

Erratum

The Plant Pathology Journal is publishing an Editorial Expression of Concern regarding the following article: “M. Sreenivasulu and D. V. R. Sai Gopal. 2010. Development of Recombinant Coat Protein Antibody Based IC-RT-PCR and Comparison of Its Sensitivity with Other Immunoassays for the Detection of *Papaya ringspot virus* Isolates from India. Plant Pathol. J. 26:25-31.” Due to a printer’s error, an uncorrected author proof of this manuscript appeared in the previous issue. The editors decide to publish the corrected version in this issue.

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Erratum

Development of Recombinant Coat Protein Antibody Based IC-RT-PCR and Comparison of Its Sensitivity with Other Immunoassays for the Detection of *Papaya ringspot virus* Isolates from India

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Papaya ringspot virus (PRSV) causes the most widespread and devastating disease in papaya. Isolates of PRSV originating from different geographical regions in south India were collected and maintained on natural host papaya. The entire coat protein (CP) gene of *Papaya ringspot virus* biotype-p (PRSV-P) was amplified by RT-PCR. The amplicon was inserted into pGEM-T vector, sequenced and sub cloned into a bacterial expression vector pRSET-A using a directional cloning strategy. The PRSV coat protein was over-expressed as a fusion protein in *Escherichia coli*. SDS-PAGE gel revealed that CP expressed as a ~40 kDa protein. The recombinant coat protein (rCP) fused with 6x His-tag was purified from *E. coli* using Ni-NTA resin. The antigenicity of the fusion protein was determined by western blot analysis using antibodies raised against purified PRSV. The purified rCP was used as an antigen to produce high titer PRSV specific polyclonal antiserum. The resulting antiserum was used to develop an immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) assay and compared its sensitivity levels with ELISA based assays for detection of PRSV isolates. IC-RT-PCR was shown to be the most sensitive test followed by dot-blot immunobinding assay (DBIA) and plate trapped ELISA.

Keywords : IC-RT-PCR, Papaya ringspot virus, polyclonal antibodies, recombinant coat protein

The papaya and watermelon biotypes of *Papaya ringspot virus* (PRSV-P&W) cause major disease in papaya and cucurbits, respectively in tropical and subtropical regions including Indian subcontinent and the disease can result in significant yield losses (Gonsalves, 1998; Varma, 1988). The disease has become a major threat to papaya cultivation throughout India by rendering orchards economically unproductive. PRSV was first reported in western India in 1958 (Capoor and Vrama, 1958) and since then it

has spread to different geographical locations irrespective of the agro-climatic conditions, resulting in crop losses of up to 85-90% (Hussain and Varma, 1994). PRSV, a definitive member of the genus *Potyvirus* of the family *Potyviridae* (van Regenmortel, 2000), is non-persistently transmitted by numerous species of aphids in nature and particles are flexuous filamentous rods of about 780×12 nm in size (Gonsalves and Ishii, 1980). The virions contain a positive sense, single stranded RNA genome approximately 10.3 kb in length surrounded by a coat protein (CP) consisting of a single protein subunit of molecular weight 36 kDa (Yeh et al., 1992). Based on the host range specificity, PRSV is classified into two types that cannot be distinguished by serological methods. PRSV type P infects papaya and has a limited experimental host range in cucurbits. PRSV type W infects wide range of economically important cucurbit crops, but, it does not infect papaya (Purcifull et al., 1984). Although both types are serologically related, observations suggested that papaya is the major source for the spread of PRSV type P (Yeh et al., 1984).

The efficient production of good quality antiserum to potyviruses is an uphill task under conventional methods due to difficulty in purification of sufficient quantities of their filamentous particles. The virus particles are relatively low stable and they tend to aggregate with plant debris or among the elongated virus particles themselves. This leads to considerable losses of virus during the purification process, thereby requiring only gentle and highly labor-intensive purification methods, with special attention to removal of plant proteins. The purification of PRSV from papaya leaf was shown to be difficult as papaya latex causes aggregation of virus particles during purification steps. At higher temperatures, especially in south Indian climatic conditions, propagation of PRSV on alternative cucurbitaceous plants for purification is also not practical. Polyclonal antibodies raised against purified virions contaminated with host tissue components, cross react with host antigens and often give variable background reactions, thus limiting their use in ELISA based diagnostic methods. The difficulties associated with the development of antibodies

