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Cloning and Characterization of Coat Protein Gene from CVMV

M. Abdul Kareem and A.S. Byadgi

Department of Plant Pathology, University of Agricultural Sciences, Dharwad - 580 005, India.

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Chilli leaf curl is the most destructive syndrome causing substantial economic losses in chilli production. Severe leaf curling, leaf distortion, dark green mottle, vein banding, reduced leaf size and stunted growth are typical symptoms of the viral infection. For a reliable detection specific primer pairs were designed, after PCR amplification using gene specific primers of CVMV, 531 bp DNA fragment was amplified in all diseased samples of chilli and no amplification was obtained in healthy samples. PCR amplicon of CVMV-*CP* gene was cloned into pTZ57R/T cloning vector and transformed into *E. coli* DH5a. Transformed white colonies were picked after blue-white colony assay and confirmed by colony PCR and restriction analysis. The sequence results clearly revealed that the coat protein gene showing 95% identity with the reported CVMV-*CP* gene sequence. Thus, the investigation focused on molecular characterization of the virus convincingly revealed potyvirus association with leaf curl disease in chilli.

Keywords: Chilli, Leaf curl, Virus, Coat Protein, Cloning, Transformation.

Chilli (*Capsicum annuum*) is an important crop grown worldwide for its use as spices and vegetables. It is an indispensable spice used as basic ingredient in a great variety of cuisines all over the world. Chilli belongs to *Capsicum* (2n=24) a new world genus belonging to *Solanaceae* family. It is an excellent source of vitamin C, A, B-complex and E. It contains seven times more vitamin C than orange.

India is the largest producer of chilli crop, grown over an area of 0.794 million hectares with an annual production of 0.13 million tonnes with the productivity of 1.5 tonnes/ha (Anonymous, 2014). The important states growing chilli are Andhra Pradesh, Karnataka, Orissa, Maharashtra, West Bengal, Rajasthan and Tamil Nadu. Karnataka ranks second in area with 100.73 ('000 ha) and production 107.00 ('000 MT) of dry chilli after Andhra Pradesh (Anonymous, 2014). In Karnataka, northern Karnataka is an important chilli growing area and it is highly concentrated in the districts like Dharwad, Haveri, Koppal, Ballari, Raichur, Kalaburagi and Belagavi.

Chilli suffers from number of viral, fungal, bacterial and nematode diseases. Chilli is highly susceptible to a large number of viruses through natural and artificial infection. Chilli is known to be affected by 42 viruses, 22 are found to occur naturally while the rest are known to infect artificially (Raju, 2010). Chilli leaf curl locally known as murda is a most destructive disease of chilli in India. Agents like thrips, mites and viruses have been reported to produce leaf curl symptoms in chilli (Puttarudraiah, 1959).

A severe leaf curl disease on chillies is associated with multiple Begomovirus components in Pakistan is reported by Akhter *et al.* (2009). In India, *Tomato leaf curl New Delhi virus* (ToLCNDV) was associated with chilli leaf curl disease occurring in Lucknow (Khan *et al*., 2006). Whitefly, *Bemicia tabaci* transmitted

* To whom all correspondence should be addressed.
E-mail: makuasd@gmail.com

Geminiviruses occur predominantly in the tropics where their vectors are most abundant (Muniyappa, 1980 and Brown, 1994).

Potyviruses are the largest group of plant viruses responsible for heavy reduction in yield and quality. Potato virus Y (PVY) (Gebre-Selassie *et al*., 1983), Tobacco etch virus (TEV) (Zitter,1972), (Atiri and Dele, 1985), *Chilli veinal mottle virus* (CVMV) (Ong *et al*., 1979), *Pepper vein banding virus* (PVBV) (Feldman and Gracia, 1977) and some other have been reported to infect pepper widely.

The detection through three different diagnostic approaches *viz.,* Electron microscopy, Serology and Polymerase chain reaction techniques inferred *chilli veinal mottle virus* which comes under *Potyvirus* genus was the only virus associated with the particular symptomatic murda complex disease (Pradeep and Byadgi, 2011).

