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Recombination study using *Radish leaf curl virus* isolates

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Begomovirus isolates, exhibiting identity of 89 and 93% with *Radish leaf curl virus* (RaLCV), were detected from the leaf curl disease affected okra (*Abelmoschus esculentus*) plants in Bihar, India. The detected isolates are supposed to be a new strain of RaLCV and therefore are referred to here as RaLCV-Hajipur1 and RaLCV-Hajipur2. Alphasatellite was identified as Cotton leaf curl Burewala alphasatellite (CLCuBuA) and betasatellite as Tomato leaf curl Bangladesh betasatellite (ToLCBDB). The aforementioned RaLCV-Hajipur isolates were used to construct infectious clones. These isolates were inoculated separately or together with alpha- and betasatellites in tobacco. The begomovirus and satellites were isolated from tobacco plants at twenty seven day post inoculation and then sequenced. Analysis of the sequences revealed the presence of a new isolate, referred to here as RaLCV-Hajipur3. Recombination analysis using recombination detection program 3 (RDP3) revealed that the RaLCV-Hajipur1, RaLCV-Hajipur2 and RaLCV-Hajipur3 had recombinant origin. RDP3 analysis of the RaLCV-Hajipur3 revealed that the two inoculated isolates (RaLCV-Hajipur1 and RaLCV-Hajipur2) were the probable major and minor parents of the newly detected isolate (RaLCV-Hajipur3).

Key words: Begomovirus, betasatellite, alphasatellite, recombination, evolution.

INTRODUCTION

Several begomoviruses (family *Geminiviridae*) have been reported to infect okra globally (Ali et al., 2000; Ghanem, 2003; Jose and Usha, 2003; Mansoor et al., 2001; Zhou et al., 1998). The begomoviruses are whitefly-transmitted geminiviruses that have emerged as a major problem for food and vegetable crops throughout the world (Moriones and Navas-Castillo, 2000; Mansoor et al., 2003, 2006). Begomoviruses are either monopartite (one component of ~2.7 kb) or bipartite (two components, each of ~2.6 kb) in genomic organisation. Bipartite begomovirus requires both components, though, only one component of monopartite begomovirus is sufficient for replication and movement (Stanley et al., 2005).

Except few tomatoes infecting begomoviruses, most of the monopartite begomoviruses are associated with betasatellite or alphasatellite or both. Betasatellite is approximately half of helper begomovirus and shares a highly conserved nonanucleotide sequence (TAATATTAC) which serves as origin of replication for betasatellite (Zhou et al., 2003). It contains an A-rich region and βC1 ORF; the former acts as stuffer and latter is responsible for symptom enhancement, virus accumulation and suppression of post-transcriptional gene silencing machinery (Briddon et al., 2001, 2003; Cui et al., 2004).

In addition to the betasatellite, one more satellite termed as alphasatellite has been found to be associated with diseases such as Ageratum yellow vein (AYVD), cot-ton leaf curl (CLCuD) and okra leaf curl (OLCD) (Mansoor et al., 1999, 2001; Saunders and Stanley, 1999). Alpha-satellite encodes replication-associated protein (Rep) which

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Figure 1. Infected plant of okra showing leaf curling and absence of fruits.

enables autonomous replication to this molecule in the cells of host plants; however, it requires the helper begomovirus for insect transmission and spread in plants (Mansoor et al., 1999). The present study was aimed at exploring the recombination event among the virus isolates and its contribution in evolution of a new isolate.

MATERIALS AND METHODS

Virus materials

Two symptomatic plants (Figure 1) from each field, and three fields each year, were selected as a source of virus which provided six samples each year and a total of twelve samples in two years (2009-10). DNA was extracted from the infected as well as healthy plants using DNAeasy plant mini kit column (Qiagen GmbH, Germany).

Detection of begomovirus and satellites

Extracted DNAs as template and specific primers F1For/Rev (5'-TTAAGAAAAGACCA GTCGGAGGG-3' / 5'-CATTTCCATCCGAACATTCAGGG-3') and F2For/Rev (5'-TTGA CATCTGAGCTTGATTTAGC-3' / 5'-TAACCTTCCGAATCTGGACGACCT-3') were used in PCR assay for amplification of begomovirus. Alphasatellite was amplified using 'nanofor' (5'-AAGTGGGTCCTGGTTCTA-3') and 'nanorev' (5'-CTGTACAGGTCTCTGG C-3') primers, while betasatellite was amplified using β01 (5'-GAAACCACTACGCTA CGCAGC-3') and β04 (5'-ACCCTCCCAGGGGTACACACCG-3') primers. PCR products of expected sizes were cloned in pDRIVE PCR cloning vector (QIAGEN, GmbH) and then sequenced.

