentrations of 100 fg per reaction (Fig. 4a), which was one-thousand fold greater than in RT-PCR, which could detect 100 pg per reaction (Fig. 4b).

The sensitivity of the STNP assay was also compared to the sensitivities of RT-PCR and ELISA assays using serial dilutions of extracts from infected papaya leaf samples (Table 1). The STNP could detect PRSV targets in samples at dilutions of at least 1:81920, whereas PRSV targets could only be detected at dilutions of 1:10,240 and 1:320 by RT-PCR and ELISA respectively (Table 1).

Diagnostic evaluations

To evaluate the suitability of STNP for routine assays, two independent experiments were conducted comparing it to regular RT-PCR and ELISA. In the first experiment, healthy plants were inoculated with PRSV and young leaves were collected for PRSV detection 7 dpi, 14 dpi, 21 dpi, 28 dpi, 35 dpi, and 42 dpi using STNP, RT-PCR and ELISA. STNP proved to be the most sensitive assay, capable of detecting PRSV 7 dpi from 16% of the PRSV inoculated plants, whereas regular PCR and ELISA could only detect virus in inoculated plants after 14 and 21 days, respectively. At 21 dpi, RT-PCR and STNP were capable of detecting PRSV positive plants in 50% and 83% of the inoculated plants, respectively. At 28 dpi, PRSV could be detected in 100%

Table 1. Comparison of sensitivities of single-tube nested-PCR, ELISA and RT-PCR for the detection of PRSV in serial dilutions of extracts from infected papaya leaf samples

Dilution (w/v)	ELISA	RT-PCR	STNP
1:10	+[a]	+	+
1:20	+	+	+
1:40	+	+	+
1:80	+	+	+
1:160	+	+	+
1:320	+	+	+
1:640	-[b]	+	+
1:1280	-	+	+
1:2560	-	+	+
1:5120	-	+	+
1:10240	-	+	+
1:20480	-	-	+
1:40960	N.T[c]	-	+
1:81920	N.T[c]	-	+

[a]PRSV positive; [b]PRSV negative; [c]Not tested.

Table 2. Detection of PRSV by STNP, RT-PCR and ELISA from symptomatic and asymptomatic samples

Type of samples	Number of positive samples (total number of samples tested)		
	ELISA	RT-PCR	STNP
symptomatic	26 (26)	26(26)	26 (26)
asymptomatic	3 (26)	12 (26)	20 (26)

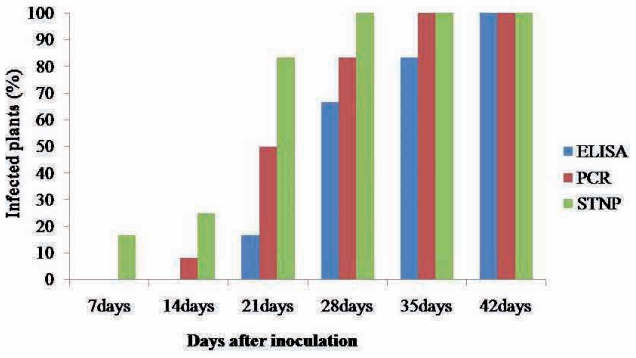

Fig. 5

Comparison of sensitivities of STNP, ELISA and RT-PCR assays for the detection of PRSV infection in inoculated plants

of the inoculated samples using STNP, whereas ELISA and RT-PCR could detect PRSV in 65% and 80% of samples, respectively (Fig. 5).

Fifty-two symptomatic and asymptomatic papaya leaf samples were collected on Oahu, Hawaii and assayed for PRSV using STNP, RT-PCR and ELISA. All the symptomatic samples were positive for PRSV by each of these three techniques. However, when asymptomatic plant samples were tested, PRSV could be detected in 20/26 (77%) using STNP; in 12/26 (46%) using RT-PCR; and in only 3/26 (12%) using ELISA (Table 2), confirming that STNP was more robust and sensitive assay.

