J Gen Plant Pathol (2005) 71:314–318
DOI 10.1007/s10327-005-0206-3

VIRAL AND VIROID DISEASES

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Possible recombination of tomato-infecting begomoviruses in Thailand

Received: September 8, 2004 / Accepted: December 14, 2004

Abstract Nucleotide sequences of the three distinct *Tomato yellow leaf curl Thailand virus* (TYLCTHV) strains (CM, NK, SK) were analyzed for recombination events. Recombination detection program analyses and a sequence alignment survey provided evidence of recombination between AC1 sequences of TYLCV, TYLCTHV-[MM], and TYLCTHV-[NK] as major parents and of ToLCLV, ToLCTWV, and TYLCTHV-[SK] as minor parents of TYLCTHV-[NK], -[SK], -[CM], respectively. The results further support the notion that interspecies recombination may play a significant role in geminivirus diversity and their emergence as important pathogens.

Key words *Begomovirus* · TYLCTHV, *Tomato yellow leaf curl Thailand virus* · Sequence analysis · Recombination.

Introduction

Geminiviruses are small, single-stranded DNA plant viruses with a unique particle morphology of twinned, incomplete icosahedra. The family Geminiviridae is divided into four genera (*Mastrevirus, Curtovirus, Topocuvirus, Begomovirus*) based on genome structure, type of insect vector, and host range (Hanley-Bowdoin et al. 1999; Pringle 1999). The *Begomovirus* genus is the largest genus of this family and comprises the whitefly-transmitted geminiviruses that infect dicotyledonous plants. Begomoviruses found in the Western hemisphere typically have bipartite genomes, whereas several monopartite begomoviruses have been identified in the Eastern hemisphere, such as *Tomato yellow leaf curl virus* (TYLCV) (Kheyr-Pour et al. 1991; Navot et al. 1991) and *Tomato leaf curl virus* (ToLCV) (Dry et al. 1993).

The genome of bipartite begomoviruses is split between two genomic components, designated DNA-A and DNA-B (Lazarowitz 1992). DNA-A encodes genes responsible for viral replication (*Rep* and *REn*), regulation of gene expression (*Trap*), and particle encapsidation (*CP*). DNA-B encodes for two proteins, MP and NSP, involved in cell-to-cell movement within the plant, host range, and symptom modulation (Hanley-Bowdoin et al. 1999). The two DNA components of a given begomovirus have little sequence identity, except for a common region (CR) of approximately 200 nucleotides. This region contains the origin of replication and *cis*-acting elements recognized by the Rep protein (Argüello-Astorga et al. 1994; Fontes et al. 1994).

Recombination contributes to the genetic diversification of geminivirus populations (Padidam et al. 1999; Sanz et al. 1999, 2000; Umaharan et al. 1998) and has been related to the emergence of some serious diseases (Rybicki and Pietersen 1999; Zhou et al. 1997). In begomoviruses, recombination occurs at the strain (Hou and Gilbertson 1996; Kirthi et al. 2002), species (Fondong et al. 2000; Navas-Castillo et al. 2000; Sanz et al. 2000; Saunders et al. 2002; Zhou et al. 1997), genus (Briddon et al. 1996; Klute et al. 1996), and family (Saunders and Stanley 1999) levels. Despite the evident importance of recombination in the begomoviruses, little work is available to document its contribution to the evolution of natural populations.

In this article we provide preliminary evidence of recombination between *Tomato yellow leaf curl Thailand virus*-CM, -NK, and -SK (TYLCTHV-[CM], TYLCTHV-[NK] and TYLCTHV-[SK], respectively) and other begomoviruses. Through analysis of full-length DNA-A nucleotide sequences, we identified the positions of recombination.

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Materials and methods

Virus isolates and extraction of DNA

Samples of tomato plants with symptoms suggesting geminivirus infection were collected from Chiang Mai, Nong Khai, and Sakon Nakhon provinces in Thailand. They were identified as TYLCTHV-[CM], -[NK], and -[SK] (Sawangjit et al. 2004). The viruses were preserved as dried leaf samples on silica gel. Total nucleic acids were extracted from infected leaf tissue using the method described by Taylor and Powell (1982).

