Journal of Agricultural and Marine Sciences 2024, 29(1): 71–77
DOI: 10.53541/jams.vol29iss1pp71-77
Received 04 Jun 2021
Accepted 01 Mar 2020

RESEARCH PAPER

Occurrence of an Old World Begomovirus Complex Infecting Radish in South of Nepal

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حدوث مجمع فيروس بيجومو في العالم القديم يصيب الفجل في جنوب نيبال

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Abstract. Radish (*Raphanus sativus*) leaf curl disease (RLCD) characterized by yellowing, color breaking, and mosaic symptoms were observed in a garden at Chitwan, Southern Nepal. Polymerase chain reaction using Taq DNA polymerase followed by rolling circle amplification using φ29 polymerase identified an Old World begomovirus complex linked with the RLCD. Pairwise sequence identity with SDT (Sequence Demarcation Tool) analysis coupled with phylogenetic analysis confirmed an *Ageratum yellow vein virus* (AYVV) and *Tomato leaf curl Java betasatellite* (ToLCJaB) was responsible for causing RLCD. This study provided initial evidence that radish plants were naturally infected with AYVV and ToLCuJaB in southern Nepal.

Keywords: *Geminiviridae*, Begomovirus, AYVV, whitefly, *Raphanus sativus*

المستخلص: تمت ملاحظة مرض تجعد أوراق الفجل (Raphanus sativus) (RLCD) الذي يتميز بالاصفرار وتكسر اللون وأعراض الفسيفساء في حديقة في شيتوان، جنوب نيبال. تفاعل البلمرة المتسلسل باستخدام بوليميريز الحمض النووي Taq متبوعًا بتضخيم الدائرة المتدحرجة باستخدام بوليميريز φ٢٩ حدد مجمع فيروسات البيجومو في العالم القديم المرتبط بـ RLCD.

أكدت هوية التسلسل الزوجي مع تحليل SDT (أداة ترسيم التسلسل) إلى جانب تحليل النشوء والتطور أن فيروس الوريد الأصفر (AYVV Ageratum) وتجعيد أوراق الطماطم (ToLCJaB Java betasatellite) كان مسؤولاً عن التسبب في RLCD. قدمت هذه الدراسة أدلة أولية على أن نباتات الفجل كانت مصابة بشكل طبيعي بـ AYVV وToLCuJaB في جنوب نيبال

الكلمات الرئيسية: الفيروسات التوأمية، الفيروس البيجومو، AYVV، الذبابة البيضاء، *Raphanus sativus*

Introduction

Begomoviruses (family; *Geminiviridae*) comprise of a single-stranded (ss) circular DNA molecules (ssDNA) and are important pathogens of vegetables, ornamentals, fruits and many other agriculture crops including weeds (Shahid et al., 2014; Al-Mabsli et al., 2021; Bananej et al., 2021; Salem et al., 2021; Shahid et al., 2021). In reference to the host range, genome orientation and insect vector *Geminiviridae* family has been divided into fourteen genera (with 520 spp.). Among them, viruses of Begomovirus genus are more prevalent and widely distributed (with 445 spp.) (Walker et al., 2021). The viruses of the genus begomovirus are additionally split into three subgroups: monopartite (having a single DNA-A molecule), bipartite (having two molecules of DNA-A and DNA-B) ~ 2.7 Kb of each, and frequent-

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ly associated monopartite DNA satellite genomes (i.e., alphasatellite, betasatellite, and deltasatellite). The DNA-A of either mono or bipartite begomovirus consisting of different Open Reading Frames (ORFs), which encodes for AC1/Rep- replication associated protein, AC2/TrAp-Transreplication associated protein, AC3/Ren- the replication enhancer protein and the AC4- viral suppressor of RNA silencing protein in the complementary sense and AV1/CP- coat protein and AV2-pre-coat protein in the virion-sense. The genome of DNA-B molecule of bipartite begomovirus consist of two ORFs, which encodes proteins as BC1/MP- function as movement protein and BV1/NSP- function as nuclear shuttle protein, which are involved in movement and intra-plant virus spread, respectively. Additionally, DNA-A and DNA-B genomes comprise of an approximately 300 nucleotides (nt.) long spacer DNA or intergenic region (IR), which includes an essential elements requisite for the extensive activity of replication as well as transcription of the begomoviral genome (Rojas et al., 2005). Frequently occurring monopartite begomoviruses spanning from South Asia, Europe, Middle East and Australia are also accompanying with small ssDNA and circular molecules, which have little or no genomic relatedness with their helper virus, exclusively identified as betasatellites (formerly called as DNA β) (Briddon and Stanley, 2006). Vegetable crops have been considered a major host for several Begomovirus species, which play a key role in spreading of these viruses into different geographical region and agriculture crops. The objective of this study was to identify the Begomovirus associated with radish host aiming to understand the role of this host in begomovirus diversity.