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of good quality against virions purified by conventional methods, can be overcome by cloning and expression of CP gene in *E. coli* and using the expressed CP as an antigen for immunization. Though immunological methods were found to be most popular, inexpensive and widely used techniques for detection of plant viruses, they are comparatively less sensitive than PCR based methods and sometimes fail to detect virus in samples at low concentrations. RT-PCR, however, is not a suggestible method for routine large scale testing of virus samples as the extraction of total nucleic acid is expensive, time consuming and not amenable for processing of a large number of samples. To overcome the difficulties and drawbacks to the application of ELISA and RT-PCR techniques in the study and diagnosis of plant viral pathogens with RNA genomes, IC-RT-PCR was developed as an alternative approach for sensitive detection and as well as for batch testing of several RNA viruses (Mumford and Seal, 1997; Nolasco et al., 1993).

The present paper reports the expression of PRSV CP in *E. coli*, the use of the purified recombinant protein as an immunogen for the production of a high titer PRSV specific polyclonal antiserum and the development of recombinant coat protein antibody based IC-RT-PCR for diagnosis of PRSV isolates. We report that the IC-RT-PCR technique is more sensitive compared to ELISA and DBIA.

Materials and Methods

Virus isolates and maintenance. PRSV P isolates used in this study were collected from commercial papaya orchards in South Indian states. All isolates were maintained on their natural host papaya in an insect-proof glasshouse by mechanical inoculation. Leaves from papaya plants showing symptoms such as vein clearing, mild mottle, leaf blistering, leaf distortion and filiformy were collected from Kodur (PRSV-APRa), Kovvur (PRSV-APKo), and Hyderabad (PRSV-APTe) in Andhra Pradesh state; Hospet (PRSV-KAHo) and Gulbarga (PRSV-KAGu) in Karnataka state;

Tiruvallur (PRSV-TATi) in Tamil Nadu state and Calicut (PRSV-KECa) in Kerala state of south India.

RNA extraction and RT-PCR. The total RNA from 100 mg of fresh healthy and PRSV (PRSV-APRa isolate) infected papaya leaf tissue was isolated using RNeasy plant mini kit according to the manufacturer's instructions (Qiagen). The resulting total RNA was incubated with oligo (dT) primer at 70 °C for 5 min and snap-chilled on ice for 2 min. cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas) at 37 °C for 1 h. The sequence and position of oligonucleotide primers used to amplify the 3' terminal region encoding C-terminal region of NIB, complete CP and 3' UTR (size ~1.7 kb) are shown in Table 1. Two to three µl of cDNA was amplified in a 50 µl reaction volume containing 2.5 units of *Taq* DNA polymerase (Fermentas), 10 pmol of forward (PRSV-F) and reverse primers (PRSV-R), 2.25 mM MgCl₂ and 0.2 mM each of the dNTPs. PCR amplification conditions included an initial denaturation cycle of 2 min at 94 °C followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 30 sec at 45 °C and extension for 1 min at 72 °C with a final extension of 30 min at 72 °C.

Cloning and nucleotide sequencing. The PCR-amplified fragments were purified using QIAquick PCR purification kit (Qiagen) and then cloned into pGEM-T easy cloning vector (Promega) according to the manufacturer's instructions. The resulting recombinant plasmid (pGEM-APRa) was transformed into *E. coli* strain DH₅α cells. The selected clones with expected size DNA inserts were sequenced using T7 and SP6 universal primers and a gene specific primer PRSV-R_i at MWG Biotech Pvt. Ltd., Bangalore.

Cloning of PRSV CP gene in an expression vector. Based on the sequence data of 3' terminal region (~1.7 kb) of PRSV-APRa isolate (Accession No. AY839863), a primer pair was designed for the site-specific amplification

Table 1. Description of oligonucleotide primers used in this study. Primers PRSV-F, PRSV-R and PRSV-R_i were designed from the PRSV genomic sequence in the GenBank (Accession No. NC_001785) while CP-F and CP-R were designed based on the sequence of PRSV-APRa isolate (Accession No. AY839863)

Primers	Sequence ^a	Orientation	Sequence Position
PRSV-F	5' ATCACAATGTATTACGC 3'	Sense	8615-8631
PRSV-R	5' CTCTCATTCTAAGAGGCTC 3'	Antisense	10311-10329
^b PRSV-R _i	5' CCGCGTTACTRAAGTGAGCCAT 3'	Antisense	9798-9819
CP-F	5' <u>CGGGATCCT</u> CAAAAAGTTTAGATGA 3'	Sense	9197-9213
CP-R	5' AACCC <u>AAGCTTT</u> TAGTTGCGCATA 3'	Antisense	10111-10123

^a Underlined sequences represent the restriction sites for BamH1 and Hind III in CP-F and CP-R, respectively.

