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Isolation, Identification and Sequencing of the *Hc-Pro* Gene of Papaya Ringspot Virus (PRSV-P)

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ABSTRACT

The study was taken up to isolate, identify and sequence the Hc-Pro gene from a papaya ringspot virus (PRSV) strain infecting papaya. The total RNA was extracted from infected papaya leaf and the *Hc-Pro* gene specific primer designed *in-silco* and the cDNA synthesized, PCR was performed to amplify the gene which was checked and the amplicon was eluted and ligated into pTZ57R/T vector. The clone was confirmed using blue white colony screening and colony PCR. Positive cloned plasmid was sequenced and analyzed. Nucleotide BLAST analysis shows that homology between cloned sequence with WB-1 Hc-Pro was 97 per cent.

PAPAYA (*Carica papaya* L.) is one of the most economically important fruit crops. Among the major diseases that limit papaya production, papaya ringspot virus (PRSV) is most important due to its severity, widespread and considerable yield loss which may extend up to 100 per cent. There are two strains of PRSV, viz., PRSV-P and PRSV-W. PRSV-P is able to infect both papaya and cucurbits, whereas PRSV-W infects only cucurbits (Jayathilake, 2004). Genome size of PRSV is 10 Kb consist VPg, P1, Hc-Pro, P3, CI, NIa and NIb. The Hc-Pro gene, codes for a proteinase, which is multifunctional and helps in the systemic and intercellular movement of the virus. Castillo *et al.,* (2011) reported that *Hc-Pro* genes from Indian strains of the virus have the highest levels of diversity, indicating an ancestral Indian origin. Genetically engineered (GE) papaya has been used to successfully control the disease caused by PRSV in Hawaii (Gonsalves *et al.,* 2010).

The total RNA was isolated from PRSV infe cted papaya leaf using LiCl maxi prep method (Sajeevan *et al*., 2014).

Primers (32 nucleotides each forward and reverse) were designed to amplify the full-length *Hc-Pro* gene from the sequence information available in NCBI database using fast-PCR software and the complete sequence of Hc-Pro gene was determined.

Hc-Pro FP	5’ GC GGA TCC ATG TCC AAA AAT GAA GCT GTG GAT 3’
Hc-Pro RP	5’ GC AAG CTT GTT GCG CAT ACC CAG GAG AGA GTG 3’

Total RNA extracted from the PRSV infected leaf material was used as template for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis. First strand cDNA was synthesized from total RNA in a 20µl reaction using MMuLV-RT enzyme (Fermentas). RT reaction was set up by adding 8 µl of RNA sample and 1ìl of Hc-Pro reverse primer (primers at 100 pm / 25 µl, Sigma), in a 0.2 ml polypropylene tube. This mixture was heated to 70°C for 5 min. and immediately chilled on ice for 5 min.

Polymerase chain reaction (PCR) was performed using the cDNA as template. The PCR was carried out to amplify Hc-Pro gene using specific primers and 2 µl of reverse transcribed product in 25 µl reaction mixture. Analysis of RT-PCR product by agarose gel electrophoresis (Sambrook and Russel, 2001), amplified DNA fragments were checked in 1 per cent agarose gel (Fig. 1)**.**

In conclusion the PRSV Hc-Pro gene amplified only in diseased and not in healthy papaya plant sample (Fig. 1). Purification of amplified PCR product was done by elution (GeneJET™ Gel Extraction kit (Fermentas) and eluted product concentration was 57.1 ng / µl.

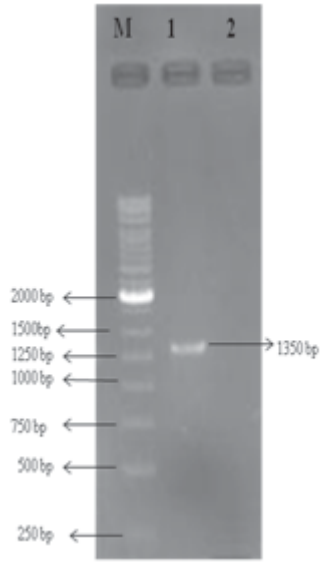

Figure 1: Agarose gel stained with ethidium bromide showing Hc-pro gene amplified from PRSV infected and healthy leaf tissue of papaya. Lane 1: Amplified DNA of infected leaf tissue of papaya, Lane 2: healthy leaf tissue cDNA as control, M: 250 bp DNA ladder (Bengaluru GeNei).

Ligation reaction mixture was prepared in 3:1 ratio of sample using concentration of (eluted product) and vector.

Component of ligation reaction mixture

Component	Volume (µl)
Vector (pTZ57R/T)	1.0
5X ligation buffer	2.0
PCR product	1.5
T_4 DNA ligase	0.5
Double distilled water	5.0

Competent cell was prepared using $CaCl_2$ method (Sambrook and Russel, 2001) and transformation was done by heat shock method (Hanahan, 1983). The transformed cells were spread Luria Bertani agar (LBA) plates with containing 30 µl of 100 µg/ml ampicillin and X-gal and isopropyl -D thiogalactoside (IPTG). The plates were inverted and incubated at 37°C overnight.

