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Molecular diagnosis of begomovirus associated with leaf curl disease of *Dahlia pinnata*

Sayed S. Sohrab*

Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

*Corresponding author E-mail: sayedsartaj@gmail.com, ssohrab@kau.edu.sa

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Abstract

Diseases caused by begomoviruses represent serious threat to crop cultivation globally. A field survey was conducted in June, 2018 and severe leaf curl disease symptoms were observed on *Dahlia pinnata* at Ballia, U.P. India. The association of begomovirus was identified by polymerase chain reaction (PCR). The viral components were amplified by Rolling circle amplification (RCA); and digested with BamH1 and ECoRV which released the 2.7 kb fragment. The betasatellites was amplified by using betasatellites specific primers. The fragments were cloned, sequenced, analyzed and submitted to GenBank under the accession numbers (MK122994-full-length) and (MK122995-betasatellites). The full genome had total 2780 nucleotides, whereas betasatellites had 1347 nucleotides. The nucleotide sequence of full genome showed the highest identity (99.5%- 98.4%) with Tomato leaf curl Patna virus (ToLCPaV); infecting *Casia tora*, tomato, and *Xanthium strumarium*. The lowest sequence identity (37.9%) was observed with Chickpea chlorotic dwarf virus infecting *X. strumarium* in Pakistan. The betasatellites sequence showed the highest identity (99.2%) with Tomato leaf curl Patna betasatellites from *C. tora*. Currently, the association of begomovirus is known for Tomato, *C. tora* and *X. strumarium* in eastern U.P. and Bihar. Based on these findings, the begomovirus was tentatively known as a strain of ToLCPaV and designated as ToLPaV-Dahlia. This is a preliminary investigation about the natural occurrence of begomovirus causing leaf curl disease on *D. pinnata* in India.

Keywords: Dahlia, leaf curl, begomovirus, genetic diversity, India

1. Introduction

Dahlia is commonly grown as garden flowering plant which belongs to family Asteraceae. It is a kind of bushy, tuberous, herbaceous and perennial plant. Currently, there are total of forty-two species of Dahlia with hybrids that are grown globally. The natural viral infection seriously affects the growth of this plant. The common viruses infecting Dahlia are known as Tomato spotted wilt virus (TSWV), Dahlia Mosaic Virus (DMV), Dahlia common mosaic virus (DMCV) and Impatiens necrotic spot

virus (INSV) (Pahalawatta *et al*., 2007; Eid *et al*., 2011). Symptoms of viral infection include; chlorosis along veins, mosaic, chlorotic spots, general chlorosis, necrotic patches, marginal necrosis leaf deformation, distortion, stunted plant growth, short flower stems, and poor flowers. According to Briddon *et al*., (2008); Briddon *et al*., (2010); Brown *et al*., (2015), the begomovirus belongs to family Geminiviridae; which is a serious pathogen causing diseases in various crops globally. The begomovirus have single stranded (ss) circular DNA genome (2.7-2.8 Kb). Zerbini *et al*., (2017) added that the family Geminiviridae has been divided currently into nine genera designated as; Becurtovirus, Begomovirus, Capulavirus, Curovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocovirus, and Turncurotovirus. The Geminiviruses are efficiently transmitted by whiteflies and cause serious threats to many economically important crops (Malathi, 2017). Additionally, the sap transmission of begomoviruses has been reported (Sayed *et al*., 2013a; Sayed *et al*., 2013b). The begomovirus associated diseases on economically important crops were reported well; and currently have emerged as potential threat to crop cultivation (Navas-Castillo *et al*., 2011; Mubin *et al*., 2012; Sohrab, 2016; Ferro *et al*., 2017; Malathi, 2017; Sayed, 2018). Recently, natural occurrence of ToLCPaV has been reported on Tomato, *C. Tora* and *X. strumarium* from Bihar and eastern U.P., India (Sohrab, 2016; Sayed, 2018). The severe leaf curl disease and whiteflies were observed on Dahlia plant at eastern U.P., India, which gave an indication that begomovirus could be associated with this disease. Based on published reports, there is no data available about the begomovirus associated disease on *D. pinnata* plants in India. In the current study and for the first time, the natural occurrence and molecular diagnosis of begomovirus leaf curl disease of *D. pinnata* has been discussed and reported from India.