^b Primer used for sequencing.

IUPAC Code for mixed bases

R=A/G

of the entire CP coding region. The specific recognition sequences for the restriction enzymes *Bam* HI and *Hind* III (underlined) were created at the 5' end of the sense (CP-F) and anti sense primer (CP-R), respectively to facilitate directional cloning of the CP gene into the expression vector. The CP gene was amplified from clone pGEM-APRa using the above primer pair under the following conditions: initial denaturation cycle of 10 min at 94°C followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 45 sec at 55°C and extension for 1 min at 72°C with a final extension of 10 min at 72°C. The amplified CP gene was cloned into the pGEM-T easy vector to produce pGEM-CP construct that was transformed into *E. coli* strain DH₅α. After digestion of pGEM-CP with *Hind* III and *Bam* HI, the released CP gene fragment was gel purified from agarose using a Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions and ligated to a similarly digested expression vector pRSET-A (Invitrogen) containing N-terminal hexa-histidine (6x His tag) which tags the expressed protein facilitating easier purification by affinity chromatography. The resulting recombinant plasmid pR-CP (pRSET vector containing the CP gene) was subsequently transformed into the *E. coli* strain DH₅α. Transformants were screened by digesting recombinant plasmids with *Hind* III and *Bam* HI and potential recombinants were sequenced using the universal forward and reverse primers to confirm the integrity of the cloned CP gene. For the expression of the CP, pR-CP construct was transformed into *E. coli* strain BL21 (DE3) pLys S (Novagen).

Expression of coat protein in *E. coli* and purification of fusion protein. Induction of the expression of CP was optimized as described by Sambrook and Russell (2001). 500 ml of Luria-Bertani broth containing 50 µg/ml ampicillin was inoculated with 1% overnight culture of BL21 cells harboring pR-CP construct and grown at 37°C. When the bacterial suspension reached OD_{600 nm} of approximately 0.6, the cells were induced with 1.0 mM IPTG and harvested 3 h later. The cells were re-suspended in lyses/binding buffer (50 mM NaH₂PO₄, 10 mM Tris Cl, 100 mM NaCl, 0.1% SDS and 0.1% Triton X-100, pH 8.0) and frozen at -20°C for overnight. After thawing to room temperature, the cells were sonicated for further disruption and the cell lysate was subjected to centrifugation at 12000 rpm for 10 min to separate soluble and insoluble fractions. The supernatant was mixed with 1 ml of Ni-NTA resin (Invitrogen), which had been pre-equilibrated with the binding buffer at 4°C for 30 min, and allowed to rotate in the shaker for 45 min to facilitate the protein binding to the resin. A column prepared with the above protein-resin complex was washed extensively with the wash buffer (20 mM NaH₂PO₄, 10 mM Tris Cl, 500 mM NaCl, 0.1% SDS and 0.1% Triton X-100,

pH 6.0) till the absorbance of the washings at 280 nm was negligible. The bound protein was then eluted with elution buffer (20 mM NaH₂PO₄, 10 mM Tris Cl, 500 mM NaCl, 0.1% SDS and 0.1% Triton X-100, pH 4.0) into 1 ml fractions. Fractions were analyzed by 10% SDS-PAGE (Laemmli, 1970) and the protein in each fraction was estimated by UV-Spectrophotometry. The fractions containing a protein concentration greater than 0.1 mg/ml were pooled and dialyzed for 24-36 h to remove free SDS against 5 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM EDTA. The renatured protein was concentrated by using centrifugal filter (PALL life sciences) with 10-kDa molecular weight cut off membrane.

Western blot analysis and production of antiserum to recombinant CP. Ni-NTA column purified protein, healthy and PRSV infected papaya total leaf proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane as described by Towbin et al. (1979) using semi-dry blotter (Millipore). The proteins on the membrane were probed with antibodies raised against purified PRSV-W virions followed by detection with alkaline phosphatase conjugated anti-rabbit antibody. The membrane blots were developed using the BCIP/NBT colorimetric system (Bangalore Genei, India). Polyclonal antibodies were raised against purified recombinant coat protein (rCP) by immunizing 3 to 4 months old New Zealand white rabbit weekly for four weeks. All injections were given intramuscularly into the thigh muscles of both legs alternatively. 1 mg of rCP in 500 µl was emulsified with an equal volume of Freund's complete adjuvant for the first injection and this was followed by three injections of 600 µg in Freund's incomplete adjuvant. The antiserum was collected from rabbit weekly for eight weeks starting from one week after the last injection and tested reactivity and specificity by SDS-immuno diffusion test using purified rCP and partially purified PRSV infected leaf antigens.

