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Serological and molecular detection of an isolate of Cucumber Mosaic Virus (CMV) infecting cucumber (*Cucumis sativus*) and cloning of its coat protein gene

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Abstract

Cucumber mosaic virus (CMV) is a widely prevalent plant virus infecting important vegetable, plantation and flower crops. Methods for early detection of viruses in plants and vectors transmitting them play a critical role in plant virus disease management. Direct plate and Dot-Enzyme Linked Immunosorbent Assay (ELISA) was standardized for detection of CMV. Optimum OD of 1.249 (1.9 ng/µl) and 1.242 (1.52 ng/µl) was observed in 1:20 and 1:50 dilution of crude and ultra purified antigen respectively, at a dilution of 1:1000 of both primary and secondary antibody. Polymerase Chain Reaction (PCR) using CMV coat protein (CMV CP) gene specific primers amplified a 657 base pair (bp) fragment, which was then cloned in pTZ57R/T cloning vector and positive clones were identified by band shift assay and colony PCR. This will aid in developing field diagnostic kits for detection of CMV in different crops and also in developing transgenics with the CP gene.

Keywords: *Cucumber mosaic* virus, direct plate ELISA, Dot ELISA, RT-PCR, CMV CP, cloning CMV CP

Introduction

Cucumber mosaic virus (CMV) is a widely prevalent important plant virus, distributed worldwide having a very large host range, infecting more than 1000 plant species. The virus infects important crops like banana, vanilla, blackpepper, tomato, geranium, chrysanthemum, carnation, gladiolus and others (Roossinck 2002, Zitter and Murphy, 2009). CMV belongs to the genus Cucumovirus and family Bromoviridae, it is a tripartite, multicomponent, positive single stranded RNA virus consisting of three genomes RNA1, RNA2 and RNA3, encapsidated individually in a 28nm diameter icosahedral particle. CMV can be transmitted from plant to plant mechanically through sap and by vectors namely aphids in a stylet borne non-persistent manner (Gildow, 2008). The current measures to manage viral diseases rely on indirect tactics like eradicating the source of infection from reaching the crop, controlling the vector, utilizing virus free planting material and breeding for disease resistance. Accurate, fast and early diagnosis of the disease is very important. Methods for detection of virus in plants and vectors transmitting the virus play a critical role in virus disease management. A rapid assay for the diagnosis of CMV, which can be employed in both laboratory and field, is essential (Zein et al. 2006). This is possible through modern serological and molecular detection techniques.

In the present study, a direct plate and Dot Enzyme Linked Immunosorbent Assay (ELISA) for serological detection and polymerase chain reaction (PCR) detection of CMV coat protein (CMV CP) as a molecular detection method was standardized. The amplified CMV coat protein gene was eluted and gel purified and cloned in pTZ57R/T cloning vector and positive clones were identified by band shift assay and colony PCR.

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Materials and Methods

Virus inoculation

The CMV isolate maintained in the Department of Plant Biotechnology, was mechanically inoculated to healthy cucumber (*C. sativus*) seedlings of Green Long variety at the two leaf stage. Plant extracts from diseased plants was extracted in chilled 0.01 M potassium phosphate buffer (pH 7.0) and rubbed onto the leaves of cucumber plants dusted with carborundum (600 mesh), which was then washed off after 2–3 min with distilled water. Plants were sprayed with 0.2% dimethaote at 15 day intervals to avoid cross contamination by aphids.

Virus purification

Leaves of inoculated *C. sativus* were harvested two weeks after inoculation and used for purification. Virus particles were purified using a slightly modified protocol (Takanami, 1981) using 0.5 M sodium citrate (pH 6.5-7.0) and 5mM sodium borate (pH 9.0).