2. Materials and methods

2.1. Collection of samples and detection of begomovirus using PCR

A field survey was conducted during June-July, 2018 around Dahlia, Tomato, and Chili growing fields. The naturally infected *D. pinnata* plants showing severe leaf curling symptoms were selected and a total of 5 leaf samples were collected from different locations at Ballia, U.P., India. The population of whiteflies was observed in and around the tomato, chili and Dahlia plants which provided an indication that begomovirus could be transmitted to Dahlia plants by whiteflies. Naturally infected leaf samples (100 mg) were used to isolate the DNA using DNAeasy plant mini kit (QIAGEN, Valencia, CA, USA). The begomovirus infection was confirmed by PCR. The betasatellites was amplified by specific primers. The PCR reaction mixture was set up with purified DNA, Taq polymerase (2.5 units) (MBI; Fermentas, USA), 10X PCR buffer (5 µl), 10 mM dNTPs (0.5 µl), forward and reverse primers (0.5 µl) (10 pmol each), and final volume was made up to 50 µl by using sterile dist. water. The PCR conditions were set as; 94°C for 5 min^{-1} cycle, and 94°C for min^{-1} cycle; 46°C 50 s; 72°C for 60 s for 30 cycles. The conditions for betasatellites amplification were set as; 94°C for 5 min^{-1} cycle, 94°C for 1 min^{-1} cycle; 50°C for 1 min^{-1} cycle and 72°C for 1 min^{-1} 30 cycles, and 72°C for 10 min^{-1} cycle were given as final extensions in both PCR's (Sohrab, 2016; Briddon *et al*., 2002).

2.2. Amplification and cloning of viral genome

Rolling circle amplification (RCA) using phi29 polymerase (MBI Fermentas) was conducted by using purified DNA (Johne *et al*., 2009). The amplification is performed by denaturation of the template at 94°C for 5 min. followed by cooling at

room temperature; finally phi29 polymerase was added in the mixture and further incubated at 30°C overnight. The amplified fragments were then digested with selected restriction enzymes like BamH1 and EcoRV. Released fragments (~2.7kb) were gel purified and finally cloned into a pUC-18 cloning vector. The betasatellites (~1.4kb) were cloned into a pGEMT-easy vector (Promega Life Corporation, USA). Initially, two clones from each sample were sequenced bi-directionally; however, only one sequence was further used for sequence analysis (Johne *et al*., 2009).

2.3. Sequencing and phylogenetic analyses

Selected clones were sequenced bi-directionally, and the obtained sequences were initially searched for sequences identity; and then compared for their similarities using BLASTn. The sequences which showed high similarities were further selected and retrieved from GenBank. The nucleotide identity matrix was analyzed using BioEdit and CLUSTALW softwares. The phylogenetic relationship was analyzed with the MEGA 7 tool (Kumar *et al*., 2016), by using generated sequences from this study such as the full begomovirus genome; as well as betasatellites sequences reported from different regions.

3. Results

During the field survey, total of five samples were collected from different localities and approximately 80% disease incidence of leaf curling was observed on *D. pinnata* plants. The association of begomovirus was identified by PCR in 4 symptomatic samples by using begomovirus specific coat protein gene primers. No amplification was observed in the healthy samples (Fig. 1). The betasatellites were amplified from the same tested samples (data not shown); and confirmed that the virus isolate was a monopartite begomovirus.

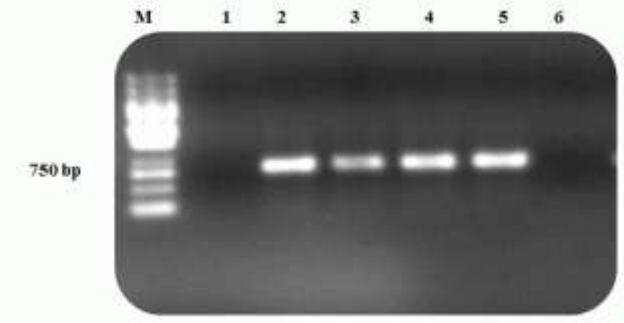

Fig. 1: Confirmation of begomovirus infection by PCR using CP gene specific primers. M: 1kb ladder (MBI, fermentas), 1: Healthy leaf, 2-6, naturally infected leaf samples.

