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Complete nucleotide sequence of the geminivirus tomato yellow leaf curl virus, Thailand isolate

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The complete genome of a Thailand isolate of the geminivirus tomato yellow leaf curl virus (TYLCV-Th) has been cloned and the nucleotide sequence determined. The genome consists of two DNAs each slightly greater than 2700 nucleotides in length and designated A-DNA and B-DNA. The A-DNA contains six open reading frames (ORFs) capable of encoding proteins with M_rs greater than 10K; two ORFs were located on the B-DNA. The predicted ORFs were found at positions on the genome similar to those identified in other geminiviruses. Based upon computer-assisted sequence comparisons with other geminiviruses, TYLCV-Th maintained the greatest degree of similarity with a tomato pathogen isolated in Australia, tomato leaf curl virus. Furthermore, sequence comparisons with a large number of geminiviruses confirm further divisions of the geminiviruses based upon geographical proximity of the viruses. Agroinoculation of *Nicotiana benthamiana* with a TYLCV-Th A-DNA clone containing a mutation in the capsid protein ORF demonstrated that the coat protein is not essential for TYLCV-Th infection but enhances the progression of the disease.

Introduction

Geminiviruses have circular ssDNA genomes encapsidated in geminate particles. The International Committee on Taxonomy of Viruses (ICTV) divided the geminiviruses into three subgroups based upon genome structure, the type of insect vector and host range (Hull *et al.*, 1991). These subgroups will become three genera in the family *Geminiviridae* in the next report of the ICTV (M. Fauquet, personal communication). Subgroup one contains the geminiviruses that infect monocotyledonous plants with maize streak virus (MSV) as the type member. These viruses are transmitted by leafhoppers and contain a single DNA molecule. Beet curly top virus (BCTV) and tobacco yellow dwarf virus (ToYDV) are the only representatives of the second subgroup and BCTV is the type member. These leafhopper-transmitted viruses infect dicotyledonous hosts and also contain a single molecule DNA genome. Bean golden mosaic virus (BGMV) is the type member of the third subgroup comprising the whitefly-transmitted geminiviruses with bipartite genomes. To date, these geminiviruses have been shown to infect only dicotyledonous plants. Another study identified subsets of viruses within this subgroup according to geographical distribution with BGMV and tomato golden mosaic virus (TGMV) as examples of 'New World' geminiviruses and African cassava mosaic virus (ACMV) and tomato yellow leaf curl virus (TYLCV) as 'Old World' geminiviruses (Howarth & Vandemark, 1989). More recently, several isolates of whitefly-transmitted geminiviruses have been described which infect tomato plants and contain only a single DNA component (Navot *et al.*, 1991; Kheyr-Pour *et al.*, 1991; Dry *et al.*, 1993). These geminiviruses include two isolates of TYLCV, isolated in Israel (TYLCV-I) and Sardinia (TYLCV-S), as well as tomato leaf curl virus (TLCV) from Australia. Geographically, these geminiviruses would be placed in the 'Old World' subset. Here we present the complete genomic sequence of the A- and B-DNAs of TYLCV-Th as well as comparisons between its putative protein products and nucleotide regulatory regions with those of other geminiviruses.

