ACTA MICROBIOLOGICA BULGARICA

Diversity and Phylogeny of Begomovirus Populations and their Management

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Abstract

Begomoviruses are plant-infecting viruses, which are transmitted by the whitefly vector *Bemisia tabaci* and have been known to cause extreme yield reduction in a number of economically important vegetables around the world. They are abundant in tropical and subtropical environments, where insects that transmit these viruses are abundant. Identification of plant viruses, monitoring for new viral diseases, understanding the vectors that transmit viruses, and determining viral and vector impacts on the growth and development of crop cultivars and lines is vital to managing and controlling these diseases. In addition to damaging crops and causing yield losses, plant viruses interact with vectors and other diseases to increase the damage from the diseases/pests. The crops and weeds growing close to the crop fields are potential reservoirs of begomoviruses, but it is not known whether the same viruses infect several host species or co-infect any of the hosts. This increases the difficulty of controlling both the plant virus and the interacting pathogen or vector.

In a survey carried out in 2011-14 we observed several cultivated crops, ornamental plants and weeds with leaf curling, vein yellowing and growth stunting - typical symptoms of begomovirus. The DNA was isolated from the infected samples and the presence of begomovirus was confirmed by PCR using component specific primers. Further, rolling circular amplification (RCA) was performed to get the full-length sequence of the isolates. The full length sequence showed genome arrangement typical of monopartite/ bipartite begomovirus. Some of the infected samples showed association of a betasatellite responsible for the epidemic diseases in the case of monopartite. Phylogenetic analysis confirmed the diversity of begomovirus in India. Our result also indicates that begomovirus has broaden its host range by recombination process. Expression of various full length or truncated or defective proteins of the virus has been effective in accomplishing pathogen-derived resistance. We analyzed the recombination parmeters of begomovirus strains and developed the RNAi strategy for the disease management.

Key words: begomoviruses, RCA, RNAi, recombination

Резюме

Бегомовирусите са растителни вируси, които се предават от вектор - белокрилката *Bemisia tabaci* и са известни, че причиняват намаляване на добива в редица икономически важни зеленчуци по света. Те са в изобилие в тропическите и субтропическите местообитания, където насекомите, техни вектори са в изобилие. Идентификацията на растителните вируси, мониторинга на нови вирусни заболявания, установяването на вирусните вектори и определянето на вирус-векторните взаимодействия върху растежа и развитието на растителните линии и сортове е от жизненоважно значение за управлението и контрола на тези заболявания. В допълнение към нанасянето на щети на посеви и причиняването на загуби на продукция, растителните вируси взаимодействат с вектори и други патогени за увеличаване на щетите от тези заболявания / вредители. Културните видове и плевели, растящи в близост до посевите са потенциални резервоари на бегомовируси, но не е известно дали същите вируси заразяват няколко гостоприемни вида или ко-инфектират някои от

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гостоприемниците.

Това увеличава трудността за контролиране както на растителния вирус, така и взаимодействието на патогена или вектора.

По време на проучване от 2011 до 2014 г. наблюдавахме типични бегомовирусни симптоми по културни видове, декоративни растения и плевели като листно завиване, жилкова хлороза и закърняване на растежа на растенията. ДНК беше изолирана от заразените проби, което потвърждава присъствието на бегомовирус чрез PCR, използвайки специфични праймери. Освен това, проведохме амплификация на въртящия се кръг (RCA), за да получим пълната дължина на геномите на вирусните изолати. Секвенциите на пълните геноми показаха типичното геномно подреждане на бегомовирусите като монопартидни / бипартидни. Някои от заразените проби показаха асоциацията на бета-сателитите, отговорни за епидемията от болести в случай на монопартиден вирусен геном. Филогенетичният анализ потвърждава разнообразието на бегомовирусите в Индия. Нашите резултати показват също, че бегомовирусите разширяват своя кръг от гостоприемници чрез рекомбинационни процеси. Експресията на пълната или скъсена дължина на различни вирусни протеини или дефектни такива е ефективен процес за осъществяването на патоген-индуцирана устойчивост. Анализирахме рекомбинационните параметри на бегомовирусни щамове и разработихме стратегия на генно мълчание за контрол на болестта.