3.2. Viral genome cloning and sequencing

The full-length of viral genome was amplified by RCA, whereas, betasatellites was PCR amplified. The RCA amplified product was gel purified and further digested with BamH1 and EcoRV enzymes, and then finally cloned into a PUC-18 cloning vector. Total of two full-length clones (~2.7kb) were confirmed by restriction enzymes. The PCR amplified betasatellites molecule (~1.4 kb) was cloned into the pGEM-T-Easy vector; however, finally only one clone from each sample was sequenced bidirectionally, and then used for phylogenetic relationship analysis. The full-length viral genome was found to have 2780 nucleotides (nt), while betasatellites had 1347 nucleotides (nt). Generated sequences were finally submitted to GenBank; thus bearing accession numbers (MK122994-full-length and MK122995-betasatellites). The identified begomovirus was tentatively designated as ToLCPaV-Dah isolate.

3.3. Analysis of sequence and phylogenetic relationship

For sequence and phylogenetic analysis, the nucleotide sequences generated in this study were BLAST search, and then significantly similar begomovirus sequences were retrieved from GenBank and used for genetic analysis.

The nucleotide sequence of ToLCPaV-Dahlia showed the highest sequence identity (99.5%) with ToLCPaV reported to cause disease on *C. tora*, followed by 98.4- 98.3% identity with the same isolate infecting tomato and *Xanthium*; respectively, reported from Ballia (Sayed, 2016; Sayed, 2018). This identity confirmed that the identified begomovirus could be a strain of ToLCPaV. The detailed genetic diversity/similarity analyses were performed with selected begomovirus sequence; based on full-length genome. Results showed that the ToLCPaV-Dah had 76.4% - 68.6% identity with previously characterized isolates as clear in Table (1). The lowest identity matrix (37.9%) was observed with Chickpea chlorotic dwarf virus recorded on *X. strumarium* plant from Pakistan. A phylogenetic tree was constructed based on the full-length genome with selected begomoviruses. The ToLCPaV-Dah isolate formed the closest cluster with ToLCPaV infecting tomato (EU862323) from Patna; ToLCPaV infecting *C. tora* (KT944082); and ToLCPaV-Xanth (KY612435) infecting *X. strumarium* from Ballia, U.P. Interestingly; there were three begomoviruses reported from Pakistan to cause diseases in *Xanthium*, but none of them formed close cluster with ToLCPaV-Dah isolate. The other begomoviruses formed clusters in multiple groups (Fig. 2).

4. Discussion

In the current study, natural occurrence; molecular diagnosis and genetic diversity of the begomovirus causing leaf curl disease of *D. pinnata* has been investigated. The highest identity was observed with ToLCPaV infecting Casia, Xanthium and tomato. This indicates that the virus has extended its host range and may spread to other economically important crops. Weeds and tomato plants were playing important roles in serving as reservoirs as well as alternative hosts to the begomovirus; in addition to whiteflies which were also capable of efficient transmission of this virus to Dahlia and other crops. Pahalawatta *et al*., (2007); Eid *et al*., (2011) pointed out that there was no begomovirus that cause disease on Dahlia except TSWV, DMV, DMCV and INSV. PCR and sequencing results of the current study confirmed the associations of begomovirus with leaf curl disease of Dahlia. However, earlier studies of Kumari *et al*., (2009); Sayed, (2016); Sayed, (2018) confirmed by PCR, reported the association of ToLCPaV on *C. Tora*, *Xanthium* and Tomato plants. The role of weeds as reservoirs of viruses/recombinant viruses; and as alternative hosts for vectors is well known and is increasing at an alarming rate. Several factors such as; whiteflies biotypes, frequent recombination, emergence of new virus strain/species, introduction of susceptible host varieties, change in cropping system, and presence of weeds as virus reservoirs; have favored begomovirus emergence as a major pathogen; which cause disease to new hosts and in new geographical regions (Lima *et al*., 2012; Mubin *et al*., 2012; Sayed, 2016; Sayed, 2018). Moreover, it has been reported that begomoviruses have DNA-B component from another species; and can infect other plants in the presence of betasatellites (Briddon *et al*., 2010; Mubin *et al*., 2012). In this study, an attempt was made to amplify DNA-B component but no positive amplification was observed in any sample. Data and results obtained in the current study provided evidence that ToLCPaV can easily infect other crops in the presence of betasatellites. Differences among phylogenetic trees have been recorded earlier for many begomoviruses reported from China, India, and Pakistan (Tahir *et al*., 2010; Mubin *et al*., 2012).

