

SHORT COMMUNICATION

**FIRST REPORT OF TOBACCO CURLY SHOOT VIRUS (TBCSV)
AND ITS ASSOCIATED SATELLITES FROM WATERMELON IN CHINA**

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Tobacco curly shoot virus (TbCSV), a monopartite begomovirus (genus *Begomovirus*, family *Geminiviridae*), is a serious threat to many crops and weeds in China and India. In this study, a begomovirus disease complex was identified from a watermelon sample displaying severe leaf curling and yellowing symptoms. The genome of this TbCSV isolate Y3560 contained 2,744 nucleotides (nt) and shared 97.9% identity with the TbCSV YN4519 isolate. The complete nucleotide sequence of the betasatellite DNA molecule associated to isolate Y3560 contained 1,342 nt and shared 91.0% sequence identity with the tomato yellow leaf curl China virus betasatellite isolate TYLCCNB-Y319. The nucleotide sequence of the alphasatellite contained 1,372 nt and shared the highest levels of identity (94.0%) with tobacco curly shoot virus associated DNA1 (TbCSA). This is the first report of a monopartite begomovirus and associated satellites from watermelon.

Keywords: *Begomovirus*, betasatellite, alphasatellite, watermelon.

The genus *Begomovirus* includes the majority of geminiviruses that are transmitted by whitefly *Bemisia tabaci* and are widespread in tropical, sub-tropical and increasingly warm temperate regions (Varma and Malathi, 2003; Nawaz-ul-Rehman and Fauquet, 2009). In the New World, begomoviruses have genomes consisting of two components (DNA-A and DNA-B). However, the Old World begomoviruses only have a single component that is homologous to the DNA-A component of bipartite begomoviruses (Harrison and Robinson, 1999). These monopartite begomoviruses are frequently associated with betasatellites (formerly DNA β) and alphasatellites (formerly DNA1), both of which are approximately a half of the DNA-A genome in size (Briddon *et al.*, 2002; Briddon *et al.*, 2003; Xie *et al.*, 2010). Recently, it has become clear that some betasatellites

affect the replication of their helper begomoviruses and contain a $\beta C1$ gene coding for a homonymous protein which has important roles in symptom induction, suppression of transcriptional and posttranscriptional gene silencing, and can affect jasmonic acid responsive genes (Zhou, 2013). Alphasatellites have also been mostly identified in plants infected with begomovirus-betasatellite complexes, however, their exact role (if any) in pathogenesis is yet to be elucidated (Zhou, 2013).

Watermelon is an important cash crop worldwide with over 81million metric tons produced annually, that is subjected to many yield-affecting adversities including diseases (Compton *et al.*, 2004). At present, five bipartite begomoviruses have been known to infect watermelon plants: watermelon chlorotic stunt virus (WmCSV) (Jones *et al.*, 1988), tomato leaf curl New Delhi virus (ToLCNDV) (Mansoor *et al.*, 2000), squash leaf curl virus (SLCV) (Abudy *et al.*, 2010), cucurbit leaf crumple virus (CuLCrV) (Guzman *et al.*, 2000), and melon chlorotic mosaic virus (MeCMV) (Romay *et al.*, 2010). However, there are no reports of any monopartite begomovirus isolated from watermelon plants.

In spring of 2014, begomovirus-centered surveys were conducted in several districts of Yunnan province, China. Three watermelon plants (Y3560, Y3561 and Y3562) showing upward leaf curling and yellowing symptoms were collected from watermelon fields located in Mangshi, a western state of Yunnan province (Fig. 1).



Fig. 1. Upward curling and yellowing of leaves observed in watermelon plants in Mangshi, Yunnan province during 2014.

Table 1. Percent sequence identities of DNA-A of the isolate Y3560 (KU198364) with isolates of tobacco curly shoot virus (TbCSV) infecting different hosts.

