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Research Article

STANDARDIZATION OF POLYMERASE CHAIN REACTION FOR AMPLIFICATION OF GEMINI VIRAL DNA

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ABSTRACT
Detection of geminivirus by conventional method is very difficult due to low titer of virus in their hosts and vectors. Majority of the geminiviruses belong to the genus *Begomovirus* and have either mono- or bipartite genome. Begomovirus being DNA containing virus, it is convenient to isolate DNA and detect the virus by polymerase chain reaction (PCR). The development of PCR allows the detection of plant viruses even if present in low titer in their hosts and vectors. The present investigation is an approach towards the standardization of PCR techniques for amplification of full length DNA-A, coat protein (CP) part of DNA-A and DNA-β molecule from tomato leaf curl viral samples. The total DNA isolated from infected plants was used for PCR amplification by using six sets of geminivirus specific primers. PCR was standardized by optimizing the volume of PCR ingredients *viz.* template, primers, $MgCl_2$ and *Taq* polymerase concentration for increasing the sensitivity of the PCR.

Key Words: *Begomovirus, full length DNA-A, coat protein, DNA- β molecule.*

INTRODUCTION
Geminiviruses (family *Geminiviridae*) are plant viruses that have a circular, single-stranded (ss) DNA genome of size ranging from 2.5 to 3.0 kilobases and are encapsulated within twinned isometric particles of 15-18 x 30 nm size (Stanley, 1983). Majority (over 80%) of the geminiviruses belong to the genus *Begomovirus* and have either mono- or bipartite genome. The genomes of bipartite begomoviruses consist of two circular ssDNA components designated as DNA-A and DNA-B (Varma and Malathi, 2003). A number of monopartite begomoviruses lack the DNA-B component and consist of one or more small circular ss-DNA molecules, having ~1.3 kb size, designated as DNA-β (Briddon *et al.*, 2002). Detection of geminivirus by conventional method is very difficult due to low titer of virus in their hosts and vectors (Eun and Wong, 2000). Begomovirus being DNA containing virus, it is convenient to isolate DNA and detect the virus by polymerase chain reaction or PCR (Sohrab *et al.*, 2006). The development of PCR allows the detection of plant viruses even if present in low titer in their hosts and vectors (Khan *et al.*, 1998). Furthermore, PCR is the most ideal approach to detect geminivirus as they replicate via a circular, double-stranded, replicative form of DNA. Since PCR amplifies viral nucleic acid, this approach is extremely useful in bypassing the problems associated with geminivirus serology. Thus the present investigation is an approach towards the standardization of PCR techniques for amplification of full length DNA-A, coat protein (CP) part of DNA-A and DNA-β molecule from tomato leaf curl viral samples.

MATERIALS AND METHODS
DNA isolation using CTAB buffer
Young leaves of tomato showing typical leaf curl symptoms were harvested and approximately 2 g of leaf tissues were ground in liquid nitrogen to isolate the DNA using the CTAB buffer (Murray and Thomson, 1980). It was then transferred to 50 ml screw cap tube and 8 ml of 2x CTAB buffer was added. The tissues were mixed well and then 10% SDS was added to make a final concentration of 0.1%. It was then incubated at 65^0C for 1 hour with periodical gentle mixing during the incubation. Equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed well, and centrifuged at 2000 g for 15 min. The

chloroform: isoamyl alcohol extraction was repeated until the interface became clean. The DNA remained in the upper (aqueous) layer. Then 2/3 volumes of isopropanol was added, mixed gently, and centrifuged for 15 min at 10,000 g. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was air dried and resuspended in 1 X TE (pH 7.4). The RNA was removed by adding 0.1% SDS and 1µg/ml DNAse-free RNAse (final concentrations) and incubating for 1 h at 37^0C. It was extracted once with an equal volume of phenol: chloroform: isoamyl alcohol and then centrifuged at 10,000 g for 10 min. It was again transferred to clean tube and precipitated with 70% ethanol by centrifuging at 10,000 g for 10 min. DNA was further purified by precipitation with 1/3 volume of 7.5 M ammonium acetate and 2.5 volumes of 70% ethanol. The precipitate obtained was then centrifuged at 10,000 g for 10 min, washed with ethanol, and air dried. The pellet was finally dissolved in 20µl of 1 X TE buffer (pH 7.4).