Development of IC-RT-PCR for detection of PRSV isolates. IC-RT-PCR was performed as described by Nolasco et al. (1993). Healthy and PRSV infected leaf extracts were prepared by grinding tissue in 500 mM Tris-HCl, pH 8.2 containing 2% polyvinyl pyrrolidone, MW 40,000; 0.01 M Na₂SO₃; 3 mM NaNO₃; 140 mM NaCl and 0.05% Tween 20 at a rate of 1 g/9 ml. The extracts were clarified by centrifugation at 10,000 rpm for 5 min. Immunocapture of virions was carried out using a coating and trapping method adapted from Clark and Adams (1977). Clarified PRSV infected and healthy leaf extract at 1:10 dilution was added to sterile PCR tubes (50 µl/tube) that had been previously coated with rCP antiserum (1:1000) diluted in coating buffer. After an overnight incubation at 4°C, the tubes were

washed thrice with PBS-T and once with sterile distilled water. Immunocaptured tubes were incubated with Oligo (dT) primer (2.5 μ M final conc.) at 80°C for 10 min and snap chilled on ice. cDNA was synthesized in a 20 μ l reaction mix using M-MuLV reverse transcriptase (Fermentas). PCR was performed using a 5 μ l aliquot of the cDNA mix as template and 10 pmoles each of the CP-F and CP-R primers under the conditions as described above for the CP amplification. The amplified products were analyzed by 1% agarose gel (Sambrook et al., 1989). Seven PRSV isolates collected from states of south India were tested as described above.

Comparison of IC-RT-PCR with ELISA and DBIA. To test the sensitivity levels of IC-RT-PCR in detection of PRSV, comparative analyses was carried out with double antibody sandwich (DAS) & direct antigen coating (DAC) ELISA and dot-blot immunobinding assay (DBIA) using serial dilutions of PRSV infected leaf sap. Healthy and PRSV-APRa isolate infected leaf extracts were prepared by grinding tissue in 0.1 M carbonate bicarbonate buffer, pH 9.6 (for DAC-ELISA and DBIA) or in 500 mM Tris-HCl, pH 8.2 containing 2% polyvinyl pyrrolidone, MW 40,000; 0.01 M Na₂SO₃; 3 mM NaNO₃; 140 mM NaCl and 0.05% Tween 20 (for DAS-ELISA and IC-RT-PCR) at a rate of 1 g/9 ml. Dilutions ranging from 10⁻¹ to 10⁻⁴ of clarified extract were tested by IC-RT-PCR as described above.

The DAC- and DAS-ELISA methods were done essentially as described by Clark and Adams (1977) and Hobbs et al. (1987), respectively. A 200 μ l aliquot was used at each step and each sample was duplicated. Plates were coated with different dilutions ranging from 10⁻¹ to 10⁻⁴ of PRSV infected leaf extracts. Healthy leaf extract of same dilutions were used as negative controls. In DAS-ELISA, the plate was initially coated with 1/1000 dilution of recombinant coat protein (rCP) antiserum and in both types of ELISA, antigens were detected with 1/10,000 dilution of antiserum. 1/10,000 dilution of Alkaline phosphatase-conjugated anti-rabbit IgG (Bangalore Genei, India) was used to reveal the antigen-antibody complex, and the substrate, di-sodium p-nitrophenyl phosphate (Sigma), diluted to 1 mg/ml in substrate buffer (10% diethanolamine, pH 9.8) was added to the plates. Colour reactions were stopped using 3 N NaOH and the absorbance was measured at 405 nm with a Bio-Rad Model 680 Microplate reader. Infected and healthy absorbance ratios (I/H ratios) were calculated on mean values of absorbance for each antigen dilution. I/H ratios were plotted against each antigen dilution. A positive-negative threshold was set for plate as two times the value of the O.D. of healthy control (I/H=2) for each dilution.

DBIA was performed as described by Berger et al. (1985). The clarified PRSV infected leaf extract dilutions

(10⁻¹ to 10⁻⁴) were dotted on nitrocellulose membrane strip. 1/10 dilution of healthy leaf extract was used as negative control. 1/10,000 dilution of rCP antibodies were used as primary antibodies and ALP- labeled goat anti rabbit antibodies as secondary antibodies. Substrate was BCIP/NBT for ALP system.