Conclusion

Finally, based on the current results; it is concluded that ToLCPaV can infect both weeds and other economically important crops. The presence of betasatellites from *D. pinnata* provided more information about the begomovirus spread to new hosts; which can thus be a threat towards the cultivation of crops in India. Therefore, it is important to screen more flowering plants, weeds and different crop species (symptomatic as well as

asymptomatic) for the presence of begomovirus and its associated satellites (alpha/beta); to get an insight of genetic diversities in the field, so that disease management strategy can be employed.

Table 1. Sequence identity matrices of full genome of ToLCPaV-Dah isolate (MK122994) with the selected begomoviruses

S. no.	Accession no.'s	Virus Acronyms	Hosts	Locations	% Identity matrices
1.	EU862323	ToLCPaV	Tomato	Patna	98.4
2.	KT944082	ToLCPaV	*Casia tora*	Ballia	99.5
3.	KY612435	ToLCPaV	*X.strumarium*	Ballia	98.3
4.	FR819708	ToLCGV	*X.strumarium*	Pakistan	72.2
5.	FR819707	CLCuBV	*X.strumarium*	Pakistan	72.7
6.	HE610413	ChCDPV	*X.strumarium*	Pakistan	37.9
7.	EF043231	ToLCNDV	Potato	Merrut	68.6
8.	GU474418	ToLCBV	Tomato	Bangalore	70.8
9.	JX987088	ToLCKV	*Zinnia elegans*	Lucknow	72.9
10.	FJ468356	ToLCNDV	Tomato	Pune	69.5
11.	DQ629102	PaLCV	Papaya	Delhi	71.6
12.	KY612431	CYVMV	*Sida cardifolia*	Ballia	70.3
13.	AJ507777	CYVMV	*C. bonplandianus*	India	71.3
14.	AM259382	SiYVMV	*Sida cardifolia*	India	71.2
15.	AJ002451	OYVMV	Okra	Pakistan	72.0
16.	FJ712189	MalCV	Malvestrum	China	76.4
17.	AY036009	HoLCrV	Holyhock	Egypt	72.7
18.	EF175733	RaLCV	Radish	India	75.1
19.	GQ245760	OKLCV	Okra	Delhi	71.4
20.	KT033710	ToLCSDV	Amaranthus	Saudi Arabia	74.3
21.	JN135233	PaLCV	Amaranthus	India	71.1

Where; ToLCPaV: Tomato leaf curl Patna Virus, ToLCPaV: Tomato leaf curl Patna Virus, ToLCPaV: Tomato leaf curl Patna Virus, ToLCGV: Tomato leaf curl Gujrat Virus, CLCuBV: Cotton leaf curl Burewala virus, ChCDPV: Chickpea chlorotic dwarf Pakistan virus, ToLCNDV: Tomato leaf curl New Delhi virus, ToLCBV: Tomato leaf curl Bangalore virus, ToLCKV: Tomato leaf curl Karnataka virus, ToLCNDV: Tomato leaf curl New Delhi virus, PaLCV: Papaya leaf curl virus, CYVMV: Croton yellow vein mosaic virus, CYVMV: Croton yellow vein mosaic virus, SiYVMV: Sida yellow vein Madurai virus, OYVMV: Okra yellow vein mosaic virus, MalCV: Malvastrum leaf curl virus, HoLCrV: Hollyhock leaf crumple virus, RaLCV: Radish leaf curl virus, OKLCV: Okra leaf curl virus, ToLCSDV: Tomato leaf curl Sudan virus, PaLCV: Papaya leaf curl virus.