The role of the coat protein (CP) in the infection cycle further differentiates the subgroups of the geminiviruses

appearance of symptoms and reduce the virus titre in systemic infection but do not prevent infection or spread of the pathogen (Stanley & Townsend, 1986; Gardiner *et al.*, 1988). In contrast, several studies of monopartite geminiviruses with dicotyledonous hosts indicated that the CP is necessary for systemic infection and cell to cell movement (Briddon *et al.*, 1989; Rigden *et al.*, 1993). In the present study we demonstrate that disruption of the CP ORF of TYLCV-Th reduces systemic infection following agroinoculation of *Nicotiana benthamiana* plants. This mutation also reduces the accumulation of

was accomplished by digesting the unique *Bgl*II restriction site in the CP gene (nucleotide number 798, Fig. 1*a*) followed by end filling with Klenow polymerase and dNTPs and subsequent religation. The mutation was present in both copies of the CP ORF in the final agroinoculation clone, pDR56. The plasmid pDR56 contains 1·6 copies of A-DNA and was constructed in a similar manner to pDR21 (1·6 copies TYLCV-Th A-DNA) as described previously (Rochester *et al.*, 1990). Mutagenesis of the TYLCV-Th CP ORF resulted in an insertion at amino acid 120 of four amino acids followed by a stop codon (TGA). The plasmid pDR37 contains 1·6 copies of the TYLCV-Th B-DNA and has been described previously (Rochester *et al.*, 1990). The plasmids used for agroinoculation were shuttled into *Agrobacterium tumefaciens* GV3111SE (Rogers *et al.*, 1986). The modified strains of *A. tumefaciens*

(*a*) TYLCV-Th-A

```
ATTGACTTGG TCAATCGGGT CCTCTCAAAC TTGGCTATGC AATCGGGGAA
TGGGTCCTTA TTATAGTTGT GGACCTAAAT GGCATAATTG TAAATAAGTG  100
TATGAAATTC AAAATTTAAA ATTCAATCGT GGCCATCCGT ATAATATTAC
CGGATGGCCG CGATTTTTTT AAAGTGGTCC CCTTGATGTG ATATGTCATC  200
CAATCAGAAC ACTCTTTGAA AGCCTAATTA TTTATGGTCC CCTATTTAAG
ACTTAGTCCC CAAGTTTCGG CGAAATTCAA AATGTGGGAT CCACTACTAA  300
                                 AV1 Start
ACGAATTTCC AGAAAACGTC CACGGTTTCC GTTGTATGTT AGCGGTGAAG
TATCTGCAAG CGGTCGAGAA GACTTATTCC CCTGATACTC TAGGGTTTGA  400
TCTCATCCGT GATCTCATCG GTGTAATTCG TGCGAAGAAC TATGTCGAAG
                                             AV2 Start
CGTCCAGCAG ATATTCTCAT TTCCACTCCC GTCTCGAAAG TACGTCGCCG  500
```

(*b*) TYLCV-Th-B

```
ATTGACTGGT CAATCGGTGT CTCTCAAACT TGGCTATGCA ATCGGTGTCT
GGTGTCTTAT TTATACCTGG ACACCAAATG GCATAATTGT AATTTAGTAA  100