PCR and cloning of the TYLCTHV genome

The polymerase chain reaction (PCR) and cloning of TYLCTHV genomic DNAs were described previously by Sawangjit et al. (2004).

Sequence analysis

Sequences of TYLCTHV-[CM], -[NK], and -[SK] DNA-A and DNA-B [GenBank accession nos. AY514630–AY514635] were obtained with an automated DNA sequencer (ABI PRISM version 3.0, model 377). The following geminivirus sequences were obtained directly from GenBank databases and were used for comparisons: *Abutilon mosaic virus* (AbMV, X15983, X15984), *Bean golden mosaic virus*-[Brazil] (BGMV-[BZ], M88686, M88687), *Cabbage leaf curl virus* (CaLCuV, U65530), *Cotton leaf curl Multan virus*-[26] (CLCuMV-[26], AJ002458), *Mungbean yellow mosaic India virus*-[Mungbean] (MYMIV-[Mg], AF416742, AF416741), *Mungbean yellow mosaic virus* (MYMV, D14703, D14704), *Mungbean yellow mosaic virus*-Thailand (MYMV-TH, AB017341), *Okra yellow vein mosaic*-[201] (OYVMV-[201], AJ002451), *Papaya leaf curl virus* (PaLCuV, Y15934), *Pepper leaf curl virus* (PepLCV, AF134484), *Potato yellow mosaic virus*-Venezuela (PYMV-VE, D00940, D00941), *Sida golden mosaic virus* (SiGMV, AF049336, AF039841), *Squash leaf curl virus* (SLCV, M38182, M38183), *Tobacco leaf curl Yunnan virus*-[Y3] (TbLCYNV-[Y3], AF240674), *Tomato golden mosaic virus*-[Yellow vein] (TGMV-[YV], K02029, K02030), *Tomato leaf curl virus*-[Australia] (ToLCV, S53251), *Tomato leaf curl Bangalore virus* (ToLCBV, Z48182), *Tomato leaf curl Bangladesh virus* (ToLCBDV, AF188481), *Tomato leaf curl Indonesia virus* (ToLCIDV, AB100304), *Tomato leaf curl Karnataka virus* (ToLCKV, U38239), *Tomato leaf curl Laos virus* (ToLCLV, AF195782), *Tomato leaf curl Malaysia virus* (ToLCMV, AF327436), *Tomato leaf curl New Delhi virus*-[Mild] (ToLCNDV-[Mld], U15016), *Tomato leaf curl New Delhi virus*-[Severe] (ToLCNDV-[Svr], U15015, U15017), *Tomato leaf curl Philippines virus* (ToLCPV, AB050597), *Tomato leaf curl Sri Lanka virus* (ToLCSLV (AF274349), *Tomato leaf curl Taiwan virus* (ToLCTWV, U88692), *Tomato mosaic Havana virus*-[Quivican] (ToMHV-[Qui], Y14874, Y14875), *Tomato mottle virus*-[Florida] (ToMoV-[FL], L14460, L14461), *Tomato yellow leaf curl virus* (TYLCV, X15656), *Tomato yellow leaf curl virus*-[Iran] (TYLCV-[IR], AJ132711), *Tomato yellow leaf curl China virus* (TYLCCNV, AF311734), *Tomato yellow leaf curl Sardinia virus* (TYLCSV, X61153), *Tomato yellow leaf curl Thailand virus* (TYLCTHV, AF141922, AF141897), *Tomato yellow leaf curl Thailand virus*-[Myanmar] (TYLCTHV-[MM], AF206674), *Tomato yellow leaf curl Thailand virus*-[Y72] (TYLCTHV-[Y72], AJ495812), and *Watermelon chlorotic stunt virus*-[IR] (WmCSV-[IR], AJ245652, AJ245653). Sequence data were analyzed using DNASTAR software (DNASTAR, Madison, WI, USA). Phylogenetic adscription was done by the Clustal method (Higgins and Sharp 1988). For detection of possible recombination events among geminiviruses, the recombination detection program (RDP) (Martin and Rybicki 2000) was used with the following setting: window size 10, highest acceptable probability 0.001, internal reference sequences.