Results

Amplification, cloning and sequencing of the 3' terminal region of PRSV genome. The 3' terminal region of the genome of PRSV isolate APRa was amplified with a primer pair "PRSV-F/PRSV-R" on the cDNA by PCR, which yielded a product of approximately 1.7 kbp fragment, and no such amplification was observed on cDNA of healthy papaya tissue. Ligation of amplicons into pGEM-T easy vector resulted in the recombinant clone (pGEM-APRa). The sequence data revealed that the 3' terminal region contained a single ORF encoding a polyprotein that included a C-terminal part of the N1b protein and complete CP. The ORF was followed by an UTR of 209 nucleotides excluding the poly (A) tail.

Expression of PRSV CP and production of its antiserum. PCR on pGEM-APRa clone with a primer pair of CP-F and CP-R yielded a product of 919 bp fragment, which contains the complete CP coding sequence and Bam HI & Hind III restriction sites at 5' & 3' ends, respectively. After sub cloning the Bam HI/Hind III digested CP coding region into the similarly digested expression vector pRSET-A, the protein was expressed in *E. coli* strain BL21 (DE3) pLys S by inducing with 1 mM IPTG. A protein of about 40 kDa in size was detected by SDS-PAGE in lysate of induced BL21 cells harboring pR-CP clone, but not in lysates of non induced bacterial cells with the same plasmid or induced cells containing the pRSET-A vector alone (Fig. 1a). Expressed CP was Ni-NTA column purified and eluted fractions were analysed on SDS-PAGE (Fig. 1b). Western blot analysis showed that the Ni-NTA column purified 40 kDa protein and 36 kDa viral CP in extracts PRSV infected leaf were reacted with antibodies produced against purified PRSV particles whereas no protein in the extracts of healthy papaya leaf was reacted, thus confirming the homology of the expressed protein to the native PRSV CP (Fig. 1c).

Polyclonal antiserum to the PRSV rCP was successfully prepared by injecting a rabbit with a total of 2.5-3.0 mg recombinant coat protein. After four weekly injections, antiserum was collected and tested its reactivity and specificity by SDS-immunodiffusion test. The antiserum reacted strongly with Ni-NTA column purified protein and with native CP of PRSV in infected papaya leaf extract, but not reacted

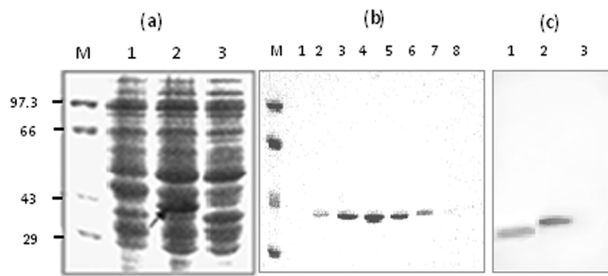


Fig. 1. (a) SDS-PAGE gel showing the expression of PRSV CP in *E. coli*: M: Protein molecular weight markers (Medium Range; Bangalore Genei, India); Lane 1, Induced *E. coli* BL21 carrying pRSET-A vector alone; 2, Induced *E. coli* BL21 with pR-CP; 3, Uninduced *E. coli* BL21 with pR-CP; (b) SDS-PAGE gel showing Ni-NTA affinity purification of expressed CP. M: Protein markers; Lane 1-8, Fractions of eluted CP; (c) Western blot analysis of the PRSV CP expressed in *E. coli*. Lane 1, PRSV-APRa infected papaya leaf proteins; 2, Ni-NTA column purified CP; 3, Healthy papaya leaf proteins.

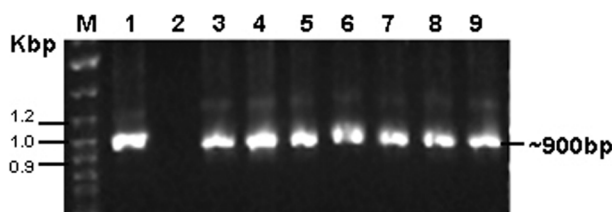


Fig. 2. Diagnosis of PRSV isolates collected from different geographical locations in south India by IC-RT-PCR using rCP antiserum and a primer pair CP-F and CP-R. Lanes 3-9. PRSV isolates APRA, APTe, APKo, TATi, KAGu, KAHo and KECA, respectively. Lane 1. Positive control. Lane 2. Healthy papaya. M: DNA ladder (GeneRuler™ DNA Ladder Mix, 100-10,000 bp; Fermentas).

with proteins in healthy leaf extract (data not shown).