ATGTAATTCA AAATTCAAAA TCCAATCGTG GCCATCCGTA TAATATTACC
GGATGGCCGC GATTTTTTTC AAGTCGTCCC ACCACTGACA TCTCAGCCCC  200
ACTGGAAAAT CATAGCCGTT GAATCGTAAC ACGCGTTACA TATCCAATTG
ATTTGCAGTG TATTTATTCT GACTATAAAT GTGATGTTAT GGTCGTCATT  300
TGTTGTTGTG AAATAGCATG AGAGTTCCTA TTAGACGACC TTTTGGCTTT
                          BV1 Start
AACAGTGATC GCCGTCCTTC CGGCGGTTCT ATGTTCCGTT ATAATTATCC  400
CTACGCCAAT TACTTTGGCA GGAGGGTTGG CAACCGTGTA TATGGAATGC
CCTTTGGTAG TACCACCTCT GTCCGGCGAC CAATTAGAAG TACAGTTCGG  500
AGGAATTTAT TTTCCGACCA GTCTTCTTCA GGTAACAAGA GCGCTAAAAC
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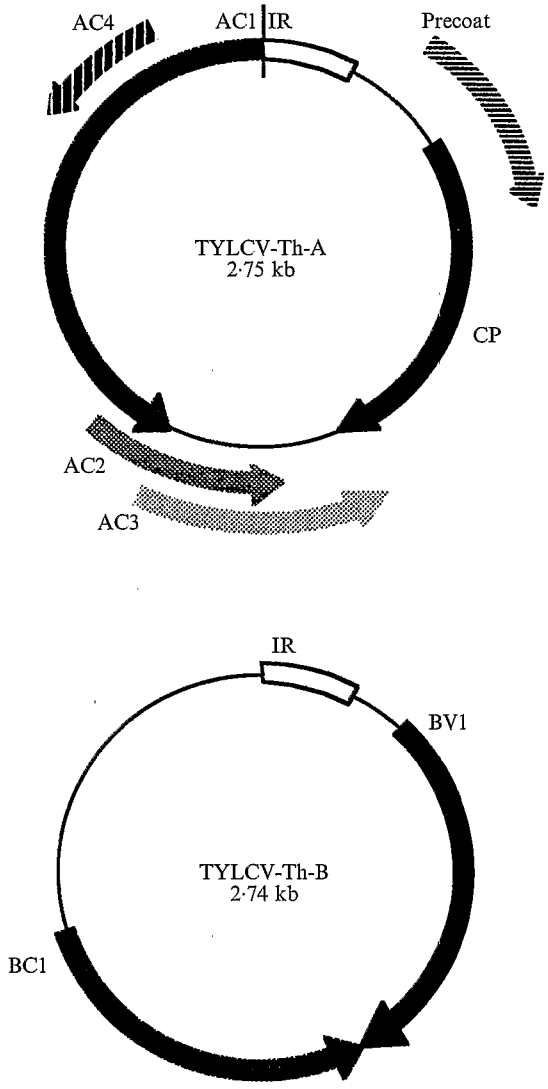


Fig. 2. Genomic organization of the TYLCV-Th A- and B-DNAs. The virion strand orientation is in the clockwise direction. Direction and relative lengths of ORFs are indicated by open boxes.

Table 1. *Predicted ORFs of TYLCV-Th*

ORF*	Nucleotides (start–stop)	M_r† ($\times 10^{-3}$)
AV1	282–620	12·9
AV2	442–1212	29·7
AC1	2743–1660	40·8
AC2	1757–1353	15·3
AC3	1612–1208	15·9
AC4	2591–2443	11·3
BV1	318–1151	31·6
BC1	1990–1158	31·2

* A and B designate whether ORF is found on the A- or B-DNA. V and C denote virion- and complementary-sense ORFs, respectively, as shown in Fig. 2.

† M_r of putative translation products.

Table 2. *Percentage similarity between the products of the different ORFs for the geminiviruses infecting tomatoes in the Old World, and ACMV*

	TLCV	TYLCV-I	TYLCV-S	ACMV
AV1				
TYLCV-Th	6·2	71	66	66
TLCV		60	58	63
TYLCV-I			82	72
TYLCV-S				72
AV2				
TYLCV-Th	74	73	69	71
TLCV		76	73	74
TYLCV-I			86	80
TYLCV-S				76
AC1				
TYLCV-Th	80	78	75	71
TLCV		83	78	76
TYLCV-I			77	72
TYLCV-S				74
AC2				
TYLCV-Th	61	57	55	60
TLCV		61	56	62
TYLCV-I			67	64
TYLCV-S				64
AC3				
TYLCV-Th	68	67	62	64
TLCV-A		47	58	63
TYLCV-I			65	73
TYLCV-S				70
AC4				
TYLCV-Th	46	63	49	23
TLCV		79	63	50
TYLCV-I			76	27
TYLCV-S				37
BV1				
TYLCV-Th				25
BC1				
TYLCV-Th				41

Comparisons of intergenic regions and entire sequences

The intergenic regions (IR) of 15 geminiviruses with members of all described subgroups were compared in a multiple alignment using Megalign as described in Methods. In the case of bipartite geminiviruses, the IR of the A-DNA was used. For the alignments, the IR included the first 200 nucleotides of the virion strand sequence beginning at the first nucleotide 3′ of the ATG start codon of AC1. A phylogenetic tree constructed from the multiple alignment analysis is shown in Fig. 3. One observation resulting from