Results and discussion

Sequence variability of intergenic regions of CM, NK, and SK isolates of TYLCTHV

The complete genome sequence of isolate CM, NK, and SK was determined for clone pTYCM, pTYNK, and pTYSK, respectively. The DNA-A sequences of TYLCTHV-[CM] and -[SK] comprise 2747 nucleotides, and TYLCTHV-[NK] comprises 2744 nucleotides. The DNA-B of the CM isolate comprises 2750 nucleotides, and NK and SK isolates were one nucleotide shorter. The first base of the 5′ end of the intergenic noncoding region was designated nucleotide 1. To investigate sequence variability among isolates of TYLCTHV-[CM], -[NK], and -[SK] in Thailand, the DNA-A were compared. These three isolates of TYLCTHV were most closely related to TYLCTHV-[MM] (95%, 92%, and 90%, respectively) than to TYLCTHV (92%, 91%, and 89%, respectively) and the new previously identified strains of TYLCTHV (Sawangjit et al. 2004).

The intergenic region (IR) separating the divergent transcription units on both components harbors the conserved stem-loop structure found in all geminiviruses and the conserved TAATATTAC nonanucleotide sequence that contains the nicking site for initiation of virion-sense DNA replication (Fig. 1) (Laufs et al. 1995; Stanley 1995). This region contains a series of *cis*-acting elements involved in DNA replication and transcription of the *AC1* gene (Argüello-Astrorga et al. 1994). All such elements are present in the sequences of all three isolates of TYLCTHV, including (1) an inverted repeat and direct repeats (binding sites for the Rep protein), (2) the TATA box for the *AC1* gene, and (3) a conserved stem-loop motif that includes the nonanucleotide sequence nicked by the Rep protein to initiate DNA replication in the origin of replication. In the origin of replication, the sequences AATCGGTGT at nucleotides 5–13 in TYLCTHV-[CM], -[NK], and -[SK] are repeated at nucleotides 33–41. These direct repeat