Accession No.	TbCSV isolate	Host	Country	DNA-A
KU934095	YN4519	Tomato	China	97.9%
AJ457986	Y41	Tomato	China	95.4%
GU199584	YN18	Mirabilis	China	96.0%
AF240675	Y1	Tobacco	China	95.1%
AJ420318	Y35	Tobacco	China	92.8%
HG003650	Y-T8	Ageratum	China	94.8%
AJ971266	Y282	Ageratum	China	92.8%
GU199583	YN20	Alternanthera	China	94.6%
JQ733557	FB-01	Common bean	India	92.6%
GU001879	SC118	Pepper	China	92.9%
JX457341	TC366	Tomato	India	92.2%
JX457342	TCb1	Tomato	India	92.0%
JN387045	To-Ag-1	Tomato	India	91.3%
HQ407395	WSF1	Sunflower	India	91.4%

Total DNA was extracted from symptomatic watermelon leaves using the CTAB method and submitted to PCR-based detection of DNA-A, beta- and alphasatellites using three previously reported primer sets to amplify partial DNA-A, betasatellite and alphasatellite, respectively (Doyle and Doyle, 1987; Briddon *et al.*, 2002; Bull *et al.*, 2003; Rojas *et al.*, 2005). Amplification products of the expected size were cloned and sequenced. Rolling circle amplification (RCA) was also carried out using a TempliPhiTM kit (GE Healthcare) to obtain enriched product (Guo *et al.*, 2009). According to the determined partial DNA-A sequence, virus-specific primers pairs TbCSV-F (5'-GCGTCACCAAAGCAAGAGCA-3') and TbCSV-R (5'-GGACAATGGGGGCAGCAGCA-3') were designed to amplify the full-length genome from the RCA product. The amplified fragments (*ca.* 2.7 kbp) were cloned into pGEM-T easy vector (Promega, USA).

Clones with the anticipated insert size were selected for sequencing in commercial facilities of Life Technologies, Shanghai, China. The nucleotide sequences were assembled with DNASTar version 7.1 (DNASTar Inc., USA) and open reading frames (ORFs) were identified by DNAMAN version 5.22 (Lynnon Biosoft, Canada). An initial sequence identity was compared by BLAST in the NCBI database (www.ncbi.nlm.nih.gov). A further comparison and multiple alignments were performed by the Clustal W method of MegAlign (DNASTar version 7.1). Phylogenetic trees were constructed using the neighbour-joining method with 1000 bootstrap replications available in MEGA5 version 5.05 (Tamura *et al.*, 2011). Recombination analysis was performed by recombination detection programs including the RDP, GENECOV, Bootscan, MaxChi, Chimaera, SiScan and 3Seq methods in the RDP version 4.46 (Martin *et al.*, 2010).

An approximately 500 bp DNA fragment was obtained using the degenerate primer pair PA/PB from a watermelon leaf sample (Y3560). Meanwhile, approximately 1350 bp-long DNA fragments were also produced

using the degenerate primer pair β 01/ β 02 and UNA101/UNA102 from the same sample. All these DNA fragments were cloned and sequenced. Sequence comparison using BLASTn showed that the sequences of amplicons generated with PA/PB primer set shared 98% identity with the CP gene of TbCSV-Y132 (data not shown), while Y3560 beta had the highest similarity with TYLCCNB-Y319 (91.0%). Clones Y3560-19 and Y3560-27 were identical to each other and shared 94.0% identity with TbCSA-Y99. However, no product of either begomovirus or satellite DNAs was obtained from the samples of Y3561 and Y3562, suggesting that observed symptoms were not caused by begomoviral infection.

The virus infecting sample Y3560 was further cloned and sequenced to generate the complete genome of TbCSV, which consisted of 2,744 nucleotides (KU198364). Comparisons with other sequences of TbCSV available in the databases showed the DNA sequences of Y3560 shared the highest levels of sequence identity (97.9%) with isolate YN4519 (KU934095) (Table 1). Similar to other TbCSV isolates, genomic DNA of isolate Y3560 encoded seven open reading frames (ORFs), including AV1 (295-1065) and AV2 (135-491) in the viral sense strand, AC1 (1514-2599), AC2 (1207-1611), AC3 (1062-1466), AC4 (2149-2448) and AC5 (273-440) in the complementary strand. Although the AC5/C5 ORF is only conserved in some bipartite and monopartite begomoviruses, an increasing number of begomoviruses have been annotated to contain an AC5/C5 ORF. Recently, research on the bipartite begomovirus mungbean yellow mosaic India virus (MYMIV) demonstrated that AC5 is a pathogenicity determinant and a strong RNA silencing suppressor that employs novel mechanisms to suppress antiviral defenses (Li *et al.*, 2015). Phylogenetic analysis of all TbCSV isolates from GenBank database along with the selected begomoviruses showed that the isolate TbCSV-Y3560 from watermelon grouped with isolate TbCSV-WSF1, and was closely related to other TbCSV isolates reported in China (Fig. 2a).

