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Emergence of a New Cucurbit-Infecting Begomovirus Species Capable of Forming Viable Reassortants with Related Viruses in the *Squash leaf curl virus* Cluster

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ABSTRACT

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Cucurbit leaf curl virus (CuLCV), a whitefly-transmitted geminivirus previously partially characterized from the southwestern United States and northern Mexico, was identified as a distinct bipartite begomovirus species. This virus has near sequence identity with the previously partially characterized *Cucurbit leaf crumple virus* from California. Experimental and natural host range studies indicated that CuLCV has a relatively broad host range within the family *Cucurbitaceae* and also infects bean and tobacco. The genome of an Arizona isolate, designated CuLCV-AZ, was cloned and completely sequenced. Cloned CuLCV-AZ DNA A and B components were infectious by biolistic inoculation to pumpkin and progeny virus was transmissible by the whitefly vector, *Bemisia tabaci*, thereby completing Koch's postulates. CuLCV-AZ DNA A shared highest nucleotide sequence identity with *Squash leaf curl virus*-R (SLCV-R), SLCV-E, and *Bean calico mosaic virus* (BCaMV) at 84, 83, and 80%, respectively. The CuLCV DNA B component shared highest nucleotide sequence identity with BCaMV, SLCV-R, and SLCV-E at 71, 70, and 68%, respectively. The *cis*-acting begomovirus replication specificity element, GGTGTCCTGGTG, in the CuLCV-AZ origin of replication is identical to that of SLCV-R, SLCV-E, and BCaMV, suggesting that reassortants among components of CuLCV-AZ and these begomoviruses may be possible. Reassortment experiments in pumpkin demonstrated that both reassortants of CuLCV-AZ and SLCV-E A and B components were viable. However, for CuLCV-AZ and SLCV-R, only one reassortant (SLCV-R DNA A/CuLCV-AZ DNA B) was viable on pumpkin, even though the cognate component pairs of both viruses infect pumpkin. These results demonstrate that reassortment among sympatric begomovirus species infecting cucurbits are possible, and that, if generated in nature, could result in begomoviruses bearing distinct biological properties.

Additional keywords: bipartite geminivirus, whitefly *Bemisia tabaci*.

Whitefly-transmitted geminiviruses have emerged as serious pathogens of agronomic and horticultural crops in subtropical and tropical regions of the Americas (2,5,39). Whitefly-transmitted geminiviruses are restricted to dicotyledonous plants and are assigned to the genus *Begomovirus* within the family *Geminiviridae* (34). Begomoviruses originating in the New World have a bipartite genome organization, whereas those from the Old World have either bipartite or monopartite genomes.

DNA A and DNA B of bipartite begomoviruses are each approximately 2.6 kb in size and share a common region (CR) of approximately 200 nt that is highly conserved among cognate components of a single virus species. The CR contains modular *cis*-acting elements of the origin of replication (*ori*) (18,29,33). Five open reading frames (ORFs) are conserved among the DNA A of the New World begomoviruses. The capsid protein (CP) is encoded by the ORF (AV1) most highly conserved among begomoviruses (34). The replication initiator protein (Rep) encoded by the AC1 ORF binds to the *cis*-acting replication specificity element of the *ori* (17), then performs cleavage and joining reactions to initiate and terminate rolling circle replication (26). A transcriptional transactivator protein (TrAP) is encoded by ORF AC2 and is required for late gene expression (40,41). The AC3 ORF encodes a replication enhancer (REn) protein necessary for optimal DNA replication (41). The fifth ORF on DNA A (ORF AC4) is completely contained within ORF AC1, but in a different frame, and appears to have no essential function in the bipartite begomoviruses (19). The begomovirus DNA B component contains two ORFs, BV1 and BC1, that encode movement proteins essential for systemic spread (38). Modifications or mutations in one or both DNA B ORFs have been shown to influence host range (23).

Squash leaf curl (SLC) disease is problematic in Arizona (6,7, 37), California (12,14,16,35), Texas (24), Sonora, Mexico (3,32, 36), Guatemala (5), and Honduras (10). SLC disease was first observed in squash in California during 1977 to 1978 (16) and in cultivated buffalo gourd in Arizona at about the same time (37). The virus complex associated with SLC disease was initially transmitted by the A biotype of *Bemisia tabaci*, which was native to the deserts of the southwestern United States and Sonora when the disease was first observed. The disease became more prevalent with the introduction of biotype B of *B. tabaci*, beginning in approximately 1987 to 1988 (13). When combined with silvering, a response by the plant caused by feeding of the B biotype vector (13), SLC disease seriously affects production of cucurbit crops in the southwestern United States. Two whitefly-transmitted begomoviruses, *Squash leaf curl virus*-E (SLCV-E) and SLCV-R, have been associated with SLC symptoms (2,27,35). SLCV-E and SLCV-R differ in experimental host range, with SLCV-E reported

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to have a broader host range compared with that of SLCV-R (27,28).

