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Molecular Evidence for the Occurrence of *Tomato Leaf Curl New Delhi Virus* and *Squash Leaf Curl China* Virus on Volunteer Pumpkin Plants in Tamil Nadu

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ABSTRACT

Volunteer pumpkin plants showing yellow mosaic from two different locations of Tamil Nadu were collected near the cultivated field. Samples analyzed were found to be infected by *Tomato leaf curl New Delhi virus* and *Squash leaf curl China virus* by PCR using coat protein gene specific primer pair (GK ToLCV F/R) of *Tomato leaf curl virus*. The nucleotide and amino acid sequence analysis reveals that, these isolates shares maximum identity towards isolates infecting cucurbits than non-cucurbitaceous plants which were previously reported from India. These virus infections on these volunteer plants might serve as a reservoir for virus inoculum to the subsequent crops under field conditions.

Key words *Begomovirus; Pumpkin; Cucurbits; Virus disease; Yellow mosaic*

Begomoviruses cause serious diseases in both agricultural and horticultural crops like pulses (Sathya, *et al*., 2013), cucurbits (Singh, *et al*., 2008), cassava (Saunders, *et al*., 2002), chilli (Khan, *et al*., 2006), Tomato (Pandey, *et al*., 2010), brinjal (Pratap, *et al*., 2011), etc., Begomoviruses are transmitted by whitefly (*Bemisia tabaci*) in a persistent manner. It constitutes a bipartite genome, DNA-A and DNA-B or sometimes with monopartite which resembles DNA-A of the bipartite begomoviruses (Dry, *et al*., 1993). Pumpkin (*Cucurbita moschata*) is one of the vital vegetable crop grown extensively in Indian subcontinent and is found to be an excellent source of vitamins and antioxidants. In Tamil Nadu state, it is cultivated in an area of around 900 ha, with major area under the districts of Coimbatore, Tiruppur and Dindigul. This crop is being reported to infected by several begomoviruses virus *viz*., *Pumpkin yellow mosaic virus, Squash leaf curl China virus* (Muniyappa, *et al*., 2003), *Tomato leaf curl New Delhi virus* (Phaneendra, *et al*., 2012), *Squash leaf curl virus* (Lazarowitz and Lazdins, 1991), *Squash mild leaf curl virus* (Brown, *et al*., 2002), *Squash leaf curl China virus*-Philippines (Kon, *et al*., 2003), *Squash leaf curl Yunan virus* (Xie and Zhou, 2003) from different parts of the world. During our survey, some of the volunteer pumpkin plants were observed with yellow mosaic on leaves. With this background, an attempt has been made to detect and characterize the viruses causing yellow mosaic disease on pumpkin in Tamil Nadu.

MATERIALS AND METHODS

Survey and Collection of plant samples

Surveys were conducted during 2012-2014 in pumpkin field of farmers holdings from Erode and Salem district to study the virus associated with yellow mosaic disease pumpkin crop. During our survey, some of volunteer pumpkin plants from Anthiyur and Mettur regions of Tamil Nadu showing mosaic, blistering and puckering of leaves observed were collected along with healthy plant samples and brought to the laboratory at the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore for detection and molecular analysis of the associated virus with the symptoms. The collected samples were stored at -80°C for further studies.

Total DNA extraction and PCR amplification

The total DNA was extracted from 100 mg of the infected leaf tissue as well as healthy leaf samples using CTAB method described by Doyle and Doyle (1990). The genomic DNA quality and quantity was checked on 0.8% agarose gel and stored at -20°C for further use. PCR amplification has been carried out with degenerate primer specific to coat protein gene of *Tomato leaf curl virus* GKToLCV F: ATGKYGAAGCGACCAGCMGA; GKToLCV R: CGCCCKCMGAYTGGGMTT TTCTT (Nagendran *et al*., 2014). PCR was carried out with the master mix (Smart Prime, India) in 50µl reaction volume containing 25 µl master mix, 5µl (10 pmole) each of forward and reverse primers and 5µl (50ng) of template PCR was made with the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 90 sec. Amplified PCR products were cloned in pGEMT vector (Promega, USA) and sequenced.