The clone of Y3560 beta (KU198365) consisted of 1,342 nucleotides, having the typical organization of betasatellites (Briddon *et al.*, 2003). It contained a single gene encoding a protein of 118 amino acids in the complementary strand (β C1; 209-565), a region of sequence rich in adenine (A-rich; 762-996, with 60% adenine content) and a highly conserved sequence present in all betasatellites known as the satellite conserved region (SCR; 1258-14). Comparison of the sequence of Y3560 beta to other betasatellites in GenBank showed that it shared the highest levels of identity (91.0%) with tomato yellow leaf curl China virus satellite DNA beta, isolate Y319 (TYLCCNB-Y319). Phylogenetic analysis of the betasatellites based upon alignments of the complete nucleotide sequences revealed that the present isolate was closely related to other isolates of TYLCCNB (Fig. 2b).

The sequence of Y3560-19 was identical with Y3560-27 and deposited in the GenBank database under accession

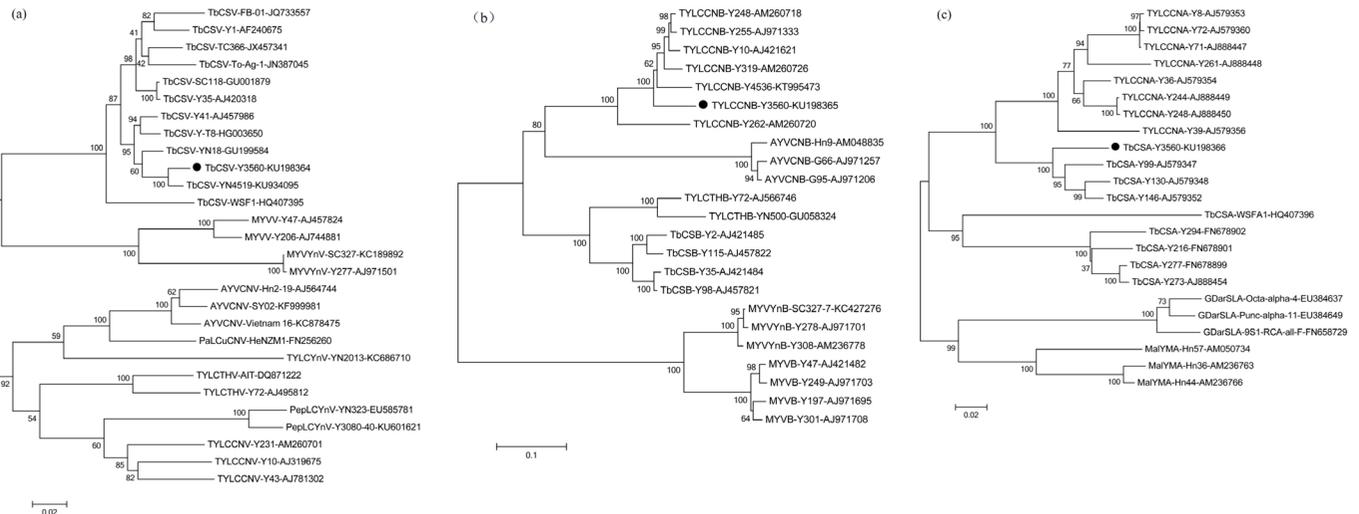


Fig. 2. Phylogenetic trees based on full-length nucleotide sequences of some begomoviruses (a), betasatellites (b) and alphasatellites (c) selected from the database. The sequences generated in this work are labeled with black circle. The phylogenetic trees were constructed using the neighbour-joining method with MEGA5, using 1,000 bootstrap replicates. The begomoviruses used to construct trees and corresponding acronyms reported in brackets are tobacco curly shoot virus (TbCSV), malvastrum yellow vein virus (MYVV), malvastrum yellow vein Yunnan virus (MYVYNV), ageratum yellow vein China virus (AYVCNV), papaya leaf curl China virus (PaLCuCNV), tomato yellow leaf curl Yunnan virus (TYLCYNV), tomato yellow leaf curl Thailand virus (TYLCTHV), pepper leaf curl Yunnan virus (PepLCYNV), tomato leaf curl China virus (TYLCCNV). The betasatellites used to construct trees and corresponding acronyms reported in brackets are tomato yellow leaf curl China betasatellite (TYLCCNB), ageratum yellow vein China betasatellite (AYVCNB), tomato yellow leaf curl Thailand betasatellite (TYLCTHB), tobacco curly shoot betasatellite (TbCSB), malvastrum yellow vein Yunnan betasatellite (MYVYNB), malvastrum yellow vein betasatellite (MYVB). The alphasatellites used to construct trees and corresponding acronyms reported in brackets are tomato yellow leaf curl China alphasatellite (TYLCCNA), tobacco curly shoot alphasatellite (TbCSA), *Gossypium darwinii* symptomless alphasatellite (GDarSLA), malvastrum yellow mosaic alphasatellite (MaLYMA).