A whitefly-transmitted geminivirus of cucurbits was discovered simultaneously in Arizona, Texas, and Coahuilla, Mexico (4), and California (20) during 1998 to 2000. The isolates were named *Cucurbit leaf crumple virus* (CLCrV) (20) and *Cucurbit leaf curl virus* (CuLCV) (4). CuLCV was recognized as distinct from SLCV-E and SLCV-R based upon distinctive yellow chlorotic spots, mosaic, and leaf curling symptoms produced in infected cucumber, cantaloupe, honeydew melon, pumpkin, and squash (Fig. 1) (data not shown). An initial comparison of *CP* (ORF AV1) gene sequences (4) further indicated that CuLCV was a new begomovirus associated with symptomatic, field-infected cucurbit species. Here we describe the biological and molecular characteristics of infectious DNA clones derived from an isolate of pumpkin from Arizona (CuLCV-AZ). Through phylogenetic and component reassortant analyses we defined relationships of CuLCV with other begomoviruses. This analysis indicates that closely related begomovirus species resident in the Sonoran agroecosystem have the potential to form new component combinations with distinct pathogenic properties.

MATERIALS AND METHODS

Virus isolates. CuLCV isolates were collected from symptomatic cucumber (*Cucumis pepo* L.), honeydew melon and muskmelon (*Cucumis melo* L.), pumpkin (*Cucurbita maxima* Duschne.), and squash (*Cucumis pepo* L.) in Arizona, Texas, or Coahuilla, Mexico during 1999 to 2001. A greenhouse-maintained culture was established for a pumpkin isolate (CuLCV-AZ) collected in Phoenix, AZ during the summer of 1999. The CuLCV-AZ culture was propagated in pumpkin by serial transmission using the whitefly vector, *B. tabaci* (Genn.) B biotype, or by biolistic inoculation (8) with RNase-treated total DNA extracts from symptomatic plants. Infectious clones of SLCV-E and SLCV-R were provided by S. Lazarowitz (21).