Confirmation of the transformed clones was based on blue-white colony assay (Ullmann *et al.*1967) and the transformation was further confirmed by colony PCR using *Hc-Pro* gene and M13 universal primer (Fig. 2), Polymerase chain reaction was performed using the colony as template. The Colony PCR (Gussow and Clackson, 1989) was carried out to amplify Hc-Pro gene and M13 universal primer using and colony in 10 ìl reaction mixture. The PRSV Hc-Pro gene was then amplified from isolated recombinant plasmid and the amplified DNA band was visualized on agarose gel (1.2 %) electrophoresis (Fig. 2).

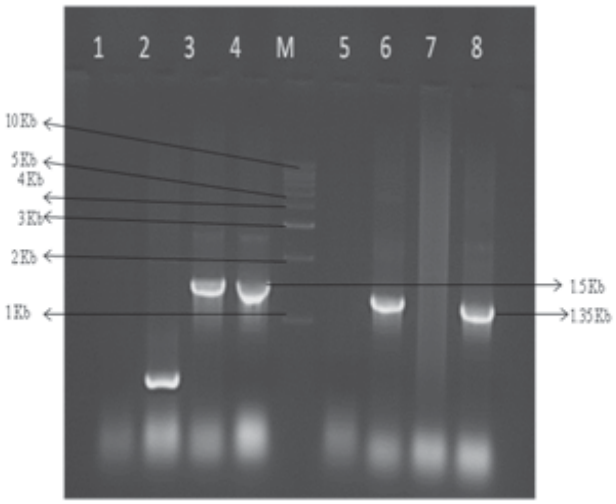

Figure 2: Confirmation of recombinant clones using colony PCR. Lane 1 to 4: M13 universal primer and Lane 5 to 8: Hc-Pro gene specific primer, M: 1 Kb DNA ladder (Bengaluru GeNei).

The identity of the Hc-Pro gene was confirmed by sequencing recombinant plasmid containing PRSV Hc-Pro gene from the PRSV strain using gene M13 primers at Chromous Biotech Pvt. Ltd., Bengaluru. The nucleotide sequence length of *Hc-Pro* gene of the PRSV strain studied was1352 bp.

The analysis of nucleotide sequences of Hc-Pro gene amplicon obtained from full length sequencing was compared to public databases using the BLAST algorithm. The Strains were confirmed to contain *Hc-Pro* gene of PRSV according to the sequence analysis (Fig. 3).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Papaya ringspot virus P isolate WB-1 helper component proteinase gene, partial cds	2246	2246	99%	0.0	97%	KC149504.1
Papaya ringspot virus isolate Rajasthan R3 helper component proteinase gene, partial cds	2069	2069	99%	0.0	94%	KJ922614.1
Papaya ringspot virus isolate Sikar (Raj) RR1 helper component proteinase gene, partial cds	2058	2058	99%	0.0	94%	KJ922615.1
Papaya ringspot virus P isolate MP helper component proteinase gene, partial cds	2047	2047	99%	0.0	94%	KC149503.1
Papaya ringspot virus W isolate W7 helper component proteinase gene, partial cds	2041	2041	99%	0.0	94%	KC149506.1

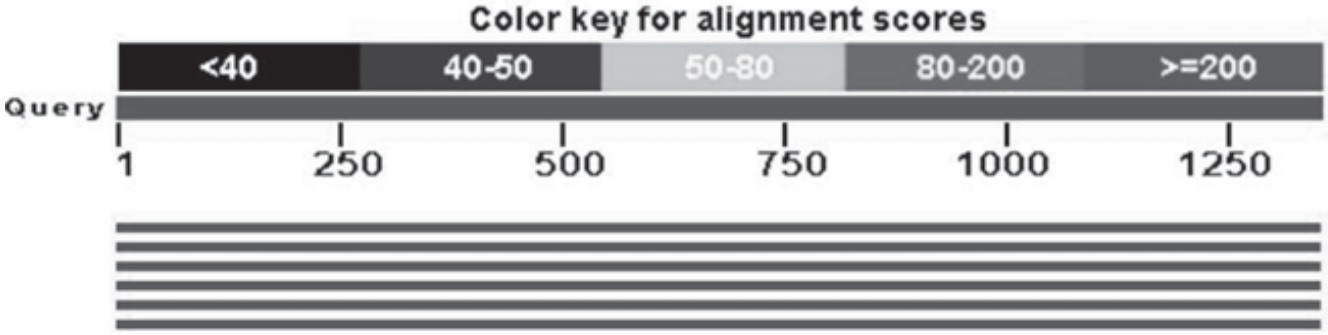

Figure 3: Graphical summary and significant alignments of BLAST homology search based on Hc-Pro gene sequences of PRSV strain by using NCBI database.

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