Polymerase Chain Reaction (PCR)

The total DNA isolated from infected tomato plants were used for PCR amplification by using six sets of geminivirus specific primers (Briddon *et al.,* 2002; Chatterjee and Ghosh, 2007; Deng *et al.,* 1994; Jose and Usha, 2000) using PX2 thermal cycler (Applied Biosystem, USA). Among the geminivirus specific primers five DNA-A specific primer sets (Chatterjee and Ghosh, 2007; Deng *et al.,* 1994, Jose and Usha, 2000) and one universal DNA-β specific primer set (Briddon *et al.,* 2002) were used in this study (Table-1). In all the PCR experiments template DNA from healthy plants were used as negative controls.

Table 1: Details of primers used in the present study.

Target genomic fragment	Primer name	Sequence	Nt. ln.	Amplicon size (~kb)
Full length DNA-A (Jose and Usha, 2000)	Bcp1.f (F)	5'-AATTAATAAAGTTTGAATTTT ATATC -3'	26	2.7
	Bcp2.r (R)	5'-TCAATTCGTTACAGAGTC- 3'	18	
Full length DNA-A (Chatterjee and Ghosh, 2007)	P1 (virion-sense)	5'-CATGAGTACGGAGATTGGGAC - 3'	21	2.7
	P2(complementary -sence)	5'-TCACACCAAAAGCATGAAGGG TCGAAGG - 3'	28	
Full length DNA-A (Chatterjee and Ghosh, 2007)	FLD-F (F)	5'-GARAGTACYCATGCYTCTAA YCC -3'	23	2.7
	FLD-R (R)	5'-AGTRTGRTTYTCRTACTTCCCA G- 3' (where K=G/T, R=A/G, S=C/G, W=A/T, Y=C/T, B=C/G/T and V=A/C/G)	23	
Coat protein (CP) part of DNA-A (Jose and Usha, 2000)	Bcp1H.f (F)	5'-AAGCTTATGTCGAAGCGAGCTG CCG -3'	25	0.77
	Bcp2.r (R)	5'-TCAATTCGTTACAGAGTC- 3'	18	
Common region and part of Coat protein of DNA-A (Deng *et al.,* 1994)	Primer A (F)	5' –TAATATTACCKGWKGVCCSC- 3'	20	0.55
	Primer B (R)	5'-TGGACYTTRCAWGGBCCTTCA CA- 3' (where K=G/T, R=A/G, S=C/G, W=A/T, Y=C/T, B=C/G/T and V=A/C/G)	23	
DNA- β (Briddon *et al.,* 2002)	β-1 (F)	5'-GGTACCACTACGCTACGCAGC - 3'	21	1.3
	β-1 (R)	5'-GGTACCTACCCTCCCAGGGGTA CAC- 3'	25	

Standardization of PCR

PCR was standardized by optimizing the volume of PCR ingredients *viz.* template, primers, $MgCl_2$ and *Taq* polymerase concentration for increasing the sensitivity of the PCR. Various concentrations of template used were 5ng, 10ng, 25ng, 50ng, 100ng and 150ng; for primer this were 5ng, 10ng, 25ng, 50ng, 100ng and 200ng; for $MgCl_2$ this were 0mM, 1mM, 1.5mM, 2mM and 2.5mM; and for *Taq* polymerase this were 0.3U, 0.45U, 0.6U, 1.0U and 5U (Fermentas, USA). The PCR cycle was standardized in a gradient thermal cycler (PX2, Thermo) with varying annealing temperatures like 50^0C, 53^0C, 55^0C, 57^0C and 60^0C.

PCR cycles

For further experiment PCR was done using 50µL volumes, each containing 50ng of template DNA, 100ng of primers, 1 X PCR buffer, 200µM dNTP, 1.5mM $MgCl_2$ and 0.6U *Taq* polymerase (Fermentas, USA). The PCR cycles for amplification with primer sets designed by Deng *et. al.* (1994) was initially one cycle of 92^0C for 2 min, 58^0C for 1 min, 72^0C for 2 min, followed by 40 cycles of 94^0C for 45 sec, 58^0C for 1 min, 72^0C for 2 min, and a final cycle of 94^0C for 45 sec, 58^0C for 1 min, 72^0C for 5 min. This primer set amplified the common region and partial coat protein (CP) gene of DNA-A. For the CP primer of okra yellow vein mosaic virus (Jose and Usha, 2000), the PCR cycle was initially for denaturation at 94^0C for 2 min, followed by 30 cycles at 94^0C for 1 min, 52^0C for 2 min, 72^0C for 3 min, and a final extension at 72^0C for 10 min. For the universal DNA-β specific primer (Briddon *et al.*, 2002) the PCR cycle was same as CP primer excepting in annealing temperature of 55^0C. Target genomic fragment of these two primer sets were the coat protein gene of DNA-A and the full length DNA-β, respectively.