Introduction

The genus *Begomovirus* (Family: *Geminivridae*) is a major group infecting weeds, ornamental plants and economically significant crops in tropical and subtropical regions (Faquet *et al*., 2008; Adams *et al*., 2013). Begomoviruses are exclusively transmitted by the white fly (*Bemisia tabaci*) and are divided into New World and Old World viruses according to their geographic origins. The New World Begomovirus consists two genomic components, DNA-A and DNA-B, whereas the Old World begomoviruses are monopartite associated with ssDNA satellite molecules betasatellites and/or alphasatellites (Seat *el al*., 2006; Brown *el al*., 2012).

Betasatellite molecules are half the size of DNA-A (~1.4Kbp) and enhance the symptoms severity along with other functions, i.e., transmission, replication, encapsidation and movements in plants (Briddon *et al*., 2001; Nawaz-ul-Raham *et al*. 2009; Nawaz-ul-Rahman *et al*. 2010; Patil and Faquet, 2010). In contrast, alphasatellites may attenuate symptoms caused by helper begomoviruses beta satellites (Idris *et al*., 2011).

Begomoviruses (family *Geminiviridae*) and their associated satellite DNAs form complexes that cause severe diseases in agricultural systems (Mansoor *et al.,* 2003; Mansoor *et al*., 2006; Zhou, 2013; Leke *et al*., 2015). These begomovirus-satellite complexes infect a wide range of dicotyledonous plants within ~37 different genera in 17 families of vegetable and fiber crops, ornamentals and uncultivated vegetation (Zhou, 2013). Various begomovirus-satellite complexes have been identified in all major dicotyledonous plant crops and evidence advocates that these complexes are rapidly increasing their host and biogeographical ranges, thus threatening agriculture in tropical and subtropical regions worldwide (Mansoor *et al*., 2003, 2006; Leke *et al*., 2015).

RNA interference (RNAi) or post-transcriptional gene silencing (PTGS) occurs in a wide variety of organisms, including animals, fungi and plants (Bass, 2000; Saunders *et al*., 2004). Virus-derived small-interfering RNAs (siRNAs) are the hallmark of an innate immune response in plants that targets invading viruses through PTGS. RNA silencing is a sequence specific RNA degradation process that is triggered either by the formation of dsRNA or alternatively by aberrant RNAs associated with transgenic viruses and transposons (Vaucheret, 2004). RNAs with hairpin with a loop structures are particularly actual inducers of PTGS in plants (Kon and Sharma, 2011).

During a survey carried out in 2011-2014, typical begomovirus symptoms, such as leaf curling, vein yellowing, growth stunting, were observed in several crops, ornamental plants and weeds. In this study, we analyzed phylogeny and recombination break points of begomovirus isolates from different host plants, using different bioinformatics tools.

Materials and Methods

DNA isolation and viral genome amplification

Plant leaves showing geminivirus-like symptoms such as leaf curling, leaf distortion, and stunted growth were collected during 2011-2014 from Sikar district, Rajasthan, India. Collected samples included rose, radish, *Petunia hybrida* and *Catharant husroseus* plant samples. Total DNA was extracted from the infected samples followed by rolling circular amplification (RCA) by using Tem-

pliPhiTM kit (GE Healthcare) as per the manufacturer's instruction. RCA products were digested with five different restriction enzymes (*EcoR* I, *Hind* III,*Pst*I, *Sal*I and *Bam*HI) and cloned in pUC19 vector followed by sequencing by Xcelris Genomics, Ahmedabad, India.