Chilli murda is a complex disease associated with thrips, mites and a virus. Curling of leaves is one of the symptoms of the murda and hence the disease is referred as Chilli Leaf curl based on symptoms by some. But the true leaf curl is caused by geminivirus and spreads with the help of whiteflies. Chilli is known to be infected by many viruses and association of begomovirus with murda complex is under question as the complex disease doesn't associate with whiteflies. Hence, role of the above viruses in the disease complex in India is not clearly understood.

Strategies for the management of viral diseases normally include control of vector population using insecticides, use of virus-free propagating material, appropriate cultural practices and use of resistant cultivars. However, each of these methods has its own drawback. Molecular techniques confer a greater reliability in the identification and characterization of viruses associated with the chilli leaf curl (murda complex).

MATERIALS AND METHODS

Polymerase Chain Reaction is versatile technique in practice and has become popular in molecular analysis including detection of plant pathogens.

Isolation and amplification

The total RNA from virus infected and healthy samples of chilli plants were isolated using RNeasy Plant Mini Kit (QIAGEN India Pvt. Ltd.) and cDNA was synthesized using oligo dT primer and reverse transcriptase enzyme. The cDNA was used as template for amplification using gene specific primer.

The primers designed and used were

S. No.	Virus	Primer sequence	Annealing temperature
1.	*Potyvirus* (CVMV)	5' AGCATGGAGAGAGCGACATT 3' 5' TGGTGACGTTCCATTCTCAA 3'	61°C

Cloning and characterization

Cloning of virus coat protein gene (CP) was done in pTZ57R/T vector by using Inst T/A cloning kit. The clones carrying the coat protein gene was further confirmed by PCR amplification using gene specific primers and also by Restriction analysis using two restriction enzymes *Xba* I and *Bam* HI and analyzed by gel electrophoresis.

The coat protein gene cloned in pTZ57R/T was sequenced using M13F/R primers employing primer walking technique at Merck Specialties Private Ltd., Bangalore. Thus obtained sequences were subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

RESULTS AND DISCUSSION

Isolation and amplification of virus causing leaf curl in chilli

Total RNA was isolated from diseased and healthy samples of chilli using RNeasy Plant Mini Kit (QIAGEN India Pvt. Ltd.) and cDNA was synthesized using oligo dT primer and reverse transcriptase enzyme. The cDNA was used as template for amplification of coat protein gene of CVMV using gene specific primer.

A set of primers were designed to amplify the CP gene of CVMV. The predicted 531 bp DNA fragment was amplified in all diseased samples and

no amplification was obtained in healthy samples (Fig. 1).

The present findings are in accordance with Tsai *et al*. (2008) who studied complete sequence of ChiVMV infecting peppers in India and reported that it shared more than 94.8% nucleotide identity with previously accomplished full length sequence of *pepper vein banding virus* (PVBV) (Anindya *et al.*, 2004).

Supporting to the above study, three left over chilli fields (previously infected with murda complex) with bare stems and free from sucking pests giving new flushes showed systemic dark green mottle, vein banding, necrotic ringspots, leaf distortion and reduced leaf size alone in all leaves. When these leaves diagnosed for ChiVMV through RT-PCR using the same above degenerate primers, all samples showed fairly a thick amplicon of the predicted size (1.2 kbp). Which implicits that the virus remained in its native form in stems and systemically infected the new flush.

Ravi *et al.* (1997) compared the N-terminal sequence of PVBV CP with all the potyviral CP sequences and observed the DAG sequence conserved in all aphid-transmitted potyviruses in the N-terminal sequence of the PVBV CP. Similar observations of conserved DAG motif was also made by Tsai *et al.* (2008) in all ChiVMV isolates including Indian isolate.