Visualization of Amplified Product by Agarose Gel Electrophoresis

PCR products were visualized using ethidium bromide under ultraviolet light after electrophoresis in a agarose gel (0.8% w/v) at 2v/cm for 1h.

RESULTS AND DISCUSSION

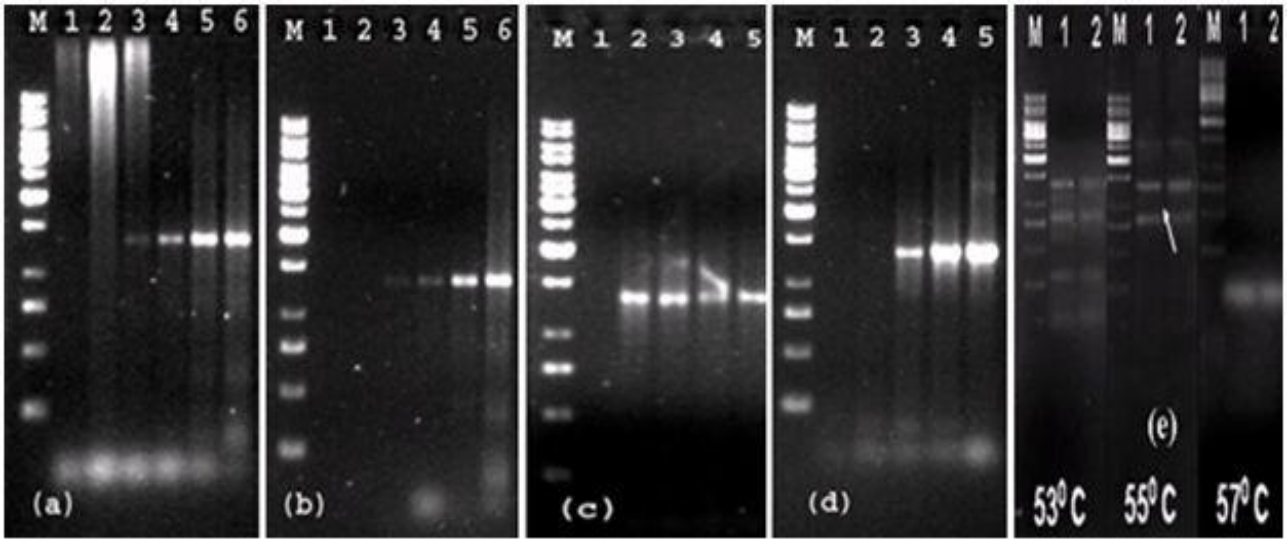

Figure 1: Standardization of PCR amplification by optimizing the volume of template (a), primers (b), $MgCl_2$ (c), *Taq* polymerase concentration (d) and varying annealing temperatures (e) in 1.5% Agarose gel electrophoresis. *Template used were 5ng, 10ng, 25ng, 50ng, 100ng and 150ng (lane 1–6) (a); primer used were 5ng, 10ng, 25ng, 50ng, 100ng and 200ng (lane 1–6) (b); $MgCl_2$ used were 0mM, 1mM, 1.5mM, 2mM and 2.5mM (lane 1–5) (c); Taq polymerase used were 0.3U, 0.45U, 0.6U, 1.0U and 5U (lane 1–5) (d). Lane M – 1kb DNA ladder.*

The expected ~0.77 kb sized coat protein part of DNA-A and ~0.55 kb amplicons, with primers used by Jose and Usha (2000) and Deng *et al.* (1994), respectively, also indicated the presence of DNA-A of begomovirus into the test plants. The leaf curl disease infected tomato leaf sample exhibited a positive amplification of ~2.7 kb size with the degenerate primers (Chatterjee & Ghosh, 2007) used to amplify full length DNA-A components in this study. The expected ~1.3 kb PCR amplicons was obtained from the leaf curl disease infected tomato sample with primer specific to DNA-β molecule (Briddon *et al.*, 2002) and showed the association of a satellite DNA-β molecule with the disease. No amplification was obtained with nucleic acid extracts from healthy control plants.

Standardization of the PCR amplification with DNA-β isolated from tomato indicated that the primers used could detect the virus even at 10ng level of infected plant DNA when other reactives remained constant (Figure 1a). Primer concentration of 50ng and even 25ng could also detect the virus with 1mM $MgCl_2$. But for a clear recognizable product, optimum concentration of template, primers and $MgCl_2$ were found to be 50ng, 100ng and 1.5mM, respectively (Figure 1a,b,c). *Taq* at 0.6U level was found sufficient for specific detection in PCR (Figure 1d). The annealing temperature of 55^0C supported good amplification with the standardized PCR mixtures (Figure 1e).

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