Detection of PRSV by IC-RT-PCR and comparison of its sensitivity with other immunoassays. The rCP antibody based IC-RT-PCR was successfully developed and tested for detection of PRSV isolates. The predicted size of DNA product (~900) was amplified from all seven isolates

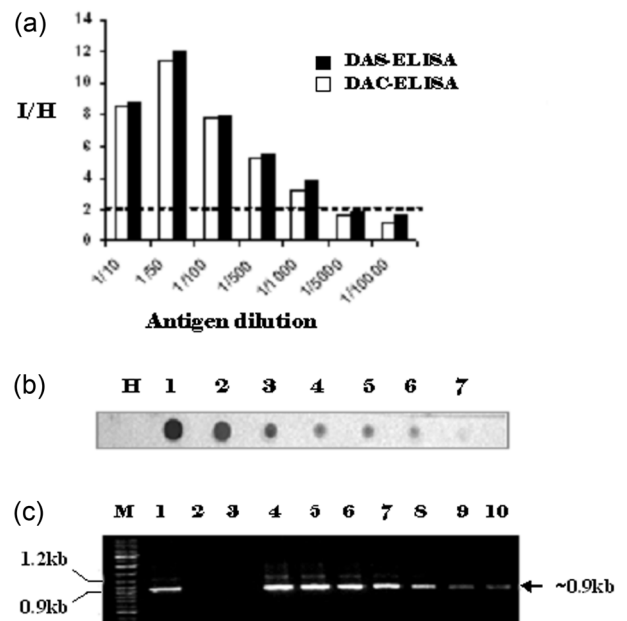


Fig. 3. Comparison of the sensitivities of assays for detection of PRSV (a) Detection limits of DAC- and DAS ELISA. Results were presented as O.D. ratios between the infected and healthy sample (I/H). The horizontal dotted line at I/H=2 represents the set positive-negative threshold. (b) DBIA analysis: H: healthy leaf extract at 1/10 dilution; Lane 1-7, PRSV infected papaya leaf extracts at 1/10, 1/50, 1/100, 1/500, 1/1000, 1/5000 and 1/10000 dilutions, respectively. (c) IC-RT-PCR analysis: M: DNA ladder (GeneRuler™ DNA Ladder Mix, 100-10,000 bp; Fermentas); Lane 1, positive control; lane 2, PCR without template DNA; lane 3, healthy leaf extract at 1/10 dilution; lane 4-10, PRSV infected papaya leaf extracts at 1/10, 1/50, 1/100, 1/500, 1/1000, 1/5000 and 1/10000 dilutions, respectively.

tested, but no such amplification was observed in extracts from healthy papaya (Fig. 2). The antiserum raised against rCP was assayed in ELISA, DBIA and IC-RT-PCR using serial dilutions (10^{-1} - 10^{-4}) of PRSV-AP Ra isolate infected papaya leaf sap. The predicted size of DNA product (~900) was seen in IC-RT-PCR analysis using PRSV infected leaf extract dilutions of up to 1/10000 and such a specific product was not found in healthy control (Fig. 3c). In DBIA,

Table 2. Comparison of the sensitivity of ELISA, DBIA and IC-RT-PCR to detect PRSV in infected leaf extract

	^a	1/10	1/50	1/100	1/500	1/1000	1/5000	1/10000
DAC-ELISA	- ^b	++	+++ ^c	++ ^d	++	+ ^e	-	-
DAS-ELISA	-	++	+++	++	++	+	-	-
DBIA	-	+++	+++	++	+	+	+	-
IC-RT-PCR	-	+++	+++	+++	++	++	+	+

^a Healthy papaya

^b Negative result or I/H ratio less than two or no dot on membrane or no band on the gel.

^c I/H ratio more than ten or strongly reacted dot on the membrane or a strong band on the gel.

^d I/H ratio greater than five but less than ten or dot on the membrane or a band on the gel with moderate intensity.