Sequence analysis

Nucleotide sequence similarity search was performed using BLAST tool. Molecular Evolutionary Genetics Analysis (MEGA4; Tamura et al., 2007) software was used for phylogenetic analysis of begomovirus and satellites.

Construction of infectious clones

Infectious clone was prepared using rolling circle amplification (RCA; TempliPhi DNA amplification kit, GE healthcare, USA). The RCA product was partially digested with *Bam*HI to obtain head-to-tail tandem repeat dimers of begomovirus and was cloned in pCAMBIA1301 (CAMBIA, Canberra, Australia). Betasatellite dimer was prepared using two primer pairs: β5For/Rev (5′-GAAACCACTACGCTACGCAGCAGCC-3′ / AGGATCC TACCCTCCCAGGGGTACACACCGCCG-3′) and β6For/Rev (5′-AGGATCCGAAACCA CTACGCTACGCAGCAGCC-3′ / 5′-TACCCTCCCAGGGGTACACACCGCCG-3′). Following PCR, both amplified products were digested with *Bam*HI restriction endonuclease to have sticky ends at the tail of β5For/Rev amplified product and at the head of β6For/Rev amplified product. Both fragments were ligated together by using T4 DNA ligase (Promega, USA). Ligated head-to-tail dimer of betasatellite was digested by *Eco*RI and then cloned into pCAMBIA1301. Alphasatellite dimer was prepared by using two primer pairs: Nano2for/rev (5′-AAGTGGGTCCTGGTTCTA-3′ / 5′-AGAGCTCCTG TACAGGTCTCTGGC-3′) and Nano3for/rev (5′-AGAGCTCAAGTGGGTCCTGGTTCTA-3′ / 5′-CTGTACAGGTCTCTG GC-3′). The PCR amplified products were digested using *Sac*I and then ligated together. The ligated head-to-tail dimer of alphasatellite was digested using *Kpn*I/*Sal*I and then ligated in pCAMBIA1301. The recombinant pCAMBIA vector, harbouring dimers of begomovirus, betasatellite and alphasatellite, were transferred into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method (Jyothishwaran et al., 2007).

Agroinoculation

Agrobacterium containing infectious clones of begomovirus and satellites were grown separately to desired OD (optical density at 550 nm = 1) and were pelleted in resuspension buffer. Five different types of inoculum were prepared by mixing agrobacterium containing; first- one begomovirus variant, second- two begomovirus variants, third- begomovirus plus alphasatellite, fourth- begomovirus plus betasatellite, and the fifth- two begomoviruses plus betasatellite plus alphasatellite. The inoculated tobacco plants were maintained in a glass house at 25°C. The begomovirus and satellites were isolated at twenty seven days post inoculation.

Recombination analysis

Recombination analysis was accomplished using recombination detection program (RDP3) which is a window based program that detects and analyses recombination signals in a set of aligned DNA sequences (Martin et al., 2010).

RESULTS

Detected begomovirus represented a new strain of RaLCV

The begomovirus isolates, detected from field (GU732203 and HQ257375), shared an identity of 89 and 93% to the *Radish leaf curl virus* (RaLCV; EF175733) and clustered with RaLCV in phylogenetic tree which was distant enough from the cluster of okra infecting begomoviruses (Figure 2). Based on the sequence identity, the aforementioned RaLCV isolates (GU732203

