RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2009.35.131

ScienceAsia 35 (2009): **131–136**

The complete nucleotide sequence of *Squash leaf curl China virus*-[Wax gourd] and its phylogenetic relationship to other geminiviruses

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Received 11 Jul 2008
Accepted 6 May 2009

ABSTRACT: Geminivirus, which causes yellow leaf curl disease of wax gourd plants, was detected using polymerase chain reaction with geminivirus-specific primers. The complete genomic sequence of a geminivirus isolated from wax gourd was determined by sequencing overlapping DNA fragments. The genome consisted of 2739 nucleotides, contained 5 open reading frames (ORFs) typical of geminiviruses, 2 ORFs on the viral sense and three ORFs on the complementary sense strand. The geminivirus isolated from wax gourd plants showed high sequence identity (94%) with that of DNA-A of *Squash leaf curl China virus*-Thailand [Thailand] (SLCCNV-TH[TH]). These data suggest that wax gourd yellow leaf curl disease in Nakhon Pathom was caused by SLCCNV. Based on sequence comparisons and phylogenetic tree analyses, the virus isolate studied was identified as a new strain of SLCCNV and named *Squash leaf curl China virus*-[Thailand:Wax Gourd:2008] (SLCCNV-[TH:Wax:08]).

KEYWORDS: *Begomovirus*, *Benincasa hispida*, Cucurbit, SLCV, whitefly-transmitted geminiviruses

INTRODUCTION

Whitefly-transmitted geminiviruses have emerged as serious pathogens of agronomic and horticultural crops in Thailand. Members of the family Geminiviridae are plant pathogens with circular single-stranded DNA genomes encapsidated in twin particles comprising the genera *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus*. They are differentiated based upon host range, genome organization, and insect vector specificity[1,2]. The *Begomovirus* genus is the largest genus of this family and comprises the whitefly-transmitted geminiviruses that infect dicotyledonous plants. Most begomoviruses have a bipartite genome that consists of a DNA-A and a DNA-B component. Notable exceptions are the tomato (yellow) leaf curl viruses from the Near East, the Mediterranean basin, Australia, and India, whose genomes are monopartite[3–5].

Severe outbreaks of a whitefly-transmitted disease have occurred in cucurbit-growing regions of central Thailand, affecting cantaloupe (*Cucumis melo* var. reliculatus), pumpkin (*Cucurbita pepo*), angled luffa (*Luffa acutangula*), bitter gourd (*Momardica hispida*), and cucumber (*Cucumis sativus* L.)[6,7]. The symptoms, namely, yellow chlorotic spot, mosaic, and leaf curling are typical of geminiviruses infections. Wax gourd (*Benincasa hispida*) is one of the important crops grown by farmers of small holdings in the central part of Thailand. Diseases caused by viruses pose serious problems for wax gourd production. Because of the type of symptoms and transmission by whitefly, geminivirus infection was suspected. In order to identify and further characterize the causal agent of wax gourd yellow leaf curl disease in central Thailand, geminiviruses were detected by polymerase chain reaction and analysed by DNA sequence analysis. The complete nucleotide sequence, the genome organization, and the relation to other reported whitefly-transmitted geminiviruses were determined.

MATERIALS AND METHODS

Virus sources

Wax gourd (*Benincasa hispida*) plants showing geminivirus-like symptoms consisting of leaf mosaic, yellowing, curling and distortion were collected from a field in Nakhon Pathom province, Thailand. Leaf tissue was desiccated with silica gel for 1 week and stored at 4 °C until use.

Table 1 Oligonucleotides used for genome amplification.