Vector-virus relationship revealed that among the insects, only aphids, *Aphis gossypii* and *Myzus persicae* were able to transmit the virus from diseased to healthy chilli seedlings and virus was identified as *Potyvirus*. However, thrips, mites and whitefly failed to transmit the virus from diseased to healthy seedlings (Gundannavar, 2006).

Fig. 1. Amplification of CVMV-*CP* gene. M: 100 bp ladder, 1 & 2: Diseased chilli samples and 3: Healthy chilli sample

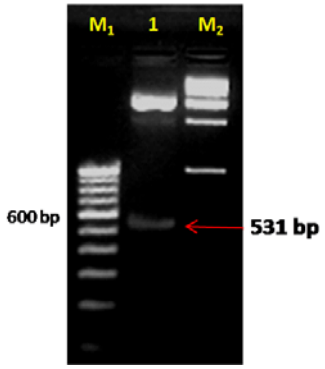

Fig. 2. Confirmation of CVMV-*CP* gene by restriction digestion. M1: 100 bp ladder, 1: CVMV-CP gene and pTZ57R/T vector backbone, M2: 1 kb ladder

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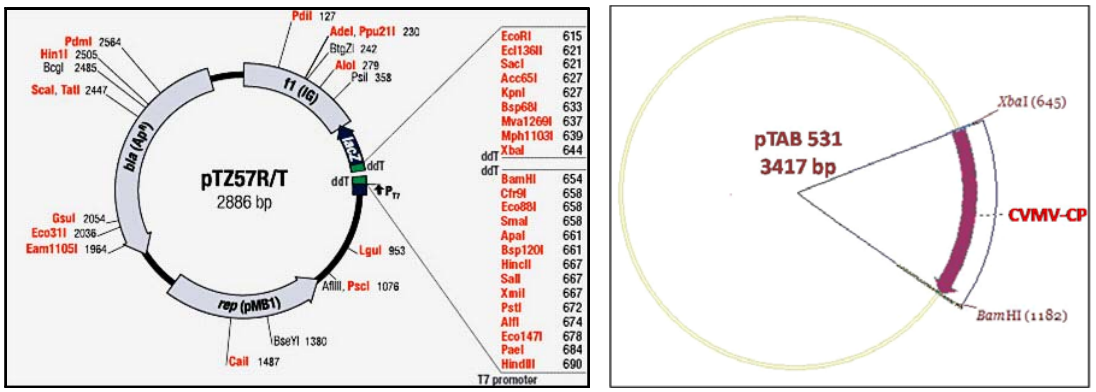


Fig. 3. Construct map of pTZ57R/T vector carrying CVMV-*CP* gene

Thus, the investigation focused on identification of the virus role in murda complex convincingly revealed the *Potyvirus* association with murda through molecular techniques at genus level. *Chilli veinal mottle virus* is the possible causative agent of murda complex through RT-PCR at species level. The vector associated with the transmission of potyvirus which causes chilli murda may be by aphids, even though their colonization was not seen in chilli plants, they may feed on chilli plants during their transitory flights and able to transmit during proboscis as reported by Byadgi *et al.* (2004) in case of papaya ring spot virus transmitted by aphid vectors.