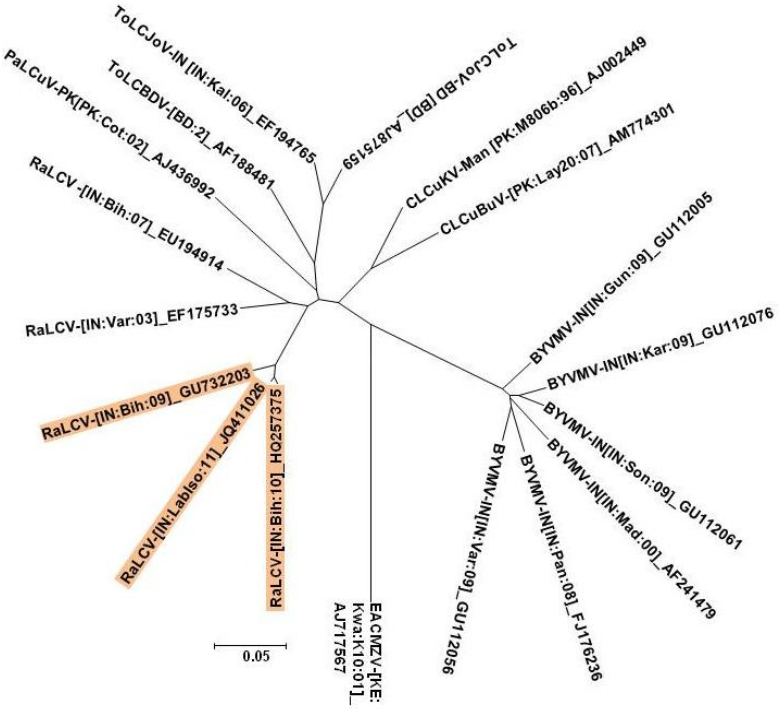

Figure 2. Phylogenetic analyses of begomovirus infecting okra (highlighted) by comparing full length nucleotide sequences of the other known begomoviruses from Genbank. The optimal tree with the sum of branch length = 1.21830170 is shown.

and HQ257375) are referred to as RaLCV-Hajipur1 and RaLCV-Hajipur2, respectively. The laboratory isolate (JQ411026) showed an identity of 91% to the RaLCV and clustered with RaLCV in phylogenetic tree (Figure 2). The laboratory isolate is hereafter referred to as RaLCV-Hajipur3. The detected begomovirus exhibited an identity of 80% with *Bhendi yellow vein Bhubhaneswar virus* among okra infecting begomoviruses in India. Beta=-satellites (HQ257376) showed an identity of 95% to Tomato leaf curl Bangladesh betasatellite (ToLCBDB; EF190215). Alphasatellites (HQ728354) exhibited an identity of 97% to Cotton leaf curl Burewala alphasatellite (CLCuBuA; GQ478667). Detected betasatellite clustered with ToLCBDB, whereas the alphasatellite grouped with CLCuBuA in phylogenetic tree. The virus acronyms used for comparison in the phylogenetic tree are according to ICTV guidelines (Fauquet et al., 2008).

Co-inoculation of two isolates resulted in emergence of a new isolate

Ten positive clones of begomovirus dimers, cloned in pCAMBIA1301, were sequenced to find the two isolates (RaLCV-Hajipur1 and RaLCV-Hajipur2). The head-to-tail repetition of the virus and satellites were confirmed by restriction fragment length polymorphism (RFLP) which yielded the expected fragments. Tobacco plants inoculated with virus only or virus plus alphasatellite showed mild symptom (Figure 3B and C), whereas the plants inoculated with infectious clones of virus plus betasatellite or virus plus betasatellite plus alphasatellite resulted into severe curling of leaves (Figure 3D and 3E). Total genomic DNA from inoculated tobacco plants served as template for amplification of begomovirus and satellites by RCA (Figure 3F). The RCA restriction products were cloned and sequenced. Sequence analysis indicated presence of one additional viral sequence RaLCV-Hajipur3 (JQ411026), in addition to RaLCV-Hajipur1 and RaLCV-Hajipur2 from the inocula-ted tobacco plants.

The new virus isolate emerged through recombination

The field isolate RaLCV-Hajipur1 were found to be recombinant and the major and minor parents of RaLCV-Hajipur1 resembled TbCSV (AJ971266) and RaLCV (EF175733), respectively. The recombination breakpoints were determined at 343 to 569 nucleotides with average probability value of 6.396×10^{-26}. The major and minor parents of RaLCV-Hajipur2 resembled RaLCV (EF175733) and RaLCV-Hajipur1 (GU732203) with average probability value of 2.362×10^{-82}. The RaLCV-