Discussion

For decades, serological and molecular methods have been used to detect PRSV in papaya samples collected from the field. The assays currently available to assess PRSV infections can be used to confirm PRSV infection in symptomatic samples (Hamim *et al*., 2018a). However, most of these assays are not able to consistently detect low levels of PRSV that occur in early stages of infection either in fields or in nurseries (Jeong *et al*., 2014). In early stages of infection, PRSV occurs in low titers and is unevenly distributed within papaya tissues, often before the development of any symptoms, or with symptoms that may be produced by abiotic stress (Jeong*et al*. 2014; Hamim personal observation). The early detection of plant viruses and careful monitoring of symptoms are essential to reduce the spread of viruses to new areas or hosts (Jeong*et al*., 2014; Llop *et al*., 2000) and therefore, improved methods to detect PRSV with enhanced reliability and sensitivity in asymptomatic samples are crucial.

Nested PCR assays are capable of detecting extremely low titers of PRSV in diseased plants; with much more sensitivity than other molecular and serological methods (Huo-gen *et al.,* 2000). However, conventional nested PCR, is prone to cross-contamination during manipulation when amplified first-round PCR products are transferred to the second-round PCR (Dey *et al.*, 2012; Llop *et al.;* 2000). These limitations can be overcome by using STNP assay (Jeong *et al.*, 2014; Dey *et al.*, 2012; Anderson *et al.*, 2003). Llop and colleagues have used STNP assay to detect *Erwinia amylovora* in infected plants at rates 20% higher than conventional PCR, and also found that the sensitivity of this assay was comparable to that of nested PCR using sequential reactions.

To improve the detection limits of the PRSV assay and to avoid problems associated with cross-contamination, we have developed an ultra-sensitive STNP assay that has the capability of detecting PRSV with high sensitivity in serially diluted cDNAs, RNAs, and plant extracts. This superior sensitivity was greater than what could be achieved using conventional RT-PCR. Shen *et al.* (2014b) have developed an RT-LAMP assay capable of detecting PRSV in about 1 pg of total RNA extracted from PRSV infected papaya. This is about 10-fold less sensitive than our newly developed PRSV STNP assay. Huo-gen *et al.* (2000) compared the detection of PRSV from PRSV inoculated plants using conventional nested PCR, immunocapture-PCR, ELISA-PCR and ELISA. They found that nested PCR assays were the most sensitive of these techniques, able to detect virus at 3 dpi. In our study, STNP was the most sensitive assay evaluated, allowing reliable detection of PRSV at 7 dpi.

This ultrasensitive STNP is capable of detecting low virus titers in asymptomatic PRSV-infected papaya, and could identify PRSV in a higher percentage of field samples than either RT-PCR or ELISA assays.

STNP provides an ultrasensitive and robust method to diagnose PRSV infection. To our knowledge, this is the first report of the use of this methodology to detect potyvirus infections in any plant host. This novel technique eliminates the potential for cross-contamination of samples by eliminating the transfer of amplification products from one PCR tube to another during two-tube nested PCR. This STNP assay is also more sensitive than regular RT-PCR or ELISA, and can detect PRSV in asymptomatic plants. This new robust assay will facilitate epidemiological studies of PRSV and allows the improvement of governments' quarantine systems by allowing the detection of extremely low infection levels of PRSV in papaya from field and other sources. Similar assays could be useful to detect other recently reported important plant viruses in Hawaii and Bangladesh such as dasheen mosaic virus (Wang *et al.,* 2017a, 2018a), taro bacilliform CH virus (Wang *et al*., 2017b,c), bean yellow mosaic virus (Wang *et al.,* 2017d, 2018b), pepper mottle virus (Wang *et al.,* 2018c), bean common mosaic virus (Green *et al.,* 2017), banana bunchy top virus (Hamim *et al.,* 2017), tomato leaf curl Bangladesh virus, tomato leaf curl Joydebpur virus and tomato leaf curl New Delhi virus (Hamim *et al.,* 2018b) very

early in non-symptomatic diseased tissues in various tropical and sub-tropical plant species resulting in more effective disease management strategies (Hamim *et al.,* 2018a).

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