Standardization of Direct Plate Enzyme Linked Immunosorbent Assay (ELISA)

Direct ELISA was standardized on polystyrene plates using the protocol described by Clark and Adams (1977). Wells were initially

coated with 100 µl of CMV specific primary antibody raised previously against the same strain of CMV in this lab (Kumar Rajeev Ranjan, 2009) and incubated overnight at 4˚C. The plates were then washed in PBS-T thrice with 3 minute intervals between washes. The wells were then coated with 100 µl antigen from infected leaf sample and purified virus prepared in PBS-T containing 2% polyvinyl pyrrolidone (PVP) and 0.2% BSA and incubated at 37^0C for 1½ hr. This was followed by the wash procedure and coating with 100 µl of secondary antibody alkaline phosphatase conjugate at 1:1000 dilution and incubated again at 37^0C for 1½ hr. Washing of plates was repeated and then the substrate p-nitrophenyl phosphate (pNPP), (Bangalore Genei), was added. The reaction was terminated 30 minutes later and absorbance recorded at 405 nm using an ELISA reader. The antiserum dilutions used were 1:500, 1:1000, and 1:1500. The dilutions of antigens used were 1:10, 1:20, 1:50 and 1:100 for both crude and ultrapurified virus with secondary antibody at a dilution of 1:1000. Control sample was the plant extract from uninoculated healthy plants.

Standardization of Direct Dot Enzyme Linked Immunosorbent Assay (Dot-ELISA)

Direct Dot-ELISA was standardized on nitrocellulose membrane (Pall Life Sciences, Bangalore). 2µl of suitably diluted primary antibody in coating buffer was dotted onto the membrane and incubated at 4^0C overnight. The membrane was washed in PBS-T thrice with 3 minute intervals between washes and then dotted with blocking solution (PBS-T+PVP+BSA), and incubated at 37^0C for 1½ hr, followed by the wash procedure. 2µl of antigen from infected leaf sample and purified virus in blocking solution was then dotted onto the membrane and later air dried and incubated at 37˚ C for 1½ hr .Washing of membrane was repeated and coated with 1:1000 dilution of secondary goat anti rabbit IgG-ALP and incubated at 37˚ C for 1½h. Wash procedure was repeated and the membrane was then dotted with the substrate Bromo Creso Indoyl Phosphate (BCIP), (Bangalore Genei), and incubated in dark for 30minutes. The antiserum dilutions used were 1:500, 1:1000 and 1:1500, the dilution of antigen used was 1:10 and 1:20 for both the crude and 1:10, 1:20 and 1:50 ultrapurified virus. Secondary antibody was used at a dilution of 1:1000.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction

RNA was isolated from the CMV infected cucumber leaves by using Tri-Reagent (Sigma Aldrich, Germany) and cDNA synthesis was carried out by RT-enzyme (RevertAid H minus First Strand cDNA Synthesis Kit, Fermentas). Primers were designed for the coat protein (CP) gene sequences of CMV (based on sequence information of CP gene available in GenBank) using FastPCR.

Primer Sequence

The forward and reverse primers used were as follows:
Cucumber mosaic virus forward primer (CMV FP): 5' GGATCCATGGACAAATCT 3'
Cucumber mosaic virus reverses primer (CMV RP): 5' ACTTTCGTGGGGCCTCCA 3'

PCR Reaction

PCR reactions were carried out as per the manufactures instructions and the amplification was performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) and the programme consisted of initial denaturation at 94˚ C for 3 minute, followed by 35 cycles of denaturation at 94˚ C for 45 s, annealing at 59˚ C for 1 minute and extension at 72˚ C for 1 minute, a final extension at 72˚ C for 20 minutes was done. The PCR products were run on 1% agarose gels in 1X TBE buffer, stained with ethidium bromide along with 1kb DNA ladder (BioLabs) and followed by photographing in a gel documentation apparatus (Alpha Infotech Corporation, CA, USA).

Cloning of CMV Coat Protein Gene

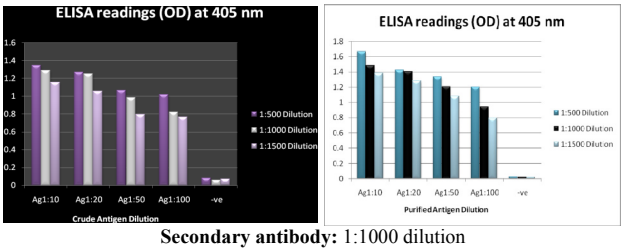
Amplified CMV CP gene was gel eluted using gel extraction kit (Cat# 28704, QIAGEN, GmbH, Hilden, Germany) and the PCR amplified cDNA fragment was cloned into the plasmid vector pTZ57R/T using Ins T/A clone TM PCR Product cloning kit (Cat# K 1214, MBI, Fermentas) following the manufacturer's instruction. The ligation mixture was prepared and the reaction was carried out as per the manufacturer's protocol. The ligation mixture was incubated at 4˚ C overnight and was used for transformation. Competent cells of *Escherichia coli* strain DH5α was prepared and transformed using standard molecular biology procedures (Sambrook, 1989).