Materials and methods

Sampling and Nucleic Acid Extraction

During 2010, cropping season, unusual growth of radish (Raphanus sativus) plants characterized as yellowing, slight curling, color breaking and mosaics symptoms on the infected plants was observed in a garden in the Chitwan (27.6291° N, 84.8542°), Nepal. Medium to high population of whitefly (Bemisia tabaci) insect vector was also noticed on the infected radish plants. Plant leaf tissues (infected and apparent healthy) were collected, kept in silica gel until transported to the laboratory and stored at -4 °C until use. Genomic nucleic acid was extracted both from symptomatic and apparent healthy radish plants using established CTAB method as described earlier (Porebski et al., 1997), with slight modifications, addition of high salt concentration and polyvinyl pyrrolidone (PVP) to eliminate polysaccharides and polyphenols, respectively.

Cloning and Sequencing

The genomic DNAs with a set of forward/reverse detection primers (AC1048/AV494) were used in polymerase chain reaction (PCR) assay, which amplified ~550 nt. of core coat protein (CP) gene only from symptomatic radish leaves tissues (Wyatt and Brown, 1996). To further obtain the complete genome of begomovirus φ29 polymerase (Thermo Fisher Scientific, USA) enzyme was used in rolling circle amplification (RCA) an isothermal amplification which generate circular DNA molecules from all nucleic acid extracts as described earlier (Haible et al., 2006). The long concatemers of RCA products were proceed in endonuclease restriction (ER) analysis and with BamH1 restriction, which released approximately 2.7kb monomer molecules of begomovirus, which were gel purified using a Gene Jet Gel Purification Kit (Thermo Fisher Scientific, USA), cloned in pUC18 provided by (Takara Bio. Inc., Japan) and sequenced bidirectionally by Macrogen Inc. (Seoul South Korea) using chain-termination PCR process, where nucleotide sequence of DNA are determined by the addition of modified dideoxyribonuleotides (ddNTPs) into the reaction. The associated betasatellite were also obtained with abutting betasatellite β01/ β02 primers (Briddon et al.2002).

Sequence Analysis

The sequences were analyzed by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the maximum similarity in the nucleotide sequences. Open reading Frames were discovered by employing ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Phylogenetic dendrogram was constructed

using MUSCLE algorithm (Kumar et al., 2016), Possible recombination events were identified in Recombination Detection Program (RDP4 v4.72) (Martin et al., 2015). Pairwise sequence identity was analyzed with Sequence Demarcation Tool (SDT v1.2) (Muhire et al., 2014).

Results

Detection of Begomovirus Complex

In initial, screening eight radish plants exhibiting viral like symptoms and two non-symptomatic individuals were collected, DNA were extracted and diagnostic PCR was run with universal begomovirus detection primers AC1048/AV494 (Wyatt and Brown, 1996). The expected size approximately 550 nt PCR amplicons were obtained only from infected samples but not from healthy control, which confirm the presence of viral particles in symptomatic radish plants.

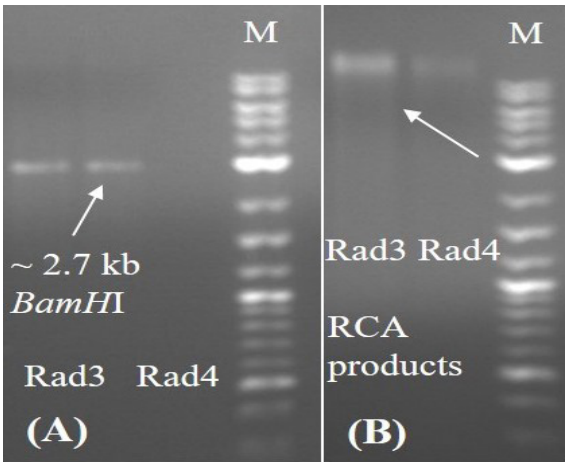

Figure 1. *Amplification of begomovirus by rolling circle amplification (RCA); monomer molecule in BamHI endonuclease restriction (A), and RCA concatemers (B)*