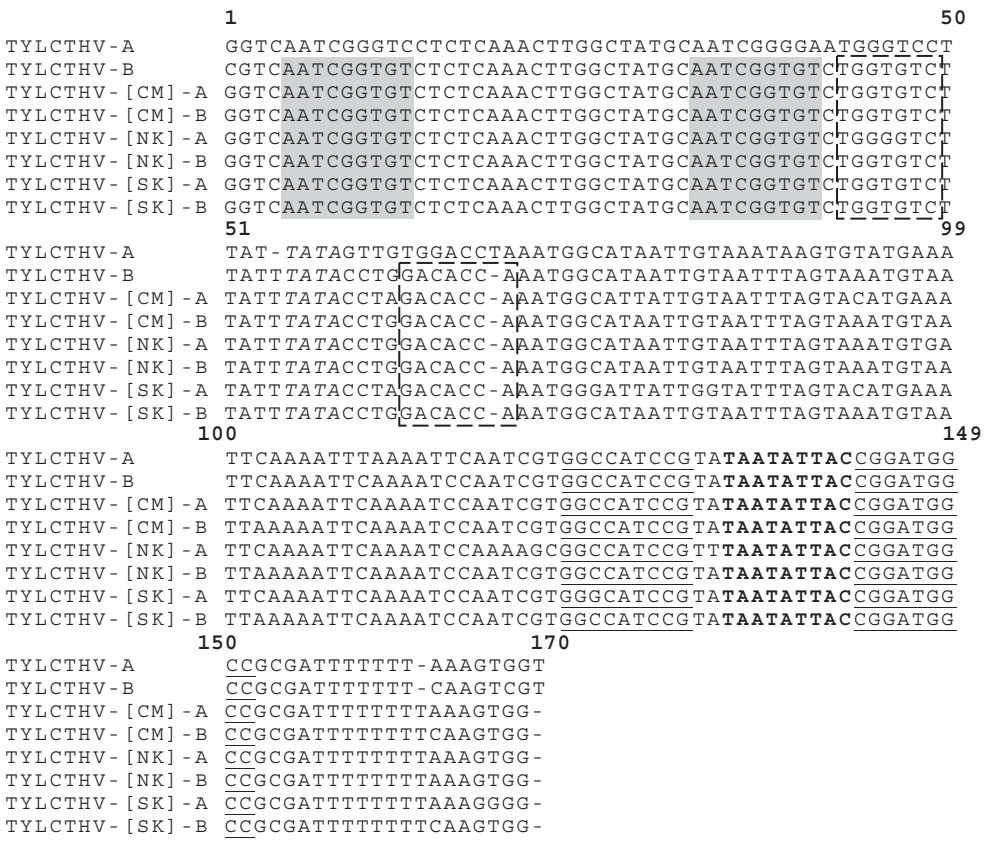

Fig. 1. Intergenic region of DNA-A and DNA-B from Thailand tomato-infecting begomoviruses. A multiple sequence alignment of common region sequences from the indicated begomoviruses was obtained with the Clustal program. The nucleotide sequences have been aligned by introducing gaps (shown as *dashes*) to maximize identity. The two direct repeats (*shaded*) contained the positive Rep protein binding site, inverted repeat *dashed-line box*, TATA box of the leftward promoter and the conserved nonanucleotide are shown by *italics* and *boldface*, respectively. The conserved stem-loop structure is *underlined*

sequences are located between the TATA box (nucleotides 55–58 in TYLCTHV-[CM], -[NK], and -[SK]) and the start codon of the *AC1* gene. The latter direct repeat sequence may contain the positive Rep protein-binding site (GGTGT) for replication, which is similar to the direct repeat sequence (GGTAG) in TGMV (Fontes et al. 1994). Two inverted repeat sequences of seven bases – TGGTGTC in TYLCTHV-[CM] and -[SK] and TGGGGTC in TYLCTHV-[NK] – are located at the 5′ end of the hairpin sequence in the left part of the stem-loop structure (Fig. 1). Comparison of the *ori* of whitefly-transmitted geminiviruses (WTGs) from the Eastern Hemisphere revealed that an arrangement of two direct repeat sequences at the part of IR close to the 5′ end of the AC1 open reading frame (ORF) sequence in TYLCTHV-[CM], -[NK], and -[SK] (AATCGGTGT) were similar to the arrangement of the direct repeat sequences of TYLCV (originally *Tomato yellow leaf curl virus*-Israel, or TYLCV-Is). However, the arrangement of the other two inverted repeat sequences in these three isolates was similar to the arrangement of the inverted repeat sequences in ToLCNDV (originally *Tomato leaf curl virus*-New Delhi, or ToLCV-Nde).

Recombination events among recently identified tomato-infecting begomoviruses

The results of studies on experimental and natural recombination indicate that recombination is a significant strategy contributing to geminivirus evolution. Previous studies of other viruses also indicate the importance of recombinations observed in these studies that were predominantly between strains of a given virus species (Chenault and Melcher 1994; Gibbs et al. 1995; Lai 1992; Revers et al. 1996; Roossinck 1997; Simon and Bujarski 1994).

Comparisons revealed that a large portion of the genome was quite well conserved among all TYLCTHV isolates. Searching for possible recombination events on TYLCTHV-[CM], -[NK], and -[SK] using the RDP software, we were able to detect statistical significance of the recombination events and to identify crossover points in these three TYLCTHV isolates. Our results showed that the *AC1* gene of TYLCTHV-[NK] could be the result of recombination between TYLCV and ToLCLV (RDP: $P = 6.761 \times 10^{-4}$), TYLCTHV-[SK] could be the result of recombination between TYLCTHV-[MM] and ToLCTWV (RDP: $P = 1.756 \times 10^{-4}$), whereas a major parent of TYLCTHV-[CM] was identified as TYLCTHV-[NK] and minor parents TYLCTHV-[SK] (RDP: $P = 4.153 \times 10^{-7}$) (Fig. 2). The *AC1* gene of TYLCTHV-[NK], -[SK], and -[CM] clearly has a backbone of TYLCV, TYLCTHV-[MM], and TYLCTHV-[NK] AC1 sequence, respectively.