Results and Discussion

Plants inoculated with a previously isolated, purified and electron microscopically confirmed strain of virus (Kumar Rajeev Ranjan, 2009) showed typical symptoms of CMV such as mosaic, mottling, chlorotic streaks, parallel venation, necrosis, leaf distortion and stunting (Figure 1 and 2).The virus was ultrapurified from these plants and used in further studies.

Figure 1 & 2: Cucumber plants infected with CMV showing characteristic mosaic symptoms

In the plate ELISA standardization experiment, colour development was observed in all combinations of the primary antibody, antigen and secondary antibody dilutions. Optimum OD reading of 1.249 (1.9 ng/µl) and 1.212 (1.52 ng/µl) was observed in the combination of 1:20 dilution of crude antigen and 1:50 dilution of ultrapurified antigen respectively and in 1:1000 dilution of primary and secondary antibody (Table 1, Figure 3).



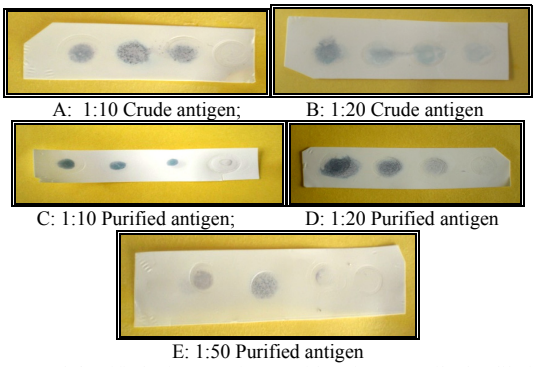
Secondary antibody: 1:1000 dilution

Figure 3: ELISA for the standardization of antibody titre using Crude antigen

CMV infects a wide variety of plants. Antibodies raised against CMV, have been used to detect the virus in various crop plants. Madhubala et al. (2005), developed a double antibody ELISA (Direct ELISA) for detection of the virus in vanilla. A titre of 1:8000 was reported for primary antibody, the enzyme conjugated specific antibody was used at a dilution of 1:2000. CMV was detected in *Viola cornuta*, detected by DAS-ELISA. On testing with

Table 1: ELISA readings (OD) at 405 nm for the standardization of antibody titer raised against CMV

Primary antibody dilutions		Crude Antigen Dilution					Purified Antigen Dilution				
		1:10	**1:20**	**1:50**	**1:100**	**Healthy Control**	**1:10**	**1:20**	**1:50**	**1:100**	**Healthy Control**
	1:500	1.346	1.267	1.063	1.016	0.031	1.667	1.423	1.334	1.205	0.025
	1:1000	1.286	**1.249** (1.9ng/µl)	0.984	0.820	0.020	1.486	1.407	**1.212** (1.52ng/µl)	0.945	0.029
	1:1500	1.157	1.056	0.792	0.767	0.012	1.385	1.285	1.081	0.796	0.022
	Secondary antibody dilution 1:1000										



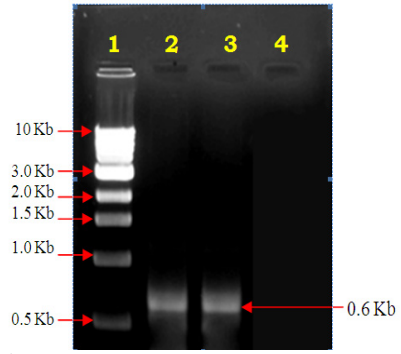
A: 1:10 Crude antigen; B: 1:20 Crude antigen

C: 1:10 Purified antigen; D: 1:20 Purified antigen

E: 1:50 Purified antigen

Dot A: Crude/Purified virus (antigen) with primary antibody dilution of 1:500; Dot B: Crude/Purified virus (antigen) with primary antibody dilution of 1:1000; Dot C: Crude/Purified virus (antigen) with primary antibody dilution of 1:1500; Dot D: Negative control;