Sequence analysis, phylogenetic and recombination analysis

Sequences of each DNA-A were analyzed and initially submitted to a BLASTn search and on the basis of similarity score Begomovirus isolates were selected (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The studied sequences were further analyzed using the ORF (open reading frame) finder tool (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the ExPASy tool (http://www.expasy.org/tools/). Phylogenetic analysis was done using other begomovirus isolates, obtained from NCBI database and trees were constructed by using the neighbor joining method in MEGA6.0 (Tamura *et al*., 2011). Bootstrap values from 1000 replicates were analyzed. The identification of potential recombinant breakpoints and parents was carried out using RDP, recombination detection methods as implemented in RDP 4.23 (Martin *et al*., 2010) with default setting.

Engineering the plant virus for silencing

Engineering the plant virus for silencing can be studied with the help of RNA-mediated resistance against Croton yellow vein mosaic begomovirus (CYVMV) infection as an example. CYVMV and its associated betasatellite from croton infested weeds have been characterized. Betasatellites are small circular ssDNA satellites containing a single open reading frame, ORF (termed as βC1), have been found to be associated with various plant diseases exclusively caused by monopartite begmoviruses in the Old World. Effective gene silencing of croton yellow vein mosaic beta satellite encoded βC1 in *Nicotiana benthamiana* infiltrated with *Agrobacterium tumefaciens* that harbours the intron hairpin RNA (ihpRNA) construct aimed at the βC1 (Sahu *et al*., 2014).

A transgene consisting of the promoter region of CYVMV DNA-β was designed to produce double stranded RNA (Fig. 1). The *Nicotiana benthamiana* plants were transformed by *Agrobacteium*-mediated gene transfer and were tested for tested for siRNA expression. CYVMV-infected transgenic lines showed small RNAs of approximate sizes, 23 nt higher expression intensities.

Results and Discussion

A total of four of DNA-A clones isolated from different host plants were sequenced and submitted to the NCBI databank. The sequence of DNA-A clone KF218188 (host chilli), KJ700653 (host *Pitunia hybrida*), KF584008 (host rose) and KP698313 (host *Catharanthusroseus*) showed 2732nts, 2732nts, 2736nts and 2741nts in length, respectively. The full length sequences of KF218188, KJ700653, KF584008 and KP698313 shared similarity with RaLCuV: HQ698591(99), ChLCuV: HM007104 (99%), RLCuV:KR052159 (93%) and PLCrV: GQ478342 (93%). Hence, they can be considered as isolates of the Radish leaf curl virus, Chilli leaf curl virus, Rose leaf curl virus and Papaya leaf crumple virus respectively.

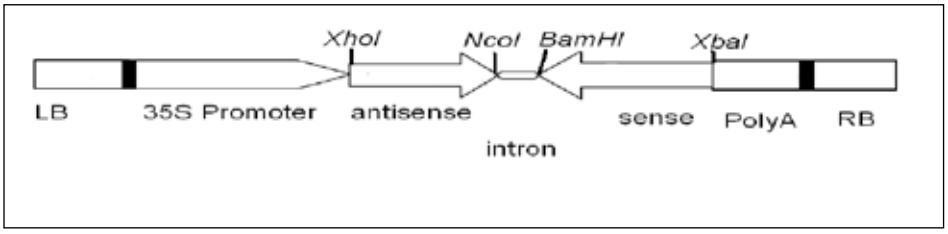

Fig. 1. Schematic diagram of the binary construct used for plant transformation. LB: Left border; RB: Right border.

The phylogenetic analysis of DNA-A clones (Fig. 2) constructed using the neighbor joining method showed three different clusters.

ChLCuV isolates KJ700653 and RaLCuV: KF218188 shared different clusters while PLCrV:KP6698313 and RLCuV: KF584008 placed in similar cluster. ChLCuV: KJ700653 show maximum similarity with ChLCuV: KJ700653 and HM007104, while RaLCuVs: KF218188 demonstrated maximum proximity with RaLCuV: HQ698591. Isolate RLCuV: KF584008 shared maximum similarity with RLCuV: GQ478342 and PLCrV: KP698313 showed similarity with PLCrV: HM140368. For detection of recombination events, all DNA-A sequences were analyzed using RDP4 package. The recombination breakpoint analysis