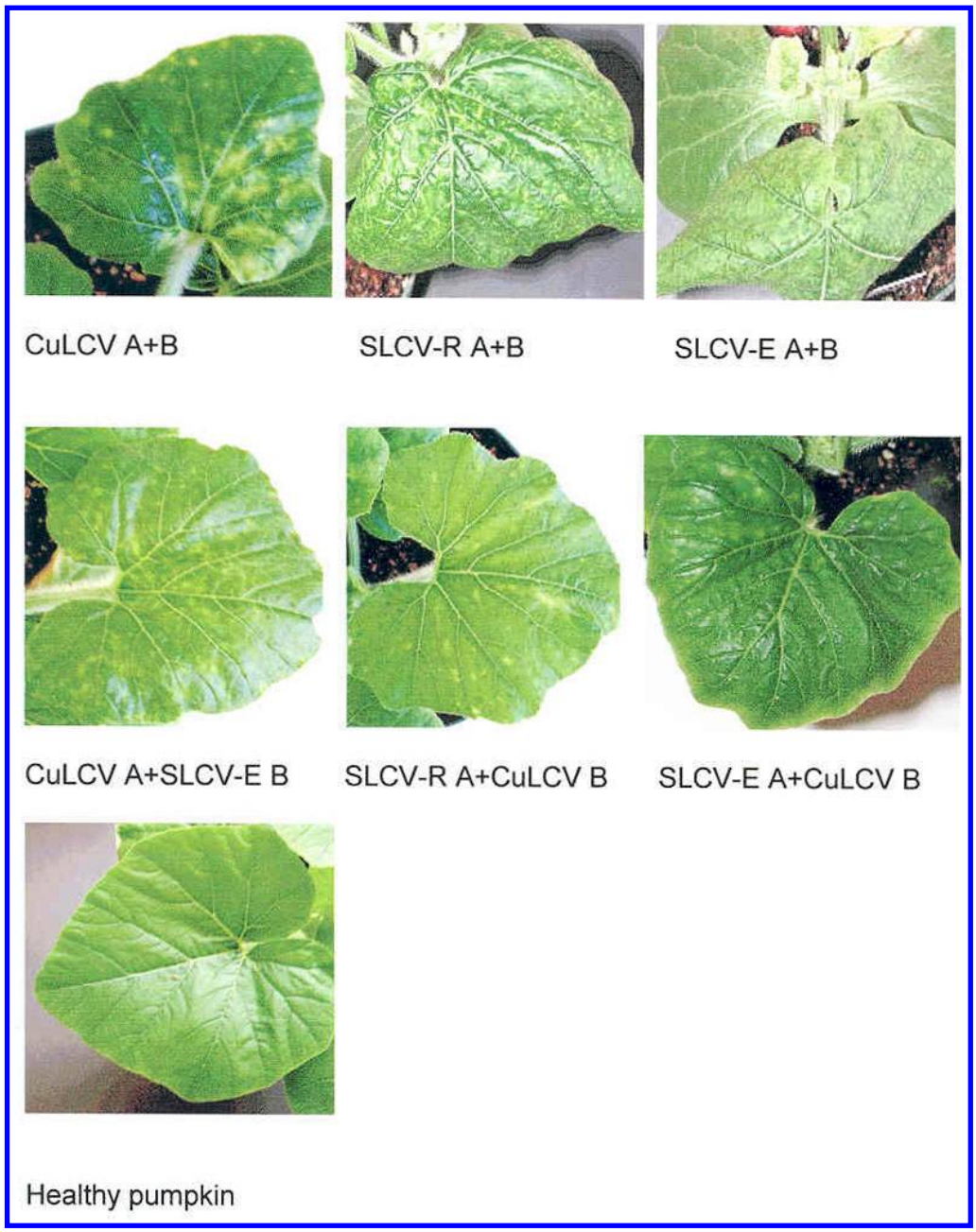

Fig. 1. Pumpkin 'Big Max' with typical symptoms of wild-type *Cucurbit leaf curl virus* (CuLCV) A + B, *Squash leaf curl virus* (SLCV)-R A + B, and SLCV-E A + B, and with symptoms of the reassortants: CuLCV A + SLCV-E B, SLCV-R + CuLCV B, and SLCV-E A + CuLCV B, and healthy control pumpkin.

Identification of natural hosts of CuLCV. Nine field isolates from symptomatic cantaloupe, cucumber, honeydew melon, pumpkin, and zucchini squash collected in Arizona and Texas in the United States and Coahuilla, Mexico were subjected to polymerase chain reaction (PCR) using degenerate primers that amplify the CP gene and adjacent sequences (22). PCR products were cloned into the pGEM7Zf+ plasmid vector and transformed into *Escherichia coli*. Recombinant plasmids of the expected size were selected and the inserted amplicon was subjected to automated sequencing. Two to three PCR-derived clones per isolate were sequenced. *CP* sequences were edited and virus identity was confirmed by multiple sequence alignment using established sequences for all begomoviruses under investigation.

Cloning and sequencing of CuLCV-AZ. Total DNA extracted (15) from CuLCV-AZ-infected pumpkin contained detectable amounts of viral single-stranded and double-stranded DNA when stained with ethidium bromide after electrophoresis in an agarose gel (data not shown). Aliquots of the total DNA sample were incubated with various restriction enzymes and the products analyzed by Southern hybridization using ^{32}P-labeled probes of the cloned DNA A and B components of SLCV-10 (an isolate of SLCV-E), as described previously (8). Digestion with *Eco*RI or *Sac*I appeared to completely linearize the ds forms of CuLCV-AZ DNA A or B, respectively; therefore, these restriction endonucleases were used to clone the components of CuLCV-AZ. Total DNA samples (20 μg) extracted from CuLCV-AZ-infected plants were digested

TABLE 1. Experimental host range and symptom phenotypes of the Arizona isolate of *Cucurbit leaf curl virus* in representative test species[a]

Test species	No. infected/inoculated (% infection)	Symptom phenotype[b]	PCR results
Arabidopsis thaliana Columbia	0/18 (0)	NS	–
Bean (*Phaseolus vulgaris*)	17/18 (94)	Lc, Mo, St	+
Cantaloupe (*Cucumis melo*)	15/18 (83)	Chl, Lc, MiMo, St	+
Cotton (*Gossypium hirsutum*)	0/17 (0)	NS	–
Cucumber (*Cucumis pepo*)	14/16 (87)	Chl, Lc, MiMo, St	+
Datura stramonium	0/17 (0)	NS	–
Eggplant (*Solanum melongena*)	0/15 (0)	NS	–
Tobacco (*Nicotiana tabacum*)	12/12 (100)	Chl, Lc, St	+
Pumpkin (*Cucurbita maxima*)	18/18 (100)	Chls, Chl, Lc, MiMo, St	+
Soybean (*Glycine max*)	0/15 (0)	NS	–
Tomato (*Lycopersicon esculentum*)	0/16 (0)	NS	–
Watermelon (*Citrullus lanatus*)	17/17 (100)	Lc, Mo, St	+
Zucchini squash (*Cucurbita pepo*)	18/18 (100)	Lc, MiMo, St	+