such an analysis is that the geminiviruses used in this comparison are grouped on the basis of the insect vector (i.e. whiteflies or leafhoppers) and whether the natural plant host is dicotyledonous (whitefly-transmitted) or monocotyledonous (leafhopper-transmitted). On the other hand BCTV, a geminivirus of a dicotyledonous host, is transmitted by the leafhopper. The IRs of the viruses

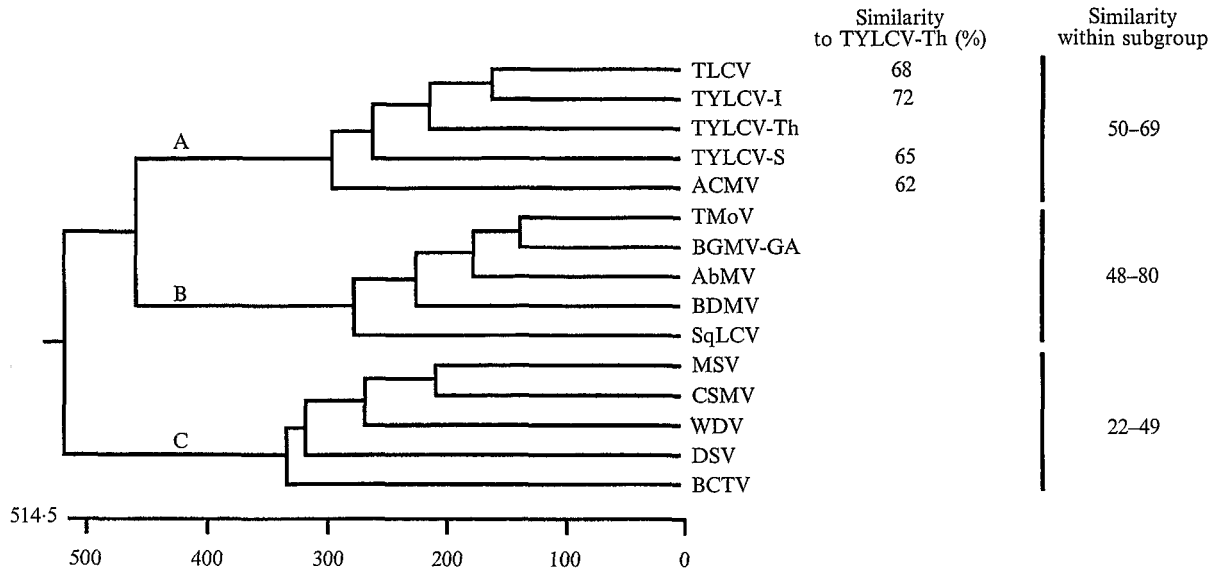


Fig. 3. Phylogenetic tree constructed from pairwise sequence comparison of the intergenic regions of fifteen geminiviruses. Percentage similarity is defined in Methods.

that infect dicotyledonous plants are further divided into two main groups, the 'Old World' and 'New World' viruses based on the geographical location where the virus was found (Howarth & Vandemark, 1989; Abouzid *et al.*, 1992). When the viruses within any of the three major branches (A, B or C, Fig. 3) are compared in a pairwise manner, the greatest overall similarity is found amongst the whitefly-transmitted geminiviruses. The IR and shown in Table 2 is derived through multiple alignments of only the Old World geminiviruses.

Comparison of AV1 (precoat) sequences

A small ORF, AV1, is found in a similar position in all Old World geminiviruses and overlaps the amino terminus of the V2 (coat) ORF. Percentage similarities

Table 3. *Agroinoculation of* N. benthamiana *with TYLCV-Th CP mutants*

TYLCV-Th DNA agroinoculated	A-DNA*	B-DNA*	Symptoms†
A	−	−	−
A	+	−	A
A	+	−	A
A+B	+	+	A+B
A+B	+	−	A
A+B	+	−	A
Acp	−	−	−
Acp	−	−	−
Acp	−	−	−
Acp+B	+	−	−
Acp+B	+	+	M
Acp+B	+	−	M
B	−	−	−
B	−	−	−
B	−	−	−

* Presence (+) or absence (−) of TYLCV-Th A- or B-DNA in extracts of upper non-inoculated leaves as determined by hybridization with the appropriate insert.

† A and A+B symptoms are described in Rochester *et al.* (1990). A+B, very severe; A, severe; M, mild.

Comparison of AC1, AC2, AC3 and AC4 sequences

Analyses of the leftward-transcribed cistrons of TYLCV-Th and counterparts of the Old World geminiviruses indicate that AC1 maintains the highest sequence conservation amongst these four polypeptides (Table 3). TYLCV-Th shares the highest similarity with TLCV in AC1, AC2 and AC3, although the differences between the viruses are, in general, small. AC4, a small ORF found within the TYLCV-Th AC1 ORF, shows the highest sequence similarity with TYLCV-I (63%). AC4 is the least conserved ORF in terms of sequence similarity amongst the Old World geminiviruses.