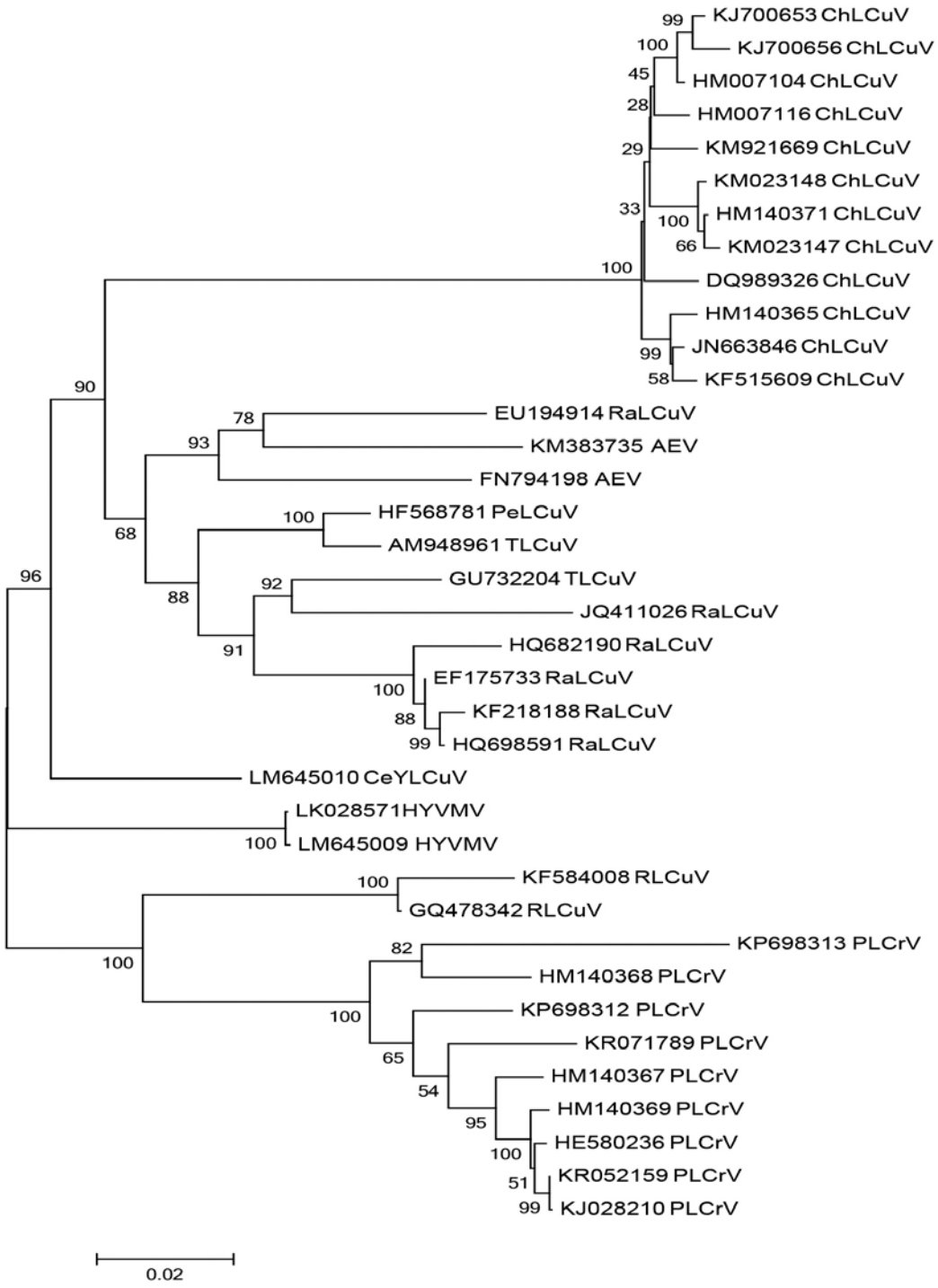

Fig. 2. Phylogenetic tree showing the relationships among the DNA-A described in this work and other begomoviruses obtained from GeneBank. The tree was constructed by the neighbor-joining method using MEGA 6 with a bootstrap values 1000 replicates. Horizontal distances are relative to calculated mutation distances, vertical branches are arbitrary. Bootstrap values are given away at nodes.

also provided strong evidence for presence of past recombination events in the analyzed sequences. A new begomovirus strain can arise by different inter and intraspecific recombination events (Patil and Faquet, 2009). For all DNA-A clones, possible breakpoints, sequence fragments, parental genotypes and supported methods with P-Value are listed in Table 1.