No. KU198366. They consisted of 1,372 nucleotides and had the typical organization of alphasatellites (Bridson *et al.*, 2004), containing a single gene in the viral strand (Rep; 71-1018), an A-rich sequence (1052-1220; with 53.2% adenine content) and a predicted hairpin structure with the loop sequence TAGTATTAC typical of alphasatellites. Comparison of the sequence of Y3560 DNA1 to other alphasatellites in the GenBank showed that it shared the highest levels of identity (94.0%) with tobacco curly shoot virus associated DNA1, isolate Y99 (TbCSA-Y99), indicating it is an isolate of TbCSA. Phylogenetic analysis of the alphasatellite with selected sequences from the databases showed the present alphasatellite isolate belongs to TbCSA and was also closely related to TYLCCNA cluster (Fig. 2c).

No recombination events were detected by RDPv4.46 in any of the three molecules sequenced from sample Y3560.

This is the first identification of TbCSV/TYLCCNB/TbCSA complex in watermelon and also the first identification of a monopartite begomovirus in watermelon. TbCSV is a monopartite begomovirus that was firstly identified from tobacco in China in 2002 (Xie *et al.*, 2002) and subsequently it was reported in India (Shilpi *et al.*, 2015). The host range of the virus extends to tomato, pepper, *Alternanthera philoxeroides*, *Mirabilis jalapa*, ageratum, sunflower and common bean. These reports indicate that TbCSV is a potential serious begomovirus threatening

crop production. The cognate betasatellite of TbCSV is TbCSB, which was previously found in a small proportion of field samples from southern China (Li *et al.*, 2005). In our study, however, TYLCCNB was also associated with TbCSV and was detected in a single sample of watermelon showing leaf curl and yellowing symptoms.

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REFERENCES

- Abudy A., Sufrin-Ringwald T., Dayan-Glick C., Guenoun-Gelbert D., Livneh O., Zaccai M., Lapidot M., 2010. Watermelon chlorotic stunt and Squash leaf curl begomoviruses—New threats to cucurbit crops in the Middle East. *Israel Journal of Plant Sciences* **58**: 33-42.
- Bridson R.W., Bull S.E., Amin I., Idris A.M., Mansoor S., Bedford I.D., Dhawan P., Rishi N., Siwach S.S., Abdel-Salam A.M., Brown J.K., Zafar Y., Markham P.G., 2003. Diversity of DNA β , a satellite molecule associated with some monopartite begomoviruses. *Virology* **312**: 106-121.