Primer	Sequence (5′ ⟶ 3′)	Target region designation
CPA5	AT G TCG AAG CGT CCA GCA GA	*CP* gene
CPR1137	GG(A)G CAG(A) T(A)T A(C)TC ATG(A) TAT TG	*CP* gene
GemF 946	GAG(A) GT(C)C(T) GGC(G) AAG TAT(C) GAG AA	*Rep* gene
PAL1960m	TGG ACT GCA GCA N*GG N*AA	*Rep* gene
GemF1802	TGG(C) GGA TCA(G) ACG TCA TCA(G) AT	IR
TYTHIR-C	GTA TGG GCT GTC AGG GTT	IR
SLC-BV1F	CAT ATA CTC CGG GAA GAC GA	BV1
SLC-BV1R	ACC AAT GTA ATT TAG CAT TAC AT	BV1

* N = A, C, T, G

Polymerase chain reaction

Total DNA was extracted from plant samples using the method described in Ref. 8. Four sets of degenerate primers were used to amplify overlapping fragments of the DNA-A and DNA-B components of geminivirus causing yellow leaf curl disease of wax gourd plants from Nakhon Pathom (WGNP). The primer pair CPA5 and CPR1137 amplified a product of 850 bp covering the *CP* and parts of *AV2* and *AC3*. The fragment covering the intergenic region (IR) and parts of *AV2* and *AC1* were amplified with GemF1802/TYTHIR-C, giving a product of 1280 bp including primers. The amplification of a product of 1035 bp covering the *AC2*, *AC3*, and parts of *AC1* was done using the primers of PAL1960m and GemF946. The primer pair SLC-BV1F and SLC-BV1R were used to amplify a BV1 fragment of the DNA-B component. The sequences of the primers are shown in Table 1. The PCR was done in 25 µl reaction mixtures: 2.5 µl of 10× PCR buffer (0.5 M KCl, 0.1 M Tris-HCl, pH 9.0 and 1% Triton X-100), 2 µl of 25 mM $MgCl_2$, 2 µl of 2 mM dNTPs, 0.1 µl of *Taq* DNA polymerase (5U/µl), 5 µl of the extracted DNA, and 1 µl of 10 µM of each primer.

Sequence determination and analysis

PCR products of the expected sizes were cloned using pJet 1.2/blunt cloning vector (Fermentas). The ligation mixture was used to transform *E.coli* DH5α competent cells by heat shock transformation. DNA was isolated and sequenced. Sequence data were assembled and analysed using DNASTAR software, and database searches were done using BLAST[9]. Virus sequences were edited using EditSeq (DNASTAR) to obtain a consensus sequence for each. For multiple alignments of viral genome sequences, Clustal W (MegAlign, DNASTAR) was used. The phylogenetic analysis was performed to determine the relationship of WGNP with other published geminiviruses by using the neighbour joining algorithm (MegAlign program). Geminivirus DNA-A sequences used for comparison and their corresponding database accession numbers are shown in Table 2.

RESULTS AND DISCUSSION

Detection of viral nucleic acid in diseased plant samples

DNA-A, but not DNA-B, was detected in nucleic acid extracts prepared from infected wax gourd leaves. Three overlapping DNA fragments containing CP (850 bp), IR (1280 bp) and Rep (1035 bp) fragments were amplified with CPA5/CPR1137, GemF1802/TYTHIR-C, and PAL1960m/GemF946 primers, respectively. All three fragments of geminivirus isolated from wax gourd DNA-A were obtained when PCR was employed (data not shown).

Genome organization

The complete nucleotide sequences of DNA-A of WGNP consisted of 2739 nucleotides (GenBank accession no. EU543562). Sequence numbering begins at base eight (A) of the conserved nonanucleotides TAATATTAC, the first base of viral (+)-strand DNA synthesized after replication initiation by the Rep protein[10]. The genome organization of DNA-A of WGNP was similar to that of other whitefly-transmitted geminiviruses containing 2 open reading frames (ORFs) on the viral sense and three ORFs on the complementary sense strand. On the viral sense strand, the *AV1* ORF (nucleotides 281–1048) was found to encode the CP that is partially overlapped by the small *AV2* ORF. WGNP *AV1* encodes a polypeptide of 255 amino acids with a calculated molecular weight of 29 674 Da. *AV2* ORF (nucleotides 121–456) separated from ORF *AC1* by an intergenic region is predicted to encode a protein of 111 amino acids with a calculated molecular weight of 12 816 Da. The complementary sense strand encodes

Table 2 Oligonucleotides used for genome amplification.