[a] Data are pooled for three replicates of four to six plants per replicate. Plants were assayed by polymerase chain reaction (PCR) to detect begomovirus DNA in leaves that developed postinoculation.
[b] Chls = chlorotic spots, Chl = interveinal chlorosis, Lc = leaf curling, MiMo = mild foliar mosaic or mottle, Mo = severe mosaic, St = stunting, NS = no symptoms.

TABLE 2. Geminiviruses used in comparative sequence analyses with their respective GenBank accession numbers

Geminivirus	Acronym	GenBank accession number	
		A component	B component
Abutilon mosaic virus-West Indies	AbMV-WI	X15983	X15984
African cassava mosaic virus-Kenya	ACMV-KE	J02057	J02058
Ageratum yellow vein virus	AYVV	X74516	…
Angled luffa leaf curl virus	ALLCV	AF102276	…
Bean calico mosaic virus	BCaMV	AF110189	FA110190
Bean dwarf mosaic virus	BDMV	M88179	M88180
Bean golden mosaic virus-Brazil	BGMV-BR	M88686	M88687
Bean golden mosaic virus-Puerto Rico	BGMV-PR	M10070	U65530
Bean golden mosaic virus-Mexico	BGMV-MX	AF173555	AF173666
Cabbage leaf curl virus	CaLCV	U65529	U65530
Chayote mosaic virus	ChMV	AJ223191	…
Chino del tomate virus	CdTV	AF226664	U57458
Havana tomato virus	HTV	Y14874	Y14875
Indian tomato leaf curl virus	IToLCV	Z48182	…
Melon chlorotic leaf curl virus	MCLCV	AF325497	…
Okra leaf curl virus-Egypt	OLCV-EG	AY036010	…
Okra yellow vein mosaic virus-Pakistan	OYVMoV-PK	AJ002453	…
Papaya leaf curl virus	PaLCV	Y15934	…
Pepper huasteco yellow vein virus	PHYVV	X70418	X70419
Pepper golden mosaic virus-Tamaulipas[a]	PepGMV-TAM	U57457	…
Potato yellow mosaic virus	PYMV	NC001934	NC001935
Rhynchosia golden mosaic virus	RhGMV	AF239671	…
Sida golden mosaic virus-Costa Rica	SiGMV-CR	X99550	X99551
Sida golden mosaic virus-Honduras	SiGMV-HO	Y11099	AJ250731
Sida golden mosaic virus-FL	SiGMV-FL	U77964	U77963
Squash leaf curl virus-China	SLCV-CH	AB027465	…
Squash leaf curl virus-E	SLCV-E	M38183	M38182
Squash leaf curl virus-R	SLCV-R	AF421552	AF421553
Tomato golden mosaic virus	TGMV	K02029	K02030
Taino tomato mottle virus	TTMoV	AF012300	AF012301
Tomato mottle virus	ToMoV	L14460	L14461
Tomato rugose mosaic virus	TRMoV	NC002555	AF291706
Tomato yellow leaf curl virus-Israel	TYLCV-IL	X15656	…
Watermelon chlorotic stunt virus-Iran	WmCSV-IR	AJ245652	AJ245653
Watermelon chlorotic stunt virus-Sudan	WmCSV-SD	AJ245650	AJ245651

[a] Formerly *Texas pepper virus.*

with *Eco*RI or *Sac*I and linearized DNA (2.4 to 2.8 kbp) recovered from an agarose gel. Size-selected DNA was ligated to *Eco*RI- or *Sac*I-digested pGEM7zf+ (Promega Corp., Madison, WI) and used to transform *E. coli* DH5α.