Figure 4: Standardization of antibody titer raised against CMV virus by Dot ELISA using 1:10 dilution of purified virus



Lane 1: 1Kb Marker
Lane 2 & 3: 0.6 Kb Amplified CMV coat protein gene
Lane 4: Healthy control

Figure 5: PCR amplification of CMV coat protein gene from cDNA

subgroup-specific antisera, the isolate was found to belong to subgroup I (Arneodo et al. 2005). Verma et al. (2006) reported an absorbance value of 0.023 and 0.438 for negative and positive controls at 405 nm in an ELISA test in geranium. In diseased plants, the values ranged from 0.346 to 0.433. Mixed infection of CMV and TAV was detected in chrysanthemum Cv. Pooja using Direct Antigen Coating-ELISA (DAC-ELISA) i.e. indirect ELISA, an OD value of 0.497 and 0.408 was recorded for the detection of the two viruses respectively (Kumar et al. 2009). A Dot Immunobinding Assay (DIBA) was also developed for detection of CMV (Zein and Miyatake, 2009). The minimum detection level was 128 ng/µl with purified preparation of Pepo-CMV and at 10^{-5} dilution of extract from infected leaves.

The result observed in the present experiment has enabled the detection of the virus in diseased plants using both direct plate and Dot-ELISA which will be useful for disease diagnosis in the field if developed into a kit. This has a practical applicability in that the virus infects several crops and early detection could help in saving crop loss and spread of virus.

PCR amplification of CMV coat protein gene produced a band of 657 kb band specific to CMV coat protein (Figure 5).

RT-PCR is a diagnostic tool for virus detection. It also aids the molecular characterization of virus, which is anticipated to aid better understanding of the genetic composition, variation caused by mutation and recombination, and correct taxonomic position of the virus. It helps in finding out how the strain under study relates to other strains of CMV reported from other parts of the world and those reported from the same local geographical region. RT-PCR is the most sensitive assay among all and might be used to confirm samples that are inconclusive in ELISA and dot blot tests (Hu et al. 1995).

CMV coat protein gene from *Amaranthus tricolor* has as size of 657 bp. CP at nucleotide level, showed 90-98% sequence identity with CMV subgroup I and less than 80% with CMV subgroup II (Srivastava et al. 2004). Madhubala et al. (2005) successfully amplified the CMV CP gene. Computer-aided analyses of the 657 bp sequence revealed the presence of a single long open reading frame capable of encoding 218 amino acids. CMV coat protein from infected paprika also had a size of 657bp (Bhadramurthy et al. 2009). In CMV CP from *Ricinus communis,* Raj et al. (2010) reported a size of ~650 bp. The sequence analysis revealed 96–98% identity and close phylogenetic relationships with several isolates of the I B subgroup of CMV. Khan et al. (2011), reported that PCR amplification of the CP gene of CMV using gene specific primers resulted in the amplification of ~657 bp fragment from three samples of naturally infected *Ocimum sanctum* (KAR, MH and UP) along with positive control, but no amplification was observed in tissue culture based healthy sample (negative control). Similar RT-PCR product was observed in inoculated *N. tabacum* Cv. 'White Burley' and *C. sativus* samples. RT-PCR on total RNA from CMV infected plant material resulted in amplification of an approximately 872 bp DNA fragment, as predicted from the nucleotide sequence of CMV strain Q, from 10 of 13 cucurbit samples (Bashir et al. 2006).

A 657 bp amplified fragment specific to CMV CP observed in the present study is similar to that reported by other scientific groups (Madhubala et al. 2005 and Yu et al. 2005). Reports with variation in size of the CP may be due to strain/isolate differences.

The CMV CP gene was eluted, cloned and ligated in pTZ57R/T vector. The clones containing recombinant molecules were selected based on blue-white colonies (Figure 6). Plasmids were isolated from white colonies and the positive clones were confirmed through band shift assay and PCR amplification by using gene specific primers (Figure 7 and 8) which reconfirmed the 657 bp fragment of CMV CP gene.