GenBank Accession No.	Geminivirus Name	Assigned abbreviation
D14703	*Mungbean yellow mosaic virus*-[Thailand1:Mungbean 1]	MYMV-[TH:Mg1]
AF134484	*Pepper leaf curl virus*-Thailand [Thailand]	PepLCV-TH[TH]
AB027465	Squash leaf curl China virus	SLCCNV
AM260206	*Squash leaf curl China virus*-China [China:Guangxi25:2005]	SLCCNV-CN [CN:Gx25:05]
AM260205	*Squash leaf curl China virus*-China [China:Hainan61:2005]	SLCCNV-CN [CN:Hn61:05]
AY184487	*Squash leaf curl China virus*-India [India:Coimbatore:Pumpkin]	SLCCNV-IN [IN:Coi:Pum]
DQ026296	*Squash leaf curl China virus*-India [India:Lucknow:Pumpkin]	SLCCNV-IN[IN:Luc:Pum]
AB330078	*Squash leaf curl China virus*-Thailand [Thailand]	SLCCNV-TH[TH]
AB085793	*Squash leaf curl Philippines virus*-Philippines [Philippines:Munoz]	SLCPHV-PH [PH:Mun]
AF195782	*Tomato leaf curl Laos virus*-[Laos]	ToLCLV-[LA]
U15016	*Tomato leaf curl New Delhi virus*-India [India:New Delhi:Mild:1992]	ToLCNDV-IN [IN:ND:Mld:92]
U15015	*Tomato leaf curl New Delhi virus*-India [India:New Delhi:Severe:1992]	ToLCNDV-IN [IN:ND:Svr:92]
AY939926	*Tomato leaf curl New Delhi virus*-India [India:Sonepat:Luffa:2005]	ToLCNDV-IN [IN:Son:Luf:05]
DQ116880	*Tomato leaf curl New Delhi virus*-India [Pakistan:Khalawal:Chili:2004]	ToLCNDV-IN [PK:Kha:Chi:04]
AJ620187	*Tomato leaf curl New Delhi virus*-India [Pakistan:Solanum nigrum:1997]	ToLCNDV-IN [PK:Sn:97]
AB330079	*Tomato leaf curl New Delhi virus*-Thailand [Thailand:Cucumber]	ToLCNDV-TH [TH:Cu]
AF102276	*Tomato leaf curl New Delhi virus*-Thailand [Thailand:Luffa]	ToLCNDV-TH [TH:Luf]
AF141922	*Tomato yellow leaf curl Thailand virus*-A [Thailand:2]	TYLCTHV-A[TH:2]
AF206674	*Tomato yellow leaf curl Thailand virus*-B [Myanmar:Yangon:1999]	TYLCTHV-B [MM:Yan:99]
AY514630	*Tomato yellow leaf curl Thailand virus*-B [Thailand:Chiang Mai]	TYLCTHV-B [TH:ChMai]
AY514631	*Tomato yellow leaf curl Thailand virus*-B [Thailand:Nong Khai]	TYLCTHV-B [TH:NoK]
AY514632	*Tomato yellow leaf curl Thailand virus*-C [Thailand:Sakon Nakhon]	TYLCTHV-C [TH:SaNa]
AJ245652	*Watermelon chlorotic stunt virus*-[Iran:1997]	WmCSV-[IR:97]

for three proteins with molecular weights greater than 10 kDa. They are designated as *Rep* gene or *AC1* ORF, *TrAP* or *AC2* ORF, and *REn* or *AC3* ORF. The *Rep* gene potentially encodes the replication-associated protein, the only viral protein absolutely required for viral DNA replication as it is responsible for initiating DNA replication during the rolling-circle amplification stage[11–15]. *TrAP* is a transcriptional activator of viral genes[16,17], whereas the REn protein enhances accumulation of ssDNA and dsDNA during replication[18]. All ORFs are described in Table 3 and their corresponding organization along the genomes is shown in Fig. 1. The DNA-A has an intergenic region of approximately 200 nucleotides, similar to that found in the IR of other geminiviruses, which is the common region of DNA-A and DNA-B. The IR contains a GC-rich inverted repeat that has the potential to form a stem-loop structure, including the conserved TAATATTAC nonanucleotide sequence that contains the nicking site (TAATATT↓AC) for initiation of virion-sense DNA replication[10]. This region contains a series of *cis*-acting elements involved in DNA replication and transcription of the *Rep* gene[19]. All such elements are present in the sequence of WGNP isolates, including: (i) the binding sites for the Rep protein, (ii) the TATA box for the *rep* gene, and (iii) a conserved stem-loop motif which includes the nonanucleotide sequence nicked by the Rep protein to initiate DNA replication in the origin of replication.