Clones bearing recombinant plasmids containing approximately 2.6-kbp inserts of CuLCV-AZ DNA A or DNA B were identified by colony hybridization using ^{32}P-labeled inserts of cloned SLCV-10 DNA A or DNA B as probes. Two recombinant plasmids containing full-length inserts of CuLCV-AZ DNA A (pCuLCV-E19) or DNA B (pCuLCV-S8) were selected for sequence analysis after verifying that infections with these clones caused symptoms identical to those observed from field isolates. Automated sequencing was performed at the Genomics Analysis and Technology Core Facility, University of Arizona. Complete sequences of both strands were obtained by primer walking. Sequences were compiled using FAKtory, an online program developed by Biotechnology Computing Facility, University of Arizona, and searched for ORFs using Editseq program (DNASTAR, Madison, WI). The complete nucleotide sequences of CuLCV-AZ DNA A and B components have been deposited as GenBank Accession Nos. AF256200 and AF327559, respectively. The complete nucleotide sequences of cloned SLCV-R DNA A and DNA B were determined on both strands by primer walking and deposited as GenBank Accession Nos. AF421552 and AF421553, respectively. Comparison of the cloned A component sequence with the PCR-amplified sequence indicated only a single nucleotide difference between the two in the coat protein coding region at nucleotide 270. The cloned B component sequence also differed from the PCR-amplified sequence within the region that was compared by one nucleotide at position 2472.

Infectivity and experimental host range of cloned CuLCV-AZ DNA. Plasmids bearing tandemly repeated copies of the cloned inserts of CuLCV-AZ DNA A (pCuLCV-E19D) or DNA B (CuLCV-S8D) were constructed in pGEM7zf+ essentially as described (39), and delivered to test plants by biolistic inoculation (8) as a mixture containing 0.5 μg of each plasmid. Seedlings inoculated with tungsten microprojectiles and water were included as negative controls. Plants used for experimental host range studies included cultivated crop or weed species common to the Sonoran Desert agroecosystem, and *Arabidopsis thaliana* (L.) Heynh. (Table 1). Test plants examined here were *A. thaliana* 'Columbia', bean (*Phaseolus vulgaris* L. 'Red Kidney'), cotton (*Gossypium hirsute* L. 'Delta' and 'Pineland 90'), cucumber (*Cucumis pepo* L. 'Bush Champion'), *Datura stramonium* L., cantaloupe (*Cucumis melo* L. 'Hales Best Jumbo'), pumpkin (*Cucurbita maxima* Duchnes 'Big Max'), soybean (*Glycine max* L. Merr. 'Bush Shell'), tobacco (*Nicotiana tabacum* L. cv. 'Samsun'), tomato (*Solanum lycopersicon* L. Karsten 'Humaya'), zucchini squash (*Cucumis pepo* L. 'Fordhook'), and watermelon (*Citrullus lanatus* L. 'Charleston Grey'). Inoculated plants were maintained in a whitefly-free greenhouse and monitored for symptom development. Seedlings (4 to 6 each) of symptomatic and asymptomatic test plants inoculated with cloned CuLCV-AZ DNAs were confirmed as positive or negative for infection 14 to 21 days postinoculation (dpi) by PCR using universal, degenerate primers that amplify a begomovirus-diagnostic fragment of 576 bp (44).

Whitefly transmission of progeny virus derived from cloned CuLCV-AZ. To confirm that progeny from infectious A and B component clones of CuLCV-AZ were transmissible by the whitefly vector, pumpkin plants infected with cloned CuLCV-AZ were used as source plants for acquisition access feeding by the B biotype whitefly vector (15 to 20 whiteflies per plant). Parameters used for whitefly transmission were a 24-h acquisition access period (AAP), followed by a 48-h inoculation access period (IAP). Transmission experiments (two replicates each with five test plants) were carried out in an environmentally controlled growth chamber (27°C, 12-h day-night cycle). The IAP was terminated by killing whiteflies with nicotine sulfate and inoculated plants were maintained thereafter in an insect-free greenhouse (27°C) with ambient light. Experimental controls were plants exposed to a virus-free whitefly colony, and whitefly-free plants. The appearance of characteristic symptoms in pumpkin 7 to 8 dpi was considered proof of CuLCV-AZ infection, and that progeny from the cloned viral genome was whitefly transmissible. Plants whitefly-inoculated with CuLCV-AZ were assayed for infection 14 to 21 dpi by PCR (44).

Phylogenetic analysis of CuLCV sequences. Phylogenetic analyses comparing CuLCV-AZ DNA A or DNA B sequences to other geminivirus sequences available from GenBank (Table 2) were based on distance and maximum parsimony methods. Distance analyses of ORFs were performed using the Clustal method of MegAlign software (DNASTAR) for multiple sequence alignment. Trees were reconstructed using parsimony and maximum likelihood methods available in phylogenetic analysis using parsimony (PAUP), version 4.0.0b8. (42). A most parsimonious tree was sought using a heuristic search method, stepwise addition, and the tree-bisection-reconnection random branch-swapping options, for 500 bootstrap iterations (10 replicates/iteration). Bootstrap values were calculated using the >70% majority rule. Maximum likelihood trees were estimated using general time reversible (GTR) and gamma distribution options and subtree pruning-regrafting (SPR) branch-swapping algorithm of the heuristic search method, and default settings for all other parameters. These analyses assume no recombination or reassortment events in the history of the taxa examined. All trees were unrooted.