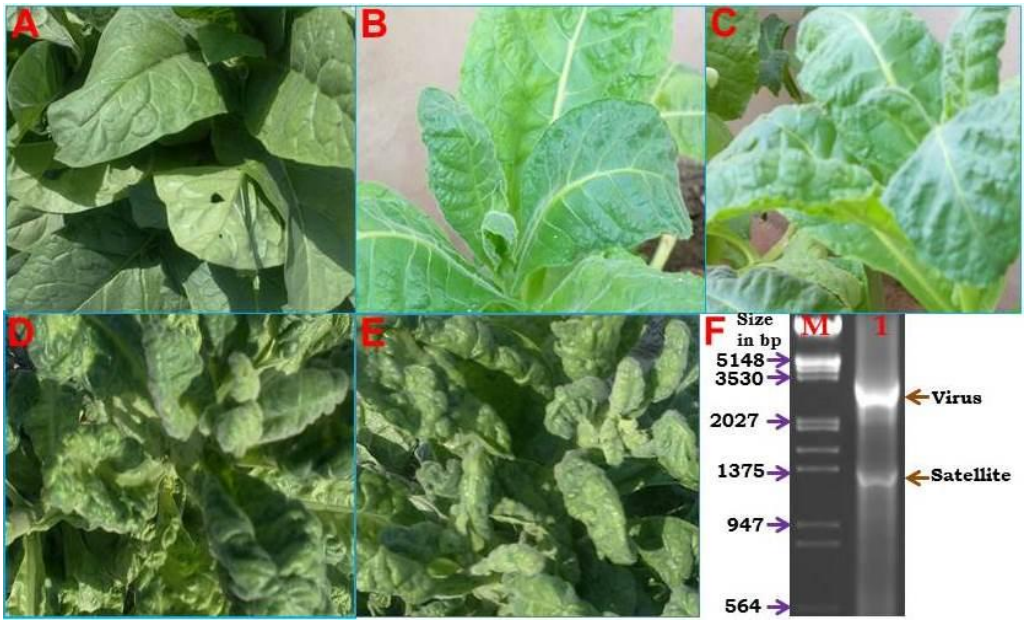

Figure 3. Tobacco plants bearing normal leaves after mock inoculation (A), exhibiting mild symptom after inoculation with virus only (B) or virus plus alphasatellite (C), and showing severe curling of leaves upon inoculation with virus plus betasatellite (D) or virus plus betasatellite plus alphasatellite (E). Rolling circle amplified product digested with *Bam*HI restriction endonuclease, showing presence of begomovirus and satellites in the inoculated tobacco plants (F). M = *Eco*RI/*Hind*III digested λ DNA, 1 = inoculated tobacco plant sample.

Hajipur3 (laboratory isolate) had major and minor parent as RaLCV-Hajipur1 (GU732203) and RaLCV-Hajipur2 (HQ257375) with average probability value of 1.543×10^{-7}. The alpha- and betasatellite also had recombinant origin.

DISCUSSION

The study reports association of a new begomovirus strain and previously known satellites with leaf curling and stunted okra plants. The detected begomovirus isolates from okra showed an identity of 89 and 93% to RaLCV, thus substantiating the presence of a new strain of RaLCV. We proposed to name the new strain as *Radish leaf curl virus*-Hajipur (RaLCV-Hajipur). Accordingly, the different isolates detected were termed as RaLCV-Hajipur1, RaLCV-Hajipur2 and RaLCV-Hajipur3 during the study. The detected alphasatellite and betasatellites were CLCuBuA and ToLCBDB, respectively.

High probability of recombination, resulting into emergence of a new pathogenic population, has been reported in geminiviruses (Hou and Gilbertson, 1996; Lefeuvre et al., 2007; Padidam et al., 1999). In addition, recombination has also been reported to affect the host specificity leading to mobility to different unusual hosts (Varsani et al., 2008). Moreover, the recombination predictions, in which RaLCV (EF175733) and RaLCV-Hajipur1 (GU732203) was detected as probable parent for RaLCV-Hajipur2 (HQ257375), indicated that recombination can occur between two isolates during mixed infection. To test this hypothesis, we did mix inoculation by inoculating RaLCV-Hajipur1 and RaLCV-Hajipur2 in a tobacco plant. Upon sequencing of the RCA restriction products from the mix inoculated tobacco plants at 27 day post inoculation, we detected a new variant RaLCV-Hajipur3 (also termed as laboratory isolate), in addition to the initially inoculated clones (RaLCV-Hajipur1 and RaLCV-Hajipur2).

We repeated the mix inoculation experiments by including the infectious clones of alpha- and betasatellite. Sequencing of the RCA restriction product, obtained from the inoculated tobacco plants revealed presence of the RaLCV-Hajipur3. Moreover, sequence alignment of the betasatellites isolated from the inoculated tobacco plants indicated few nucleotide changes in A-rich reason of betasatellite (at nucleotide positions 815 to 1125), which supports the hypothesis advocating that, A-rich region may help in adaptation of betasatellite with different helper begomoviruses (Nawaz-ul-Rehman et al., 2009). However, alphasatellite did not reveal significant differrences in nucleotide sequences upon sequence analysis, suggesting that alphasatellites is dependent on helper virus for encapsidation and movement, but not for replication. Outcome of this study strengthens the probability of recombination in evolution of a new

geminivirus strains or isolate. It also warns that recombination may lead to widening of host range for a virus which may lead to serious implications on biosecurity.

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