Comparison of BC1 and BV1 sequences

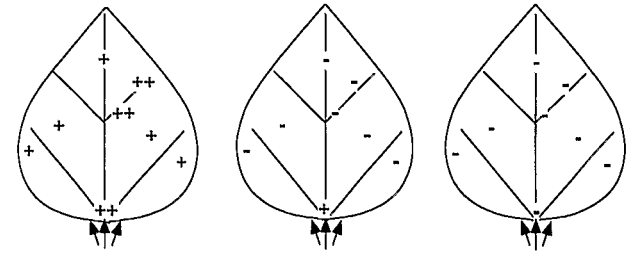
To date, TYLCV-Th is the only bipartite geminivirus of the Old World subgroup that readily infects tomato plants. TYLCV-Th BC1 maintains weak sequence similarity with the homologous ORFs of ACMV (43%), TGMV (45%), BGMV (41%) and SqLCV (42%). The most impressive similarity is found in BC1 sequences of the geminiviruses of the New World with several large blocks of identical amino acids (alignments not shown). Overall, the similarities between the BV1 ORFs are less than those found in any of the other ORFs. TYLCV-Th and ACMV BV1 maintain very little similarity with each other (25%), and no blocks of more than four identical amino acids were found. The phylogenetic tree constructed from multiple alignments of each of the ates the Old World and New World geminiviruses (data not shown).

Infectivity of a TYLCV-Th CP mutant

Studies of geminiviruses with a single genomic DNA have shown that the CP is necessary for systemic spread of infection. Therefore, these studies were undertaken to determine whether the CP of TYLCV-Th was required for local and systemic infection. Eleven days after agroinoculation, three of five tomato plants (UC82b) and 13 of 15 *N. benthamiana* plants agroinoculated with wild-type TYLCV-Th:A displayed disease symptoms. All plants with symptoms contained viral DNA (data not shown). On the other hand, at 11 days post-infection, plants agroinoculated with TYLCV-Th:Acp did not display any of the symptoms observed on TYLCV-Th:A infected plants.

None of the tomato plants inoculated with TYLCV-Th:Acp contained detectable amounts of viral DNA. By contrast, three *N. benthamiana* that were similarly agroinoculated accumulated viral DNA at levels five-to-tenfold lower than that found in TYLCV-Th:A infection (data not shown). Because of this result, *N. benthamiana* were then agroinoculated with TYLCV-Th:A, TYLCV-Th:Acp, TYLCV-Th:B and all possible combinations (three plants each treatment). Symptoms were detected on plants infected with TYLCV-Th:A (two of three) and mixed infections of TYLCV-Th:A and TYLCV-Th:B (three of three) at 28 days post-inoculation as expected. Approximately 75 days after inoculation, mild symptoms were observed on two of three plants agroinoculated with a combined culture of TYLCV-Th:Acp and TYLCV-Th:B. Dot blot analysis was carried out on samples from all plants and results are shown in Table 3. In five of six plants agroinoculated with TYLCV-Th:A alone or in combination with TYLCV-Th:B, TYLCV-Th A-DNA was detected in the upper leaves. One of three plants agroinoculated with a mixture of TYLCV-Th:A and TYLCV-Th:B supported B-DNA replication; this plant displayed severe disease symptoms as described before (Rochester *et al.*, 1990). Two of three plants that were agroinoculated with TYLCV-Th:Acp and TYLCV-Th:B contained TYLCV-Th A-DNA sequences. The presence of TYLCV-Th B-DNA was also detected in one of these plants. In these experiments, no significant differences in the amounts of accumulated viral DNA were observed.

These results indicated that the presence of TYLCV-Th CP enhances progression of disease. Excised *N. benthamiana* leaves were subsequently used in other experiments to determine the role of CP in the local movement of TYLCV-Th A-DNA. Leaves were removed



the accumulation of viral DNA was due to the spread of *A. tumefaciens* since B-DNA was not detected. Furthermore, *A. tumefaciens* did not overgrow the plates.