Full-length begomovirus genome were obtained in rolling circle amplification (RCA) with BamH1 endonuclease restriction (Figure 1). The complete nt. sequences of two virus isolates (Rad3/Rad4) and two betasatellite isolates (Rad5/Rad7) were generated by the reassembly of the virus and betasatellite contigs. Further, Blast and Open reading frames (ORFs) analysis showed the arrangement of genes of the begomovirus genome to be feature of the Old World reported viruses containing (AC1-AC4, AV1/CP and AV2) ORFs.

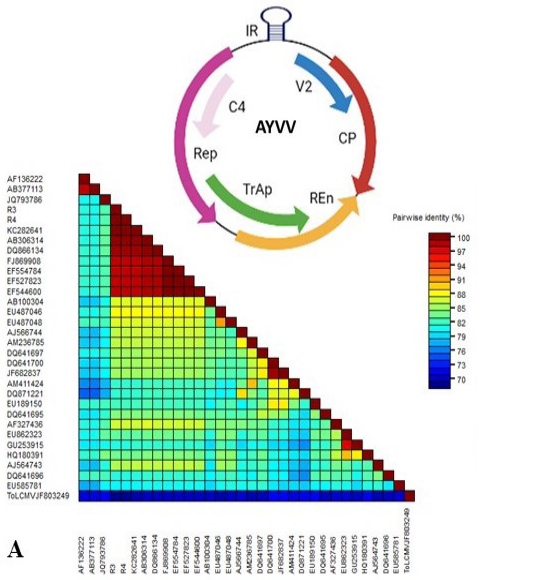

Figure 2A. *Genome structure of begomovirus, betasatellite along with SDT analysis for begomovirus (A), betasatellite (B)*

Characterization of Virus

The complete sequences 2,750 and 2,751 nt. of the begomovirus isolates (Rad3/Rad4) discovered after chain termination sequencing which were submitted in NCBI GenBank (acc. no. MZ393962-MZ393963). Pairwise analysis with Sequence Demarcation Tool (SDT v1.2) (Muhire et al.2014), revealed the sequences of RAD3 and/or RAD4 exhibited highest nt. identity (99.9%) to the isolate of Papaya infecting Ageratum yellow vein virus (AYVV) (KC282641) reported in Nepal (Shahid et al., 2013). Whereas the radish isolates exhibited (99.4 and 99%) nt. identity percentage with AYVV and Ageratum yellow vein Taiwan virus (AYVTWV), respectively. Moreover, these isolates showed 98% nt. identify with the genomic isolates of other AYVV but below than 87.0 % nt. identity percentage to the whole genomic sequences of other begomovirus species (Figure 2A). In phylogenetic dendrogram using MUSCLE algorithm (Kumar et al., 2016), RAD3/RAD4 isolates clustered with AYVV isolates, being maximum related to the isolates originating from Nepal, Japan,