^e I/H ratio greater than twice but less than five or dot on the membrane with less intensity or a weak band on the gel.

the antiserum gave a positive reaction in dots of infected leaf tissue and no color development in dot of healthy plant tissues (Fig. 3b). Though the dilution end point of the infected leaf sap in reacting with rCP antiserum in both types of ELISA was same, in DAS-ELISA high I/H values were obtained for all dilutions (Fig. 3a). The results demonstrate the higher sensitivity of IC-RT-PCR when compared to two types of ELISA methods and DBIA. The detection limit for ELISA and DBIA were found to be 1/1000 and 1/5000 respectively, while IC-RT-PCR was able to detect PRSV at all dilutions including the highest dilution of 1/10000 (Table 2). The sensitivity of DBIA was found to be five times more than that of ELISA and the sensitivity of IC-RT-PCR was appeared to be ten-fold and two-fold more than those of both types of ELISA and DBIA, respectively.

Discussion

The increasing number of viral sequences available in databases and wide spread use of PCR has enabled many scientists to amplify and clone viral genes into expression vectors, purify the recombinant proteins and use them for producing antibodies. In plant virology, this approach has been applied for detection and localization of several non structural viral proteins (Kallender et al., 1988; Kormelink et al., 1994; van Poelwijk et al., 1993; Mullineaux et al., 1988) and also for production of polyclonal antibodies against several viruses that are less stable and difficult to purify from plant material (Abouzid et al., 2002; Hema et al., 2003; Hourani and Abou-Jawdah, 2003; Ling et al., 2000; Nikolaeva et al., 1995; Targon et al., 1997). In this study, we also successfully cloned the PRSV CP gene, efficiently expressed in a bacterial expression system and used it to produce a high titre PRSV-specific polyclonal antiserum. The cloning strategy used for construction of pR-CP clone resulted in the addition of 36 amino acids to the N-terminus of CP sequence (34 from pRSET-A vector and 2 from sense primer). Hence the CP was expressed as a fusion protein with an estimated MW of about 40 kDa, which is about 4 kDa more than expected MW of 36 kDa. The additional 36 amino acids at the N-terminus of the expressed CP did not affect its antigenicity, which is indistinguishable from the native viral CP.

The bacterially expressed PRSV CP was proved to be good immunogen, yielding antiserum comparable to the antiserum produced against purified PRSV virions (data not shown). The titer of the antiserum against PRSV, as determined by indirect DAC-ELISA, reached approximately 10^{-7} four weeks after the last injection and the results obtained with the above three detection methods showed that the antibodies produced against recombinant CP fusion protein are of good quality and specificity for diagnosis of

PRSV. The antiserum at 1/10,000 dilution could detect PRSV in infected plant extracts by both plate trapped and dot ELISA methods without perceptible background and this dilution was selected for routine application. The IC-RT-PCR was the method of choice for detecting low concentrations of PRSV in papaya and in various cucurbits as this method was reported to be more sensitive than ELISA and DBIA and could detect the virus in leaf extracts of at least 10^{-4} dilution. Though the ELISA methods were found to be less sensitive than DBIA and IC-RT-PCR in detection of PRSV, still the ELISA methods were able to detect PRSV in infected sap of dilution 1/1000. This demonstrates the applicability of ELISA for batch testing of samples. In addition to field diagnosis, the rCP antiserum can be useful in breeding programs, both traditional and transgenic approaches that are used for production of PRSV resistant papaya varieties.

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References

- Abouzid, A. M., Freitas-Astua, J., Purcifull, D. E., Beckham, K. A., Crawford, W. E., Peterson, M. A., Peyser, B., Patte, C. and Heibert, E. 2002. Serological studies using polyclonal antisera prepared against the viral coat protein of four Begomoviruses expressed in *Escherichia coli*. *Plant Dis.* 86:1109-1114.
- Berger, P. H., Thornbury, D. W. and Pirone, T. P. 1985. Detection of pictogram quantities of potyviruses using dot-blot immunobinding assay. *J. Gen. Virol.* 12:31-36.
- Capoor, S. P. and Varma, P. M. 1958. A mosaic disease of papaya in Bombay. *Indian. J. Agri. Sci.* 29:225-233.
- Clark, M. F. and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.
- Gonsalves, D. 1998. Control of *Papaya ringspot virus* in papaya: a case study. *Ann. Rev. Phytopathol.* 36:415-437.
- Gonsalves, D. and Ishii, M. 1980. Purification and serology of *Papaya ringspot virus*. *Phytopathology* 73:1028-1032.
- Hema, M., Kirthi, N., Sreenivasulu, P. and Savithri, H. S. 2003. Development of recombinant coat protein antibody based IC-RT-PCR for detection and discrimination of *Sugarcane streak mosaic virus* isolates from Southern India. *Arch. Virol.* 148: 1185-1193.
- Hobbs, H. A., Reddy, D. V. R., Rajeswari, R. and Reddy, A. S. 1987. Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. *Plant Dis.* 71:747-749.