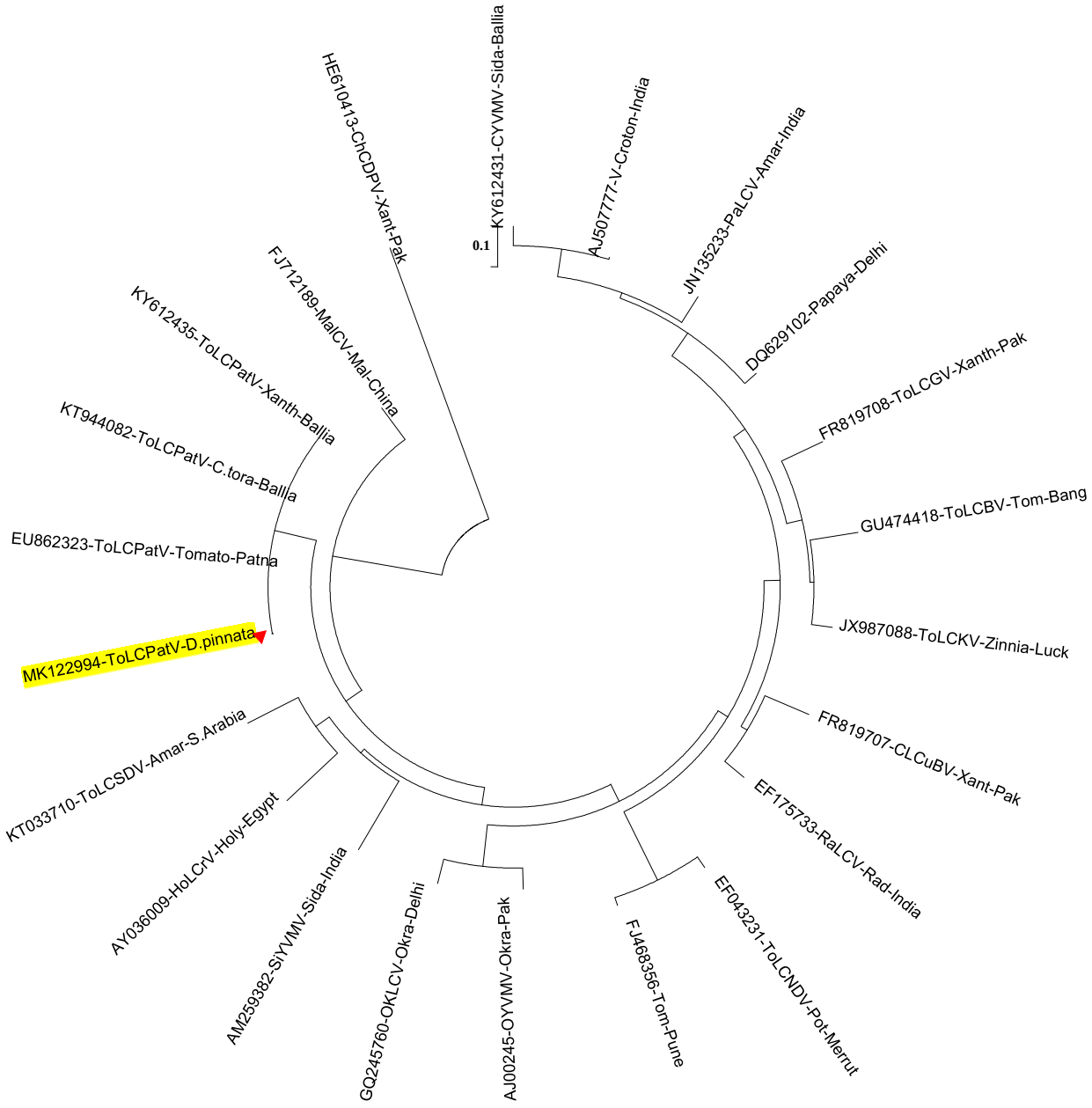

Fig. 2: Phylogenetic analysis; based on full genome of ToLCPaV-Dahlia isolate causing leaf curl disease of *D. pinnata*, with genomes of other selected begomoviruses

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