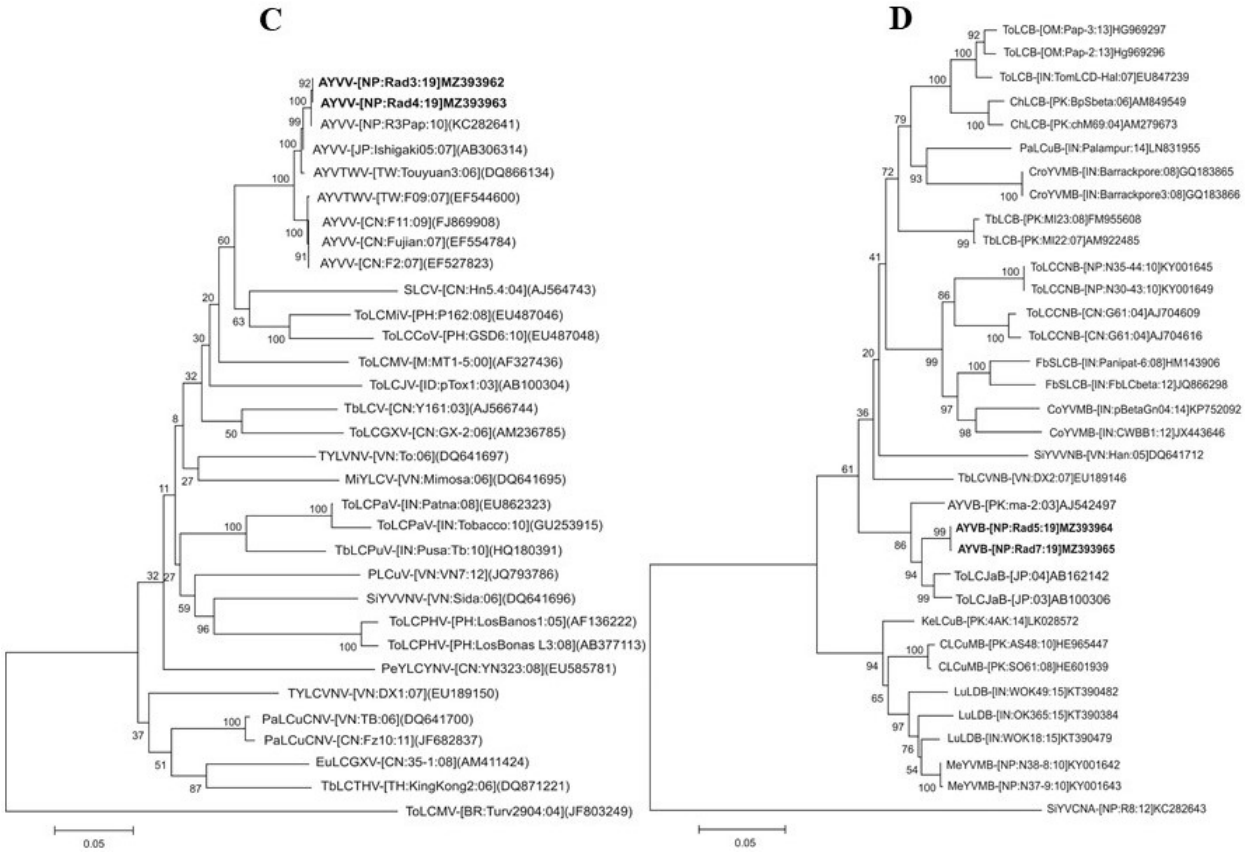

Figure 3. *Phylogenetic tree of monopartite begomovirus (C) and betasatellites (D) including refence sequences extracted from GenBank for comparison. The virus/betasatellite trees were rooted as outgroup on tobacco leaf curl Malaysia virus and Sida yellow vein China alphasatellite sequences, respectively. The R. sativus isolates highlighted as bold text.*

Taiwan and China (Figure 3C). In line with the recommendations by ICTV indicates for species cut off value the virus detected in R. sativus is an isolate of AYVV. Possible recombination events were identified in RDP4 (v4.72) by exploiting using algorithms as RDP (v4.72) program (2.27E-03), GENECONV (3.27E-03), Bootscan (3.42E-02), Maxchi (1.06E-05), Chimaera (5.22E-04), SiSscan (1.28E-02) and 3Seq (2.94E-05) (Martin et al.2015). In our study six algorithms confirms the recombinant which are reliable for recombination, Where AYVTWV, Pingdong isolate from Taiwan -Taiwa (AYVV-TW [TW: PD] AF327902) and Ageratum yellow vein China virus (TYVCNV-ZI: JX911332) reported as major and minor parents, respectively (Xiong et al.2007).

Characterization of Betasatellite

The associated betasatellite were obtained with abutting betasatellite β01/ β02 primers and cloned in pTZ257R/T (Thermo Fisher Scientific, USA) (Briddon et al., 2002). The sequences of associated betasatellite isolates (Rad5/Rad7) were found to be 1,355 and 1,357 nt long with NCBI GenBank acc. no. (MZ393964-MZ393965). The betasatellite sequences contained structures characteristic of reported betasatellite (Table 1); comprising of a single βC1 gene which encodes 118 amino acids long protein which functions as pathogenicity determinant as well as suppressors of gene silencing in different monopartite begomovirus complexes; a satellite conserved region which is present in all reported betasatellite molecules and a region which is rich in adenine nt. sequences (Briddon et al., 2002).