Intermolecular reassortment experiments. Pumpkin seedlings were inoculated with all possible pairs of A and B component clones of CuLCV-AZ, SLCV-E, and SLCV-R (Table 3). Six seedlings were inoculated with each combination in each of three replicates. Controls lacking DNA were included in all replicates. After inoculation, seedlings were placed in a growth

TABLE 3. Results of reassortment experiments in pumpkin plants inoculated with all possible combinations of cloned DNA A and DNA B components for the Arizona isolate of *Cucurbit leaf curl virus* (CuLCV-AZ), *Squash leaf curl virus* (SLCV)-E, and SLCV-R[a]

Reassortants	No. infected/inoculated	Symptom[b]	dpi[c]	PCR results
CuLCV DNA A + DNA B	18/18	Chls, Chl, Lc, MiMo, St	6	+
SLCV-R DNA A + DNA B	16/18	MiMo, Chls, Lc	6	+
SLCV-E DNA A + DNA B	18/18	GYMo	4	+
CuLCV DNA A + SLCV-R DNA B	0/18	NS	–	–
CuLCV DNA B + SLCV-R DNA A	5/18	Chls	6	+
CuLCV DNA A + SLCV-E DNA B	16/18	GYMo, Lc	5–6	+
CuLCV DNA B + SLCV-E DNA A	13/18	Chls, Lc	5–6	+
No DNA control	0/18	NS	NS	–

[a] Data shown are pooled for three replicates in which six plants were inoculated per replicate. Virus DNA presence or absence was confirmed by polymerase chain reaction amplification (PCR) of a 576-bp fragment of viral capsid protein.

[b] Chls = chlorotic spot, Chl = interveinal chlorosis, Lc = leaf curling, MiMo = mild foliar mosaic or mottle, Mo = severe mosaic, St = stunting, GYMo = green and yellow mottle, NS = no symptoms.

[c] dpi = days postinoculation.

chamber (27°C, 12-h day-night cycle) and monitored for symptom development. Total nucleic acids were extracted and assessed by PCR for presence or absence of begomovirus DNA as described.

RESULTS

Biological properties of CuLCV. During 1999 to 2001, samples from naturally infected plants were collected from fields in Arizona (AZ) and Texas (TX) in the United States, and Coahuilla (COA) in Mexico. Species in which CuLCV infection was initially detected by PCR and verified by sequencing of cloned *CP* PCR products included cucumber (COA, TX), honeydew melon (COA, TX), cantaloupe (COA, TX), pumpkin (AZ), watermelon (AZ), and zucchini squash (AZ). CuLCV sequences derived from natural infections were compared with the *CP* sequence for CuLCV-AZ obtained from the infectious clone. Nine field isolates of CuLCV shared 98.7 to 100% nucleotide sequence identity among themselves and cloned CuLCV-AZ (data not shown).