Table 3 Open reading frames (ORFs) of *Squash leaf curl China virus*-[Thailand:Wax Gourd:2008] (SLCCNV-[TH:Wax:08])

ORF	Frame	Nucleotide number[a]	Number of amino acids	Protein mol. wt. (Da)
AV1	+2	281–1048	255	29 674
AV2	+1	121–456	111	12 816
AC1	−1	1506–2585	359	40 547
AC2	−3	1190–1594	134	15 156
AC3	−2	1045–1455	136	16 048

[a] Sequence numbering begins at base eight (A) of the conserved nonanucleotide TAATATTAC, according to Ref. 10.

Relationships and sequence comparison of WGNP to other geminiviruses

Comparison of complete sequences of DNA-A and partial sequences of the A component including *AV1*, *AV2*, *AC1*, *AC2*, *AC3*, and the intergenic region (IR) revealed that geminivirus infected wax gourd in Nakhon Pathom (WGNP) formed a closely related group with a previously characterized SLCCNV-TH[TH] described in Ref. 20 and showed a greater sequence homology among them than with other Thailand reported geminiviruses infected tomato such as TYLCTHV-A[TH:2], TYLCTHV-B[TH:ChMai], TYLCTHV-B[TH:NoK], and TYLCTHV-C[TH:SaNa]. WGNP was placed in the same clade as SLCCNV, whereas four isolates of TYLCTHV were clearly separated from the clade containing those geminiviruses. The difference may be due to host specificity since cucurbits were found to be the natural hosts of

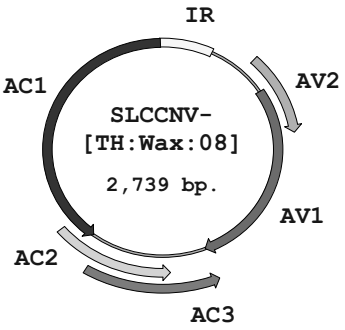

Fig. 1 Genome organization of *Squash leaf curl China virus*-[Thailand:Wax Gourd:2008] (SLCCNV-[TH:Wax:08]). Open reading frames (ORFs) are shown as arrows. The ORFs of viral sense strand are designated *AV1* ORF and *AV2* ORF. The ORFs of the complementary sense strand are designated *AC1*, *AC2*, and *AC3* ORF. IR indicates the intergenic region of DNA-A.