- Hourani, H. and Abou-jawdah, Y. 2003. Immunodiagnosis of *Cucurbit yellow stunting disorder virus* using polyclonal antibodies developed against recombinant coat protein. *J. Plant Pathol.* 85:197-204.
- Hussain, S. and Varma, A. 1994. Occurrence of *Papaya ringspot virus* from Amritsar (Punjab) India. *J. Phytopathol. Res.* 7:77-78.
- Kallender, H., Petty, I. T. D., Stein, V. E., Panico, M., Blench, I. P., Etienne, A. T., Morris, H. R., Coutts, R. H. A. and Buck, K. W. 1988. Identification of the coat protein gene of *Tomato golden mosaic virus*. *J. Gen. Virol.* 69:1351-1357.
- Kormelink, R., Storms, M., van Lent, J., Peters, D. and Goldbach, R. 1994. Expression and sub cellular location of the NSm protein of *Tomato spotted wilt virus* (TSWV), a putative viral movement protein. *Virology* 200:56-65.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680-685.
- Ling, K. S., Zhu, H. Y., Jiang, Z. Y. and Gonsalves, D. 2000. Effective application of DAS-ELISA for detection of Grapevine leafroll associated closterovirus-3 using a polyclonal antiserum developed from recombinant coat protein. *Eur. J. Plant Pathol.* 106:301-309.
- Mullineaux, P. M., Boulton, M. I., Bowyer, P., van der Vlugt, R., Marks, M., Donson, J. and Davies, J. W. 1988. Detection of a non-structural protein of Mr. 11 000 encoded by the virion DNA of maize streak virus. *Plant Mol. Biol.* 11:57-66.
- Mumford, R. A. and Seal, S. E. 1997. Rapid single tube immuno capture RT-PCR for the detection of two Yam potyviruses. *J. Virol. Methods* 69:73-79.
- Nikolaeva, O. V., Karasev, A. V., Gumpf, D. J., Lee, R. F., Garnsey, S. M. 1995. Production of polyclonal antisera to the coat protein of *Citrus tristeza virus* expressed in *Escherichia coli*: application for immunodiagnosis. *Phytopathol.* 85:691-694.
- Nolasco, G., de Blas, C., Torres, V. and Ponz, F. 1993. A method combining immunocapture and PCR amplification in a microtitre plate for the detection of plant viruses and subviral pathogens. *J. Virol. Methods* 45:201-218.
- Purcifull, D. E., Edwardson, J. R., Hiebert, E., Gonsalves, D. 1984. *Papaya ringspot virus*. CMI/AAB Descriptions of Plant Viruses no: 292. Wallingford, UK: CAB International.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
- Sambrook, J. and Russell, D. W. 2001. Molecular Cloning: a laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
- Targon, M. L. P. N., Nikolaeva, O., Manjunath, K. L. K., Lee, R. F. and Machado, M. A. 1997. Cloning and expression in *E. coli* of CTV coat protein gene and production of polyclonal antisera. *Fitopatologia Brasileira* 22:99-102.
- Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4354.
- Varma, A. 1988. The economic impact of filamentous plant viruses-The Indian sub-continent. In: *The plant viruses*, ed. by R.G Miline, pp. 371-376. Plenum Press, NY, USA.
- van Poelwijk, F., Boye, K., Oosterling, R., Peters, D. and Goldbach, R. 1993. Detection of the L protein of tomato spotted wilt virus. *Virology* 197:468-470.
- van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E., Lemon, S., Maniloff, J., Mayo, M. A., McGeoch, D., Pringle, C. R. and Wickner, R. B. 2000. Virus taxonomy. Seventh report of the International committee on taxonomy of viruses. Academic press, New York, USA.
- Yeh, S. D., Jan, F. J., Chiang, C. H., Doong, T. J., Chen, M. C., Chung, P. H. and Bau, H. J. 1992. Complete nucleotide sequence and genetic organisation of *Papaya ringspot virus* RNA. *J. Gen. Virol.* 73:2531-2541.
- Yeh, S. D., Gonsalves, D. and Providenti, R. 1984. Comparative studies on host range and serology papaya ringspot and watermelon mosaic virus. *Phytopathol.* 74:1081-1085.