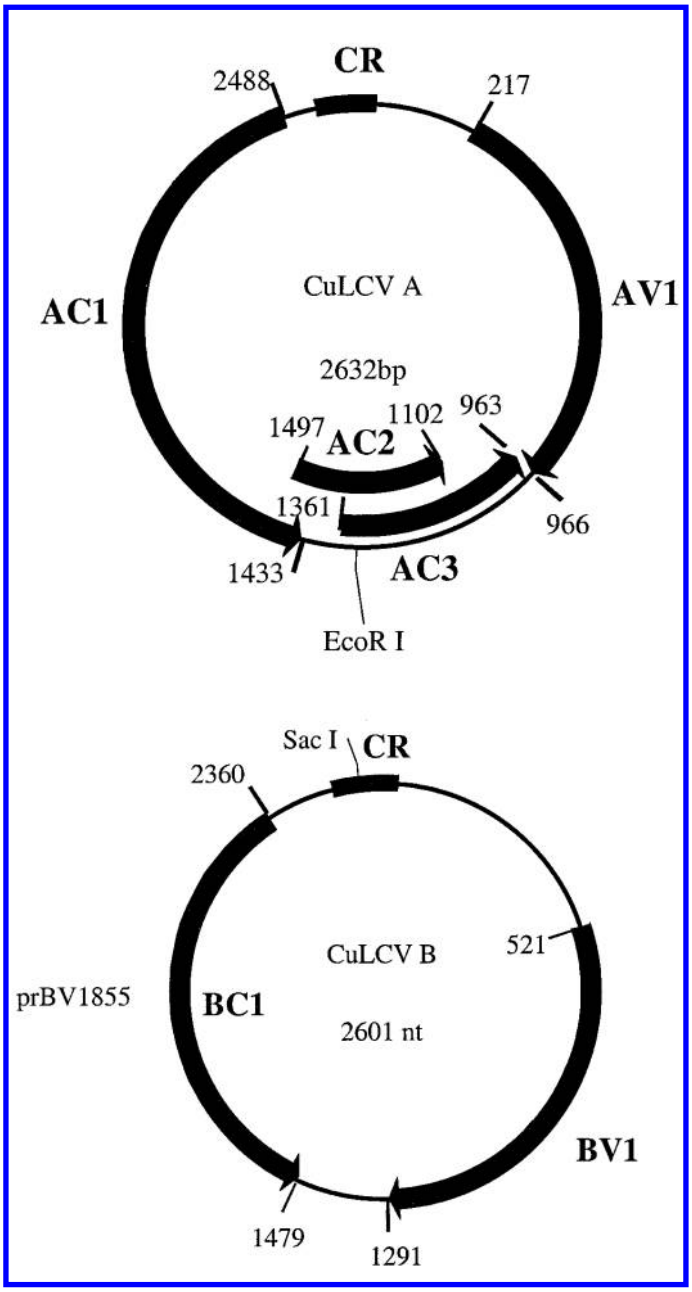

Fig. 2. Physical maps of the Arizona isolate of *Cucurbit leaf curl virus* (CuLCV-AZ) DNA A and B components. Arrows denote polarity and location of open reading frames (ORFs), arc denotes the location of common region (CR) sequences. Numbers indicate start and stop coordinates for each ORF. Nucleotide one is defined as the A residue immediately 3′ of the replication initiator protein nick site within the CR. *Eco*RI and *Sac*I cloning sites for CuLCV DNA A and DNA B, respectively, are indicated.

Experimental hosts of CuLCV-AZ reside in three plant families: *Cucurbitaceae*, *Fabaceae*, and *Solanaceae* (Table 1). Pumpkin, zucchini squash, cantaloupe, cucumber, common bean, watermelon, and tobacco were successfully infected with cloned CuLCV-AZ. Symptoms observed in natural host species were indistinguishable from those observed experimentally. Infection status assessed by PCR indicated that only symptomatic test plants were infected with CuLCV-AZ; no asymptomatic hosts were identified (data not shown). Nonhost species tested were *A. thaliana*, *D. stramonium*, eggplant, soybean, tomato, pepper, okra, and cotton.

The B biotype vector transmitted progeny virus of cloned CuLCV-AZ from source plants to test plants for three serial passages. Of 10 pumpkin plants whitefly inoculated with progeny virus, 9 developed typical CuLCV-AZ symptoms and were positive for begomovirus when assessed by PCR (data not shown).

Relationships of CuLCV-AZ to other begomoviruses. The complete nucleotide sequences of CuLCV-AZ DNA A and B were determined to be 2,632 and 2,601 nt, respectively. The CuLCV-AZ genome organization was typical of a bipartite begomovirus, encoding all ORFs conserved among bipartite begomoviruses (Fig. 2).

Trees reconstructed using maximum likelihood and parsimony methods were indistinguishable; hence, only maximum likelihood trees are presented. Phylogenetic relationships for CuLCV-AZ DNA A and DNA B with reference geminiviruses are shown in Figure 3A and B. Comparison of DNA A and monopartite viral genome sequences (Fig. 3A) indicated that CuLCV-AZ is a member of the SLCV cluster containing the New World begomoviruses *Bean calico mosaic virus* (BCaMV), *Cabbage leaf curl virus* (CaLCV), *Melon chlorotic leaf curl virus* (MCLCV), SLCV-E, SLCV-R, and *Pepper golden mosaic virus*-Tamaulipas (PepGMV-TAM).