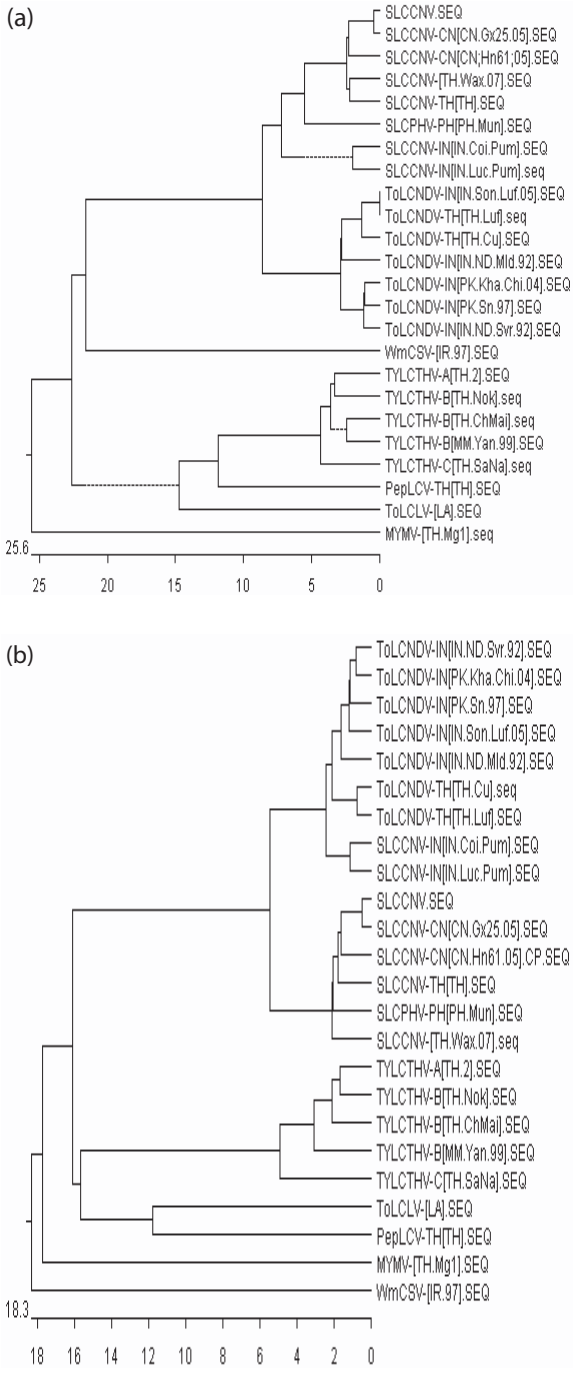

Fig. 2 Phylogenetic tree obtained from the alignment of (a) DNA-A and (b) AV1 sequences of SLCCNV-[TH:Wax:08] and 24 other whitefly-transmitted geminiviruses.

WGNP and the previously described SLCCNV, whereas tomato was reported to be a natural host of TYLCTHV. Phylogenetic relationships for WGNP DNA-A and *AV1* with reference geminiviruses are shown in Fig. 2. Results the from phylogenetic tree indicate that WGNP is a member of the SLCCNV cluster containing *Squash leaf curl China virus* (SLCCNV), *Squash leaf curl China virus*-Thailand [Thailand] (SLCCNV-TH[TH]), *Squash leaf curl China virus*-China [China:Hainan61:2005] (SLCCNV-CN[CN:Hn61:05]), *Squash leaf curl China virus*-China [China:Guangxi 25:2005] (SLCCNV-CN[CN:Gx25:05]), *Squash leaf curl Philippines virus* Philippines [Philippines:Munoz] (SLCPHV-PH [PH:Mun]), *Squash leaf curl China virus*-India [India:Coimbatore:Pumpkin] (SLCCNV-IN[IN:Coi:Pum]), *Squash leaf curl China virus*-India [India:Lucknow:Pumpkin] (SLCCNV-IN[IN:Luc:Pum]).

Due to difficulties inherent in species identification, the ICTV Geminiviridae Study Group proposed new species demarcation criteria, the most important of which is an 89% nucleotide identity threshold between full-length DNA-A component nucleotide sequences for begomovirus species. This threshold has since been used with general satisfaction. More recently, an article has been published to clarify the terminology used to describe virus entities below the species level[21]. The present publication is proposing demarcation criteria and guidelines to classify and name geminiviruses below the species level. Using the Clustal V algorithm (DNAStar MegAlign software), the distribution of pairwise sequence comparisons, for pairs of sequences below the species taxonomic level, identified two

Table 4 Percentage nucleotide and amino acid similarities predicted by the Clustal option of MegAlign for open reading frames of the *Squash leaf curl China virus*-[Thailand:Wax Gourd:2008] (SLCCNV-[TH:Wax:08]) compared with the most closely related geminiviruses or isolates of tomato-infecting geminiviruses in Thailand (TYLCTHV).