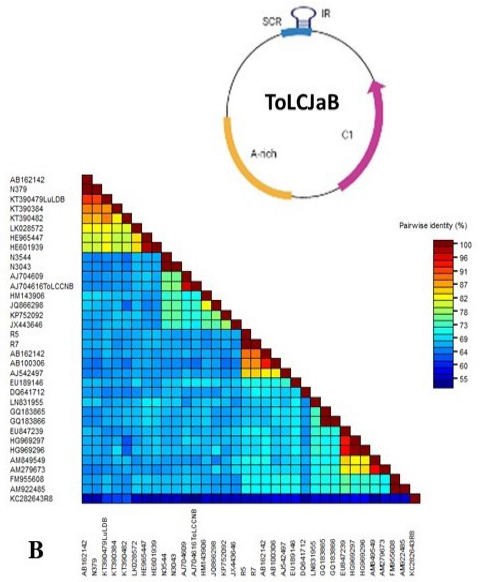

Figure 2B. *Genome structure of begomovirus, betasatellite along with SDT analysis for begomovirus (A), betasatellite (B)*

Further, SDT analysis displayed Rad5/Rad7 to have the greatest nt. identity 89-87% to the sequences of tomato leaf curl Java betasatellite (ToLCJaB; AB162142 and AB100306) whereas 84% nt. identity with Ageratum yellow vein betasatellite (AJ542497) and less than 71% to all other reported betasatellites (Figure 2B). The evolutionary relation using phylogenetic analysis segregate the radish isolates with ToLCJaB and AYVV isolates, confirming the identification of the sequences form R. sativus as isolates of ToLCJaB (Figure 3D).

Discussion

The findings of this study exhibited for the first time that Raphanus sativus with yellowing, color breaking and mosaic symptoms in South of Nepal is linked with the newly discovered monopartite begomovirus species AYVV and the betasatellite ToLCJaB. Radish is a major vegetable crop grown for the domestic purposes in Nepal. Comparatively only few begomoviruses, such as papaya leaf curl virus, Radish leaf curl virus (RaLCV), Croton yellow vein mosaic virus (CrYVMV), Papaya leaf curl virus (PaLCuV), Cotton leaf curl virus (CLCuV), Pedilanthus leaf curl virus (PeLCV), Tomato yellow leaf curl virus (TYLCV) and Chilli leaf curl virus (ChiLCV) have been identified to infect R. sativus from India, Pakistan and Oman (Mansoor et al., 2000), (Singh et al., 2012), (Ismail et al., 2017), (Al-Shihi et al., 2018), and (Kumar et al., 2021). The extensive study has not been done in Nepal for the occurrence of Geminiviruses, nevertheless few begomovirus species infecting various host species have been discovered recently (Shahid et al., 2012; Shahid et al., 2013; Tahir et al., 2015; Shahid et al., 2017). The discovery of AYVV and accompanied by ToLCB in R. sativus host demonstrates the possible significance of R. sativus in the appearance of begomoviral diseases of fruits, vegetable and ornamental crops in Nepal, that may expedite their drive into distinct host plant species. Due to the pervasive nature and broad host range, the begomoviral diseases are the main concern to the agriculture industry throughout the world. However, application of conventional agricultural practices such as quarantine regulation, incorporating of resistance gene to the elite cultivars, integrated pest management (IPM) can help to control the begomovirus diseases in Nepal (Al-Shihi et al., 2018). Further, ssDNA viruses are transferred through its insect vector (whitefly), so the management of whitefly density might be beneficial in inhibiting outbreak of such disease, which can be achieved by the use of agryl covers followed by the application of recommended insecticides would help to control the vector infestation (Rojas et al., 2005).

Overall, the findings of this study should provide as the source for further analysis of infected and non-infected fruits, vegetable and ornamental crops that could be possible sources and can hold diverse numerous begomovirus strains which may lead to the production of new begomovirus strains or species due to frequent recombination and component exchange phenomenon.

Authors contribution

MSS concive the idea, excecute the study and write the manuscript. Sampling and DNA extraction was done by BJP.

Acknowledgements

We are grateful to the farmers assisting us in plant sample collections

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