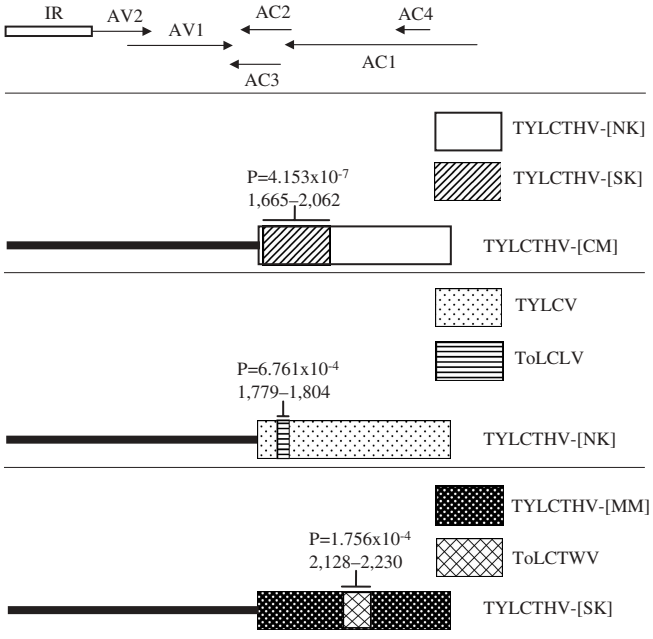

Fig. 2. Recombinant regions of Thailand tomato-infecting begomoviruses. Potential recombination breakpoints and the probability (*P*) that the indicated regions do not have a recombinant origin are presented on the linear map of the DNA. The *shading patterns* indicate the viral origin of the recombinant region. *TYLCV*, *Tomato yellow leaf curl virus*; ToLCLV, *Tomato leaf curl Laos virus*; ToLCTWV, *Tomato leaf curl Taiwan virus*; *TYLCTHV-[CM]*, -[*NK*], and -[*SK*], *Tomato yellow leaf curl Thailand virus*-[Chaing Mai], -[Nong Khai], and -[Sakon Nakhon], respectively

In these recombination events, regions from nt 1779–1804, 2128–2230, and 1665–2062 of TYLCTHV-[NK], -[SK], and -[CM] were replaced with the corresponding sequences of ToLCLV, ToLCTWV, and TYLCTHV-[SK], respectively (Fig. 2).

The analysis of the three related strains TYLCTHV-[CM], -[NK], and -[SK] supports the hypothesis that new begomoviruses evolve by recombination between previously existing species (Padidam et al. 1999; Rybicki 1994; Torres-Pacheco et al. 1993; Zhou et al. 1997). The recombination involved a region of the AC1 ORF, which is a hot spot for recombination in geminiviruses (Navas-Castillo et al. 2000; Sanz et al. 1999; Stanger et al. 1991; Stanley 1995). Three factors could contribute significantly to recombination: mixed infections, high levels of viral replication, and an increased host range of the vector (Padidam et al. 1999). First, mixed infections, a prerequisite for recombination, are common in geminivirus diseases (Padidam et al. 1995; Umaharan et al. 1998). Second, geminiviruses replicate via a double-stranded replicative form and achieve extremely high copy numbers (Accotto et al. 1993; Kanevski et al. 1992). Third, with the emergence of the B biotype whitefly that can feed on hundreds of species (Bedford et al. 1994), the host range of geminiviruses has expanded greatly. As a result, there is greater opportunity for a recombinant virus to emerge by infecting a new host.

Recombination is not a rare phenomenon among begomoviruses (Harrison and Robinson 1999; Padidam et al. 1999; Sanz et al. 1999; Zhou et al. 1997), providing additional sources of variation with unpredictable effects on virus pathogenicity. Our results further support the conclusion of others (Bananej et al. 2004; Chatchawankanphanich and Maxwell 2002; Harrison et al. 1997; Kitamura et al. 2004; Sanz et al. 1999; Zhou et al. 1998) that recombination may play a significant role in geminivirus diversity. Knowledge of sequence variants of TYLCTHV-[CM], -[NK], and -[SK] present in a given geographical region is essential for the establishment of effective control measures, particularly because breeding is the best way to control this virus.

Acknowledgments Financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program to Sopid Sawangjit and Associate Professor Dr. Supat Attathom is acknowledged. The authors thank Dr. Peter Revill and Dr. Susan Porchun for technical assistance.

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