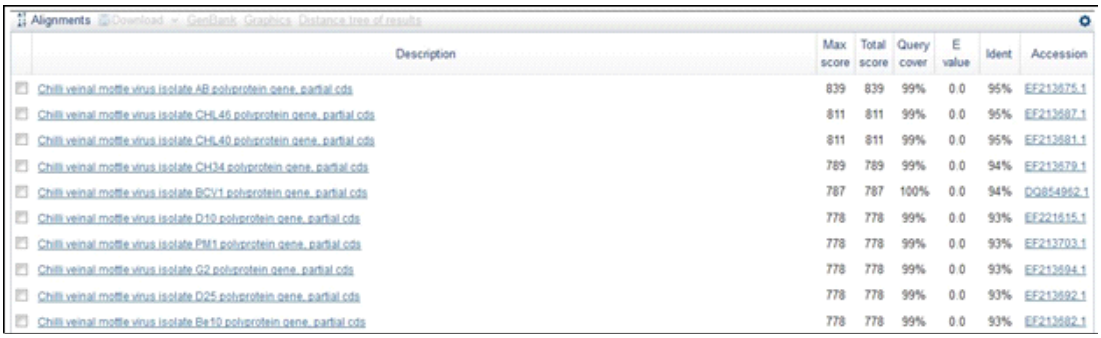
Cloning of CVMV-*CP* gene

PCR amplicon of CVMV-*CP* gene with 531 bp was cloned into pTZ57R/T (2886 kb) a cloning vector. The ligated recombinant vector pTZ57R/T carrying CVMV-*CP* gene was transformed to *E. coli* DH5a. Transformants carrying the ligated product were screened for blue-white colony assay to identify the recombinant clones carrying the coat protein gene. White colonies were produced due to the insertion of CVMV-*CP* gene in multiple cloning sites of pTZ57R/T, which disrupts the expression of *lacZ* and these colonies were picked and maintained on LA medium containing ampicillin (100 ìg/ml). The pTZ57R/T clones carrying CVMV-*CP* gene were named as pTAB531 (Fig. 3).

The transformed *E. coli* DH5a carrying CVMV-*CP* gene was initially confirmed by colony PCR using gene specific primers which yielded 531 bp amplicon when resolved on 1.2 per cent agarose gel. Plasmid isolated from the PCR confirmed colonies were further confirmed for presence of CVMV-*CP* gene by restriction analysis using *Xba* I and *Bam* HI restriction enzymes, which released a fragment of 531 bp CVMV-*CP* gene (Fig. 2).

Characterization of CVMV-*CP* gene

Sequencing of CVMV-*CP* gene was carriedout at Bangalore GeNei Pvt. Ltd. Sequence having a DNA fragment of 531 bp was obtained. Further, the sequence was analysed to find the homology and identity by subjecting them to NCBI-BLAST programme (www.ncbi.nih.gov/BLAST). The sequence clearly demonstrated the


Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chilli veinal mottle virus isolate AB polyprotein gene, partial cds	839	839	99%	0.0	95%	EF213675.1
Chilli veinal mottle virus isolate CHL46 polyprotein gene, partial cds	811	811	99%	0.0	95%	EF213687.1
Chilli veinal mottle virus isolate CHL40 polyprotein gene, partial cds	811	811	99%	0.0	95%	EF213681.1
Chilli veinal mottle virus isolate CH34 polyprotein gene, partial cds	789	789	99%	0.0	94%	EF213679.1
Chilli veinal mottle virus isolate BCV1 polyprotein gene, partial cds	787	787	100%	0.0	94%	DQ854962.1
Chilli veinal mottle virus isolate D10 polyprotein gene, partial cds	778	778	99%	0.0	93%	EF221615.1
Chilli veinal mottle virus isolate PM1 polyprotein gene, partial cds	778	778	99%	0.0	93%	EF213703.1
Chilli veinal mottle virus isolate G2 polyprotein gene, partial cds	778	778	99%	0.0	93%	EF213694.1
Chilli veinal mottle virus isolate D25 polyprotein gene, partial cds	778	778	99%	0.0	93%	EF213692.1
Chilli veinal mottle virus isolate Be10 polyprotein gene, partial cds	778	778	99%	0.0	93%	EF213682.1

Fig. 4. BLAST analysis of CVMV-*CP* gene

coat protein gene showing 95% homology with the reported CVMV (EF213675.1) coat protein gene sequence (Fig. 4).

The sequence analysis showed CVMV-*CP* gene was inserted in sense direction in pTZ57R/T. Further restriction analysis using Vector NTI Advance 10 software was carriedout to map the restriction sites (Fig. 3). The compatible restriction sites were searched for further cloning into binary vector from the restriction map. *Xba* I and *Bam* HI restriction sites were found in flanking to the gene in the multiple cloning sites of vector.

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