Sequence comparison and Phylogenetic analysis

The sequences of virus isolates under this study were compared with the sequences of selected begomoviruses obtained from the GenBank database. Multiple sequence alignment was done using Clustal W (www.ebi.ac.uk) followed by phylogenetic analysis using MEGA 6.0 (www.megasoftware.net) and constructed with the neighbor-joining algorithm,

Table 1. Percentage identity of nucleotide and amino acid sequences of CP gene encoded by *Begomoviruses* of volunteer pumpkin plants in Tamil Nadu with those other selected *Begomovirus* for the study

Accession no.	Virus	TN MET PUM2		TN ANT PUM1	
		nt	aa	nt	aa
AY184487	*Squash leaf curl China virus*-[Pumpkin:Coimbatore]	94.4	96.1	97.5	98.0
KF188433	*Squash leaf curl China virus* isolate KP1	93.5	95.7	97.1	98.0
AY396151	*Squash leaf curl China virus* - [Pumpkin: Lucknow]	95.5	96.4	97.0	98.4
DQ026296	*Squash leaf curl China virus* - [Pumpkin: Lucknow]	95.5	96.4	97.0	98.4
AM286794	*Squash leaf curl China virus* - [*Cucurbita pepo*: Lahore] clone CPoAL4(3)	95.0	95.7	96.4	97.6
GQ225735	*Squash leaf curl China virus* isolate SQLCCNV:Var:Pum:08:4	94.6	95.7	96.2	97.6
FJ232922	*Squash leaf curl China virus*	94.9	95.7	96.2	97.6
GQ225734	*Squash leaf curl China virus* isolate SQLCCNV:Var:Pum:08:3	94.5	95.7	96.1	97.6
FN645905	*Tomato leaf curl New Delhi virus*, clone 4-RCA-AI-F	97.0	96.8	94.6	98.0
AM747291	*Tomato leaf curl New Delhi virus* - Bitter Gourd isolate Mn-05	96.8	96.8	94.8	98.0
AM286434	*Tomato leaf curl New Delhi virus*-[Pumpkin:New Delhi] isolate 2	96.3	96.1	94.5	98.0
KF551576	*Tomato leaf curl New Delhi virus* isolate TC309	96.1	96.1	94.5	98.0
AM286433	*Tomato leaf curl New Delhi virus*-[Pumpkin:New Delhi] isolate 1	96.1	95.3	94.2	97.2
EU366163	*Tomato leaf curl New Delhi virus* strain AKT	95.9	96.4	94.6	98.4
KC513822	*Tomato leaf curl New Delhi virus*-India isolate Lucknow	95.8	96.4	94.8	98.4
AY939926	*Tomato leaf curl New Delhi virus*	95.8	96.1	94.5	98.0
KC545812	*Tomato leaf curl New Delhi virus* isolate India:Delhi:Cucumis:2012	95.7	95.7	94.0	97.2
KC207815	*Tomato leaf curl New Delhi virus* isolate India:UP:Bahraich:*Luffa cylindrica*:2012	95.7	96.1	94.6	98.0
JN208136	*Tomato leaf curl New Delhi virus* isolate India:Ash gourd:2011	95.7	94.5	93.9	96.4
KM269363	*Tomato leaf curl New Delhi virus* TN MET PUM 2	100.0	100.0	93.7	94.9
KM269340	*Squash leaf curl China virus* TN ANT PUM 1	93.7	94.9	100.0	100.0

nt – nucleotide aa – amino acid

bootstrapped with 1000 replicates. Similarity percentage were calculated for the viruses under this study with other genbank accessions using BIO-EDIT.

RESULTS AND DISCUSSION

In 2012-2014, during our survey on virus diseases of cucurbits in Tamil Nadu, yellow mosaic diseases observed on volunteer pumpkin plants i.e., isolate TN MET PUM2 from Mettur (Salem) and TN ANT PUM1 Anthiyur (Erode) (Fig. 1). Similarly, chlorotic spots, yellowing, mottling, vein clearing and mild mosaic were associated with the virus infection in Squash (Abdalla and Ali, 2013). Saritha, *et al*. 2011 reported that SLCCNV infection cause mosaic and puckering of leaves on summer squash in India. Sohrab, *et al*., 2010 described the association of ToLCNDV with chlorosis and curly stunt disease of bottle gourd in North India.