- Briddon R.W., Bull S.E., Amin I., Mansoor S., Bedford I.D., Rishi N., Siwatch S.S., Zafar M.Y., Abdel-Salam A.M., Markham P.G., 2004. Diversity of DNA 1: a satellite-like molecule associated with monopartite begomovirus-DNA β complexes. *Virology* **324**: 462-474.
- Briddon R.W., Bull S.E., Mansoor S., Amin I., Markham P.G., 2002. Universal primers for the PCR-mediated amplification of DNA beta-a molecule associated with some monopartite begomoviruses. *Molecular Biotechnology* **20**: 315-318.
- Bull S.E., Briddon R.W., Markham P.G., 2003. Universal primers for the PCR-mediated amplification of DNA 1: a satellite-like molecule associated with begomovirus-DNA β complexes. *Molecular Biotechnology* **23**: 83-86.
- Compton M.E., Gray D.J., Gaba V.P., 2004. Use of tissue culture and biotechnology for the genetic improvement of watermelon. *Plant Cell Tissue and Organ Culture* **77**: 231-243.
- Doyle J.J., Doyle J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* **19**: 11-15.
- Guo W., Yang X.L., Xie Y., Cui X.F., Zhou X.P., 2009. Tomato yellow leaf curl Thailand virus-[Y72] from Yunnan is a monopartite begomovirus associated with DNA β . *Virus Genes* **38**: 328-333.
- Guzman P., Sudarshana M.R., Seo Y.S., Rojas M.R., Natwick E., Turini T., Mayberry K., Gilbertson R.L., 2000. A new bipartite geminivirus (Begomovirus) causing leaf curl and crumpling in cucurbits in the Imperial Valley of California. *Plant Disease* **84**: 488.
- Harrison B.D., Robinson D.J., 1999. Natural genomic and antigenic variation in whitefly-transmitted geminiviruses (Begomoviruses). *Annual Review of Phytopathology* **37**: 369-398.
- Jones P., Sattar M.H.A., Al Kaff N., 1988. The incidence of virus disease in watermelon and sweetmelon crops in the Peoples Democratic Republic of Yemen and its impact on cropping policy. *Annals of Applied Biology* **17**: 203-207.
- Li F.F., Xu X.B., Huang C.J., Gu Z.H., Cao L.G., Hu T., Ding M., Li Z.H., Zhou X.P., 2015. The AC5 protein encoded by Mungbean yellow mosaic India virus is a pathogenicity determinant that suppresses RNA silencing-based antiviral defenses. *New Phytologist* **208**: 555-569.
- Li Z.H., Xie Y., Zhou X.P., 2005. Tobacco curly shoot virus DNA β is not necessary for infection but intensifies symptoms in a host-dependent manner. *Phytopathology* **95**: 902-908.
- Mansoor S., Khan S.H., Hussain M., Mushtaq N., Zafar Y., Malik K.A., 2000. Evidence that watermelon leaf curl disease in Pakistan is associated with *Tomato leaf curl virus-India*, a bipartite begomovirus. *Plant Disease* **84**: 102.
- Martin D.P., Lemey P., Lott M., Moulton V., Posada D., Lefevre P., 2010. RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**: 2462-2466.
- Nawaz-ul-Rehman M.S., Fauquet C.M., 2009. Evolution of geminiviruses and their satellites. *FEBS Letters* **583**: 1825-1832.
- Rojas M.R., Hagen C., Lucas W.J., Gilbertson R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. *Annual Review of Phytopathology* **43**: 361-394.
- Romay G., Chirinos D., Geraud-Pouey F., Desbiez C., 2010. Association of an atypical alphasatellite with a bipartite New World begomovirus. *Archives of Virology* **155**: 1843-1847.
- Shilpi S., Alok K., Biswas S., Anirban R., Bikash M., 2015. A recombinant Tobacco curly shoot virus causes leaf curl disease in tomato in a north-eastern state of India and has potentiality to trans-replicate a non-cognate betasatellite. *Virus Genes* **50**: 87-96.
- Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S., 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.
- Varma A., Malathi V.G., 2003. Emerging geminivirus problems: A serious threat to crop production. *Annals of Applied Biology* **142**: 145-164.
- Xie Y., Zhou X.P., Zhang Z.K., Qian Y.J., 2002. Tobacco curly shoot virus isolated in Yunnan is a distinct species of Begomovirus. *Chinese Science Bulletin* **47**: 197-200.
- Xie Y., Wu P.J., Liu P., 2010. Characterization of alphasatellites associated with monopartite begomovirus/betasatellite complexes in Yunnan, China. *Virology Journal* **7**: 178.
- Zhou X.P., 2013. Advances in understanding begomovirus satellites. *Annual Review of Phytopathology* **51**: 357-381.

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