Discussion

The A- and B-DNAs of TYLCV-Th were cloned and sequenced. By comparing the genomic organization and similarities of the amino acid sequences of the ORFs predicted from the nucleotide sequences we concluded

causing symptomatic infections in tomato and *N. benthamiana*. Nevertheless, symptoms of TYLCV-Th disease are more severe following agroinoculation of both A- and B-DNAs.

Agroinoculation of *N. benthamiana* plants with TYLCV-Th A-DNA constructs containing a mutation in the CP cistron resulted in delayed and decreased disease symptoms and reduced accumulation of A-DNA. These findings are similar to the results of studies where disruptions in the V2 (CP) cistron of TLCV greatly reduced virus spread following agroinoculation (Rigden *et al.*, 1993). Systemic infection by geminiviruses with bipartite genomes is not severely affected by disruption of their CP genes (Brough *et al.*, 1988; Etessami *et al.*, 1989). Where studied, geminiviruses with a single genomic DNA have no capacity to cause disease in the absence of the CP (Lazarowitz *et al.*, 1989; Boulton *et al.*, 1989; Briddon *et al.*, 1989). Initial infection and movement of the TYLCV-Th A-DNA following agroinoculation is not completely eliminated by a mutation in the CP since systemic infection and mild symptoms occurred in whole plants, although delayed relative to infection by wild-type A-DNA. Interestingly, in one plant which supported TYLCV-Th:Acp and TYLCV B-DNA, accumulation of both viral components was equivalent to the levels found following agroinoculation of both wild-type viral DNAs. In this case, function(s) lost owing to the mutation in the CP may have been provided by the B-DNA. Two research groups demonstrated previously that efficient systemic infection by the bipartite geminiviruses ACMV and TGMV was only possible when both A- and B-DNAs were present (Stanley, 1983; Hamilton *et al.*, 1983). In the case of TYLCV-Th, infection of *N. benthamiana* with both A- and B-DNAs resulted in a more severe infection than infection by A-DNA alone (Rochester *et al.*, 1990). However, infection by A-DNA alone led to significant disease symptoms. In contrast, plants infected with only the A-DNA of ACMV did not develop symptoms and contained 20-fold less viral DNA than those infected with both A- and B-DNAs (Klinkenberg & Stanley, 1990). More recently it was shown that agrobacterium-mediated inoculation of the DNAs of TLCV and two TYLCV isolates on tomato leads to systemic infection indistinguishable from natural whitefly-transmitted disease (Navot *et al.*, 1991; Kheyr-Pour *et al.*, 1991; Dry *et al.*, 1993). In this regard, TYLCV-Th has characteristics somewhat intermediate among the whitefly-transmitted geminiviruses.

Excised intact leaves were used to analyse the role of the CP in the local movement of TYLCV-Th. Inoculation of TYLCV-Th:A at the petiole or the base of the leaf resulted in a more efficient systemic spread than infection by TYLCV-Th:Acp (Fig. 4). Agroinoculation of excised leaves indicated that the location of the initial site of infection dictated and possibly enhanced the degree of infection by TYLCV-Th:Acp. Infection at the petiole resulted in spread of the mutant virus throughout the leaf, although much less viral DNA accumulated than after inoculation with the wild-type A-DNA. However, when TYLCV-Th:Acp was inoculated at the leaf base, there was little or no spread of the mutant virus. A small amount of viral DNA was detected at the site of inoculation possibly due to initial infection and replication. These results are similar to those obtained with TLCV CP mutants (Rigden *et al.*, 1993). The results indicate that the CP of these viruses is not necessary for viral DNA synthesis, yet is needed for cell to cell spread. When excised leaves were inoculated with TYLCV-Th:Acp at the petiole, there was apparently systemic movement through the vascular tissue and limited cell-to-cell movement within the interveinal areas.

TYLCV-Th has genomic and biological characteristics that place it in an intermediate position among the geminiviruses. The bipartite genome structure and arrangement of ORFs of this virus are similar to those of the well characterized whitefly-transmitted geminiviruses. Furthermore, the CP of TYLCV-Th enhances but is not completely necessary for efficient systemic infection yet appears to enhance cell-to-cell movement and accumulation of viral DNAs.

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