DNA extracted from the symptomatic and non-symptomatic leaves were subjected to PCR for screening of presence of *Tomato leaf curl virus* using GKToLCV F/R primer pairs (Nagendran, *et al*., 2014) amplifying the complete coat protein. Symptomatic leaves showed an amplification of ~930bp but not in non-symptomatic leaves (Fig. 2) which confirms the presence of *Begomovirus* associated with the yellow mosaic diseases of pumpkin. Zepeda, *et al*., 2007 used the complete coat protein (CP) gene nucleotide sequence for provisional identification of species in *Begomovirus*. Similarly, amplified products were cloned in pGEMT vector. Two clones per ligation were sequenced and the clones shared 99.8% nucleotide sequence identity between them and sequences were analyzed. In a BLAST search, sequence from Mettur (TN MET PUM2) shares 97 % similarity towards ToLCNDV infecting cucurbits reported from India (FN645905, KC914896 and AM747291) and 96% identity with AM286434 and KF551576 of cucurbits and Tomato respectively. Our Anthiyur (TN ANT PUM1) isolate shares 98% identity with AY184487 and 97% with

TN MET PUM 2 (Mettur) TN ANT PUM 1 (Anthiyur)

Fig. 1. Yellow mosaic symptoms of *Begomovirus* infection on volunteer pumpkin plants

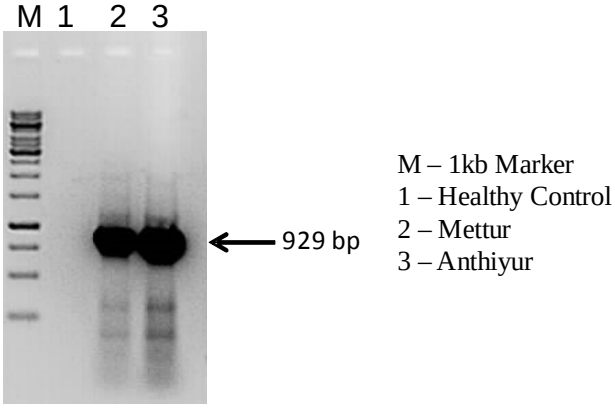

Fig. 2. Gel photograph showing amplification of ToLCNDV and SLCCNV using coat protein gene specific primer pairs GK ToLCV F/R (B)

KF188433 and DQ026296 of *Squash leaf curl China virus* (SLCCNV) infecting cucurbits from India. Nagendran *et al.* (2014) reported that association of ToLCNDV and SLCCNV infection on ash gourd in Tamil Nadu causing severe mosaics; chlorotic spots and curling and deformation of leaves. In this study, both ToLCNDV and SLCCNV infection cause yellow mosaic on pumpkin plants. Interestingly, in BLASTn analysis, our ToLCNDV isolate infecting pumpkin is found to have close relationship towards the ToLCNDV isolates infecting cucurbits in GenBank than isolates infecting other crops.

The nucleotide and amino acid sequence analysis of the complete coat protein of our ToLCNDV isolate TN MET PUM 2 (KM269363) revealed a maximum identity of 97% and 96.8% respectively with ToLCNDV (FN645905) infecting bottle gourd from India followed by other cucurbits infecting ToLCNDV ranging from 96.8-95.7% at nucleotide level and 96.8-94.5% at amino acid level identity. But this isolate is sharing nucleotide identity ranging from 95.5-93.5 % towards SLCCNV (Table 1). Similarly, the nucleotide and amino acid sequence analysis of the complete coat protein of our SLCCNV isolate TN ANT PUM 1 (KM269340) revealed a maximum identity of 97.5% with SLCCNV (AY184487) at nucleotide level and 98.4% identity at aminoacid level with AY396151 and DQ026296 of SLCCNV infecting pumpkin from India followed by other cucurbits infecting SLCCNV ranging from 97.1-96.1% at nucleotide level and 98.0-97.6% at amino acid level identity (Table 1). But this isolate is sharing nucleotide identity ranging from 94.8-93.6 % towards