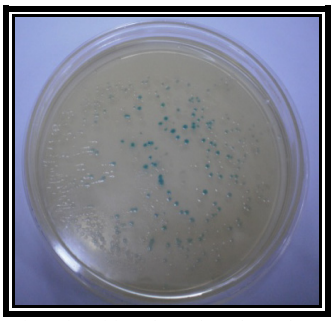
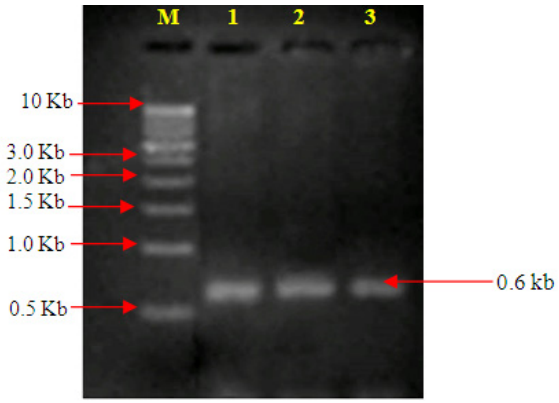
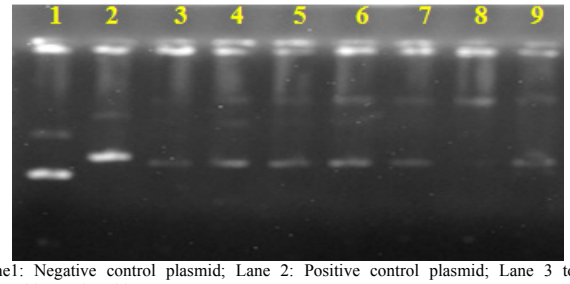


Figure 6: Screening of positive clones based on Blue-White selection



Lane M: 1Kb ladder
Lane 1, 2 & 3: Amplified CMV coat protein gene from plasmid

Figure 7: Confirmation of positive clones of CMV coat protein gene by PCR



Lane1: Negative control plasmid; Lane 2: Positive control plasmid; Lane 3 to 9: Recombinant plasmid

Figure 8: Confirmation of positive clones through band shift assay

Cloning of CMV CP gene has been reported by several groups. The coat protein genes for CMV-22 and CMV-PG were amplified by RT-PCR and cloned to obtain pMON18804 and pMON18825, respectively, CMV-22 coat protein sequence was identical to the CMV-D isolate while the CMV-PG coat protein gene has a two nucleotide difference from CMV-WL (Kaniewski et al. 1999).

CMV-CP gene has been cloned in several other crops namely *Amaranthus tricolor* (Srivastava et al. 2004), vanilla (Madhubala et al. 2005), betel vine and pepper (Hareesh et al. 2006), paprika (Bhadramurthy et al. 2009), *R. communis* (Raj et al. 2010), basil (Khan et al. 2011) and banana (Shahanavaj et al. 2010).

Conclusion

Mechanical sap infection of cucumber plants using carborundum was effective and resulted in true characteristic symptoms of the disease. Direct-ELISA and Dot-ELISA were standardized and successfully used to detect the virus and to fix the antibody titers raised against CMV.

Serologically, ELISA and Dot-ELISA were standardized for the detection of the virus. Molecular diagnosis was carried out to confirm the presence of CMV. Total RNA was isolated from the infected plants and RT-PCR was done to synthesize cDNA using gene specific reverse primer. Expected size (657 bp) of the CMV coat protein gene was amplified, which confirmed the presence of CMV in infected cucumber plants.

The expected amplified PCR product was gel eluted and cloned in a pTZ57R/T cloning vector. The recombinant plasmid was transferred into *E. coli* DH5α cells. The transformed cells were plated on LB plates containing ampicillin, X-gal and IPTG. Recombinant white clones were selected and plasmid was isolated and positive clones were further confirmed by band shift assay and colony PCR.
Future work will have to focus on development of immunodiagnostic strips for rapid detection of the virus in the field and in tissue cultured plants, characterizing and grouping of strains of the virus, monoclonal antibodies can be developed for strain specific identification of virus and development of transgenic cucumber plants resistant to CMV using the coat protein gene.

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