Table 1. Detection of recombination breakpoints

№	Breakpoints	Major parent	Minor parent	Recombinant	ORF	P-value	Supporting methods
1	1871-585	EU194194	JQ411026	KF218188	CR, V1, V2,C1	**3.909×10^{-36}**	R, G,**B**, M,C,S
2	2553-246	GQ478342	FN794198	KJ700653	CR, V2,C4	**1.395×10^{-04}**	R, **B**,M,C
3	1522-2099	GQ478342	LM645009	KJ700653	C1,C2	**4.493×10^{-13}**	**R**, G,B,M,C,S
4	486-987	HM140368	LK028571	KP698313	V1	**1.132×10^{-38}**	**R**,G,B,M.C,S
5	1053-1419	GQ478342	AM948961	KP698313	C3	**7.420×10^{-31}**	**R**,G,B,M,C,S
6	1430-1869	LK028571	FN794198	KP698313	C2,C1	**7.907×10^{13}**	R,GB,M,C,**S**
7	1911-2130	HM140368	GQ478342	KP698313	C1	**7.962×10^{-20}**	**R**,G,B,M,C,S
8	1385-1893	LK028571	FN794198	KF584008	C1,C2,C3	**7.907×10^{-13}**	R,G,B,M,C,**S**

R-RDP, G-GENECONV, B-BOOTSCAN, M-MAXIMUM CHISEQURE, C-CHIMERA, S-SISSCAN
P-values are shown in bold.

There is evidence for recombination events in all six ORFs V1, V2, C1, C2, C3, C4 and CR regions. Our analysis also indicates that the highest frequency of recombination apparently occurs in the portion of the Rep encoding C1/AC1 ORF, with minimum breakpoint located in the N-terminal portion and/or the 5′-end of the common region. In begomoviruses, recombination breakpoints are nonrandomly distributed amongst mono- and bipartite genomes, with hot spots in the Rep N-terminal portion and in the 5′-end of the common region (Lefeuvre *et al*., 2007; Lefeuvre *et al*., 2007a]. The recombination analysis in our study also shows that the most unique recombination event occurs in the C1/Rep and conserved region of the genome. This explains that studying DNA-A is contributed by other begomovirus isolates and increasing its host range by recombination process.

The available data and our results reveal the parallel importance of the bC1 gene in the bipartite begomovirus life cycle. Producing siRNA in *N. benthamiana* plants may be significantly important for producing antiviral resistance. We evaluated the silencing of bC1 in transgenic plants by analysis of siRNA. RNA isolated from a wild type *N. benthamiana* plant and from transgenic plants (challenged with over 30 viruliferous whiteflies per plant and after 3 wpi) and subjected to siRNA detection by northern blot hybridization by using specific probe to bC1 transgene. This led to easy detection of resistance in these transgenic lines, i.e. no symptoms. The siRNA was absent in the non-transgenic plant showing disease symptoms.

Conclusion

Considerable resistance in transgenic plants against viruses can be created by exploiting the phenomenon of RNAi. Silencing specific genes by RNAi is a desirable natural solution to this problem as disease resistant transgenic plants can be produced within a regulatory framework. Transgenic plants expressing RNAi vectors, as well as dsRNA containing crop sprays have been successful for efficient control of plant pathogens affecting economically important crop species. Begomoviruses are increasing their host range by a recombination process which is a major threat to economically important plant species.

Acknowledgements

The author/s are expressing thanks to the Council of Scientific & Industrial Research (file no 09/1064 (0002)/2013-EMR-1) for financial support.

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