Virus	Nucleotide/amino acid						
	AV1	AV2	AC1	AC2	AC3	IR	DNA-A
SLCCNV	92/93	93/90	90/82	94/89	95/95	88	92
SLCCNV-TH[TH]	94/93	93/91	93/92	96/92	93/97	92	94
SLCCNV-CN[CN:Gx25:05]	93/94	93/89	93/92	97/86	93/95	91	93
SLCCNV-CN[CN:Hn61:05]	93/91	93/91	91/90	96/93	91/97	90	93
SLCPHV-PH [PH:Mun]	93/93	91/87	81/80	93/88	92/90	68	85
SLCCNV-IN[IN:Coi:Pum]	87/91	82/77	91/90	90/84	91/91	87	89
SLCCNV-IN[IN:Luc:Pum]	87/92	82/77	90/88	91/86	90/91	86	89
ToLCNDV-TH[TH:Luf]	87/92	77/67	81/85	54/10	81/10	68	81
ToLCNDV-TH[TH:Cu]	87/92	83/79	82/87	82/78	82/85	66	82
TYLCTHV-A[TH:2]	68/75	69/62	67/71	59/46	67/62	38	63
TYLCTHV-B[TH:ChMai]	68/76	69/64	69/93	59/50	69/60	45	65
TYLCTHV-B[TH:NoK]	68/76	68/62	69/72	60/50	69/63	50	65
TYLCTHV-C[TH:SaNa]	70/77	73/70	68/73	60/50	68/60	43	65
TYLCTHV-B[MM:Yan:99]	68/75	69/64	69/74	62/51	69/63	47	65

peaks: one at 85–94% nucleotide identity that is proposed to correspond to "strain" comparisons and one at 92–100% identity that corresponds to "variant" comparisons[22]. Comparison of nucleotide sequence identities of WGNP genome components with those of reference geminiviruses are presented in Table 4. WGNP DNA-A shares more than 92% nucleotide sequence identity with the A component of its four closest relatives, SLCCNV-TH[TH] (94%), SLCCNV-CN[CN:Gx25:05] (93%), SLCCNV-CN[CN:Hn61:05] (93%), and SLCCNV (92%). WGNP DNA-A was distinct from other tomato-infecting geminiviruses from Thailand (TYLCTHV) at 63–65% nucleotide identity (Table 4). Therefore, we concluded that this geminivirus is strain of SLCCNV and named it *Squash leaf curl China virus*-[Thailand:Wax Gourd:2008] (SLCCNV-[TH:Wax:08]).

The IR is the part of the genome that shows the greatest variation among different begomoviruses. Apart from the conserved nonanucleotide sequence and the TATA boxes, IRs of different viruses show little similarity to one another. Although iterons are always present, they vary in length, sequence, number, and orientation. Iteron sequences can differ in closely related viruses[23] and conversely, identical sequences are found in some more distantly related viruses[19,24]. Comparison of nucleotide identities among the IR of SLCCNV-[TH:Wax:08] and its closest relatives (also members of the SLCCNV cluster) shared the following percentages of identities: SLCCNV-TH[TH] (92%), SLCCNV-CN[CN:Gx25:05] (91%), SLCCNV-CN[CN:Hn61:05] (90%), SLCCNV (88%), SLCCNV-IN[IN:Coi:Pum] (87%), and SLCCNV-IN[IN:Luc:Pum] (86%), whereas less than 50% sequence identities were found in comparisons with the other geminiviruses infecting tomato in Thailand (Table 4).

AV1 gene is the most conserved region of the begomovirus genome, with AV1 amino acid sequences being most similar among viruses from the same geographical area[25]. AV1 sequence of SLCCNV-[TH:Wax:08] was highly conserved with SLCCNV, SLCCNV-TH[TH], SLCCNV-CN [CN:Hn61:05], SLCCNV-CN[CN:Gx25:05], and SLCPHV-PH [PH:Mun] ranging from 92–94% nucleotide sequence identity but only 68–70% for the geminiviruses infected tomato in Thailand, indicating that AV1-regions of SLCCNV-[TH:Wax:08] and its three closest relatives had originated from the same parental virus.

Comparisons of nucleotide identities for individual ORFs of SLCCNV-[TH:Wax:08] with other viruses of the SLCCNV and TYLCTHV cluster are presented in Table 4. Examination of the similarity of individual ORFs of SLCCNV-[TH:Wax:08] with other geminiviruses shows that all ORFs of SLCCNV-[TH:Wax:08] are most closely related to SLCCNV-TH[TH].

Acknowledgements: This research work was supported by grants from the Research and Development Institute, Suan Sunandha Rajabhat University.

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