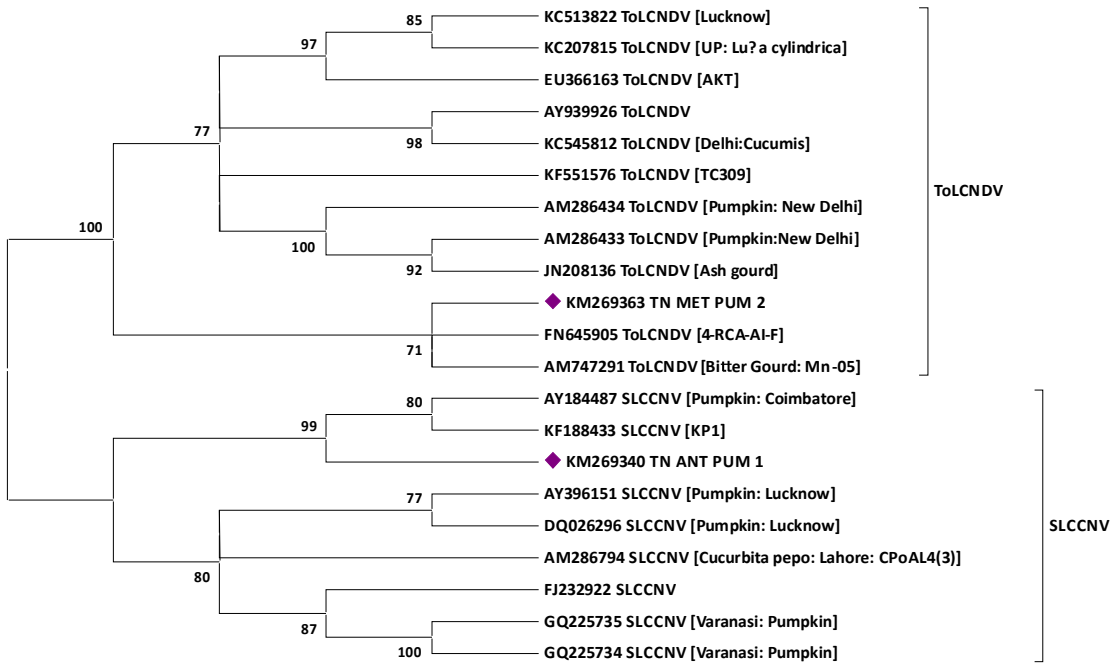

Fig. 3. Phylogeny of complete nucleotide sequence of CP region of ToLCNDV (TN MET PUM2) and SLCCNV (TN ANT PUM1) with other *Begomovirus* isolates. The tree was generated using the Neighbour-joining method in MEGA 6. A bootstrap analysis with 1000 replicates was performed and bootstrap percent values more than 70 are numbered along branches.

ToLCNDV. Identity of 93.7% at nucleotide and 94.9% at amino acid level were observed between our ToLCNDV (TN MET PUM2) and SLCCNV (TN ANT PUM1) isolates.

The phylogenetic analysis of coat protein nucleotide sequence, ToLCNDV-TN MET PUM2 formed a separate cluster under ToLCNDV isolates of India infecting cucurbits showing close relationship with FN645905 and AM747291 isolates of ToLCNDV and SLCCNV- TN ANT PUM1 form separate cluster along with other SLCCNV isolates showed closest relationships with the isolates of *Squash leaf curl China virus* (AY184487 and KF188433) (Fig. 3). SLCCNV and *Pumpkin yellow vein mosaic virus* (PYVMV) causes yellow vein mosaic disease of pumpkin in North India (Singh et al., 2008) and southern India (Muniyappa et al., 2003). But PYVMV was later identified as *Squash leaf curl China virus*-(Pumpkin: Coimbatore) on the basis of its complete nucleotide sequence (Muniyappa, *et al.*, 2003). Similarly Phaneendra *et al.*, 2012 reported the occurrence of ToLCNDV on pumpkin causing upward leaf curl with chlorotic patches and stunting of plant from North India. In this present study, we can evidence the simultaneous occurrence of both SLCCNV and ToLCNDV on pumpkin in Tamil Nadu causing similar kind of yellow mosaic disease. Since these samples were collected from the volunteer pumpkin plants, these plants may act as a virus reservoir to the cultivated crops. This study provides the strong platform for development of management strategies to prevent the transmission of virus from volunteer plants to healthy crop under field conditions. For the effective management of these virus diseases in crop plants, infected volunteer plants in and around cultivated field have to be removed on for prevention in spreading of virus disease.

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