Comparisons of nucleotide sequence identities of the CuLCV-AZ genome components with those of reference geminiviruses, calculated by PAUP (maximum likelihood analysis), are presented in Table 4. CuLCV-AZ DNA A shares 84.1, 82.8, and 80.2% nucleotide sequence identity with the A components of its three closest relatives, SLCV-R, SLCV-E, and BCaMV, respectively. CuLCV-AZ DNA A was distinct from other cucurbit-infecting begomoviruses from both the New World (MCLCV from Guatemala at 74.1% nucleotide identity) and the Old World (SLCV from China at 58.8% nucleotide identity, and WmCSV from Iran and Sudan at 57.5% nucleotide identity). The CuLCV DNA B shares 70.6, 68.7, and 66.7% nucleotide sequence identity with the B components of its three closest relatives, BCaMV, SLCV-R, and SLCV-E, respectively, while diverging from all other New World begomovirus B components included in this analysis by 35.9 to 40.3%.

Comparisons of nucleotide identities for individual ORFs of CuLCV-AZ with other viruses of the SLCV cluster are presented in Table 5. The high nucleotide sequence identity of CuLCV-AZ with a virus isolate from California designated *Cucurbit leaf crumple virus* (CuLCrV) by Guzman et al. (20) indicates that CuLCV-AZ and CuLCrV are isolates of the same species. Examination of the percent identity of individual ORFs of CuLCV-AZ with other begomoviruses shows that the AC1 and AV1 ORFs of CuLCV are most closely related to SLCV-R. Interestingly, the CuLCV-AZ ORFs AC2 and AC3 are more closely related to SLCV-E than to SLCV-R.

CR sequences of CuLCV-AZ DNA A and B were highly conserved (99.4%), confirming that the genome components cloned represent a cognate pair. Comparison of nucleotide

sequence identities among the CR of CuLCV-AZ and its closest relatives (also members of the SLCV cluster) shared the following percent identities: BCaMV (76.9%), CaLCV (67.3%), MCLCV (AF325497) (72.2%), SLCV-E (64.2%), and SLCV-R (76.9%), with variation most notable at sites in the CR not shown to have crucial roles in *ori* function or gene expression. The begomovirus *cis*-acting element conferring replication specificity (GGTGTC-CTGGTG) (8) was identical among all components of CuLCV-AZ, SLCV-E, SLCV-R, and BCaMV.

Intermolecular reassortment experiments. Systemic symptoms developed in pumpkin plants biolistically inoculated with both possible reassortants of CuLCV-AZ and SLCV-E. The CuLCV-AZ A/SLCV-E B reassortant caused systemic symptoms in 16 of 18 plants 5 to 6 dpi. The reciprocal combination, SLCV-E A/CuLCV-AZ B was nearly as infectious, with 13 of 18 plants developing symptoms 5 to 6 dpi (Table 3). Wild-type SLCV-E symptoms in pumpkin were characterized by a green and yellow mottle, severe leaf curling, and shortened internodes 4 to 5 dpi. Wild-type CuLCV-AZ symptoms are characterized by a foliar mosaic with chlorotic spots, moderate leaf curling, and shortened internodes. The reassortants exhibited symptoms that were distinguishable from either parent virus (Fig. 1). The CuLCV-AZ A/SLCV-E B reassortant caused green and yellow mottle and leaf curl symptoms in pumpkin 5 to 6 dpi, while the reciprocal reassortant (CuLCV-AZ B/SLCV-E A) caused chlorotic spot and moderate leaf curl symptoms that also developed 5 to 6 dpi (Table 3).

Inoculation of pumpkin plants with reassortants of SLCV-R and CuLCV-AZ resulted in systemic infection only when SLCV-R DNA A was coinoculated with CuLCV-AZ DNA B. Furthermore, the infection efficiency of this reassortant was lower (3 of 10 plants) than that observed for SLCV-E/CuLCV-AZ reassortants. Mosaic, moderate foliar curling, and shortened internode symptoms was observed in pumpkin infected with SLCV-R. The reassortant SLCV-R A/CuLCV-AZ B produced chlorotic spot and leaf curl symptoms, which first appeared at 6 dpi but were milder than symptoms produced by SLCV-R, SLCV-E, CuLCV-AZ, or SLCV-E/CuLCV-AZ reassortants.

All plants that exhibited systemic symptoms after inoculation with cognate or reassorted viral components were positive for presence of begomovirus DNA, based on detection of a 576-bp diagnostic begomovirus *CP* gene fragment, whereas all asymptomatic plants tested negative (Table 3).

DISCUSSION

CuLCV is a new begomovirus species of the SLCV cluster. CuLCV is unique in that it is the first highly virulent begomoviral pathogen of cucumber and melons, as well as all *Cucurbita* spp. grown in the region. In contrast, although SLCV-E is highly virulent in gourd, pumpkin, squash, and watermelon, it causes mild to no discernable symptoms in cucumber and melon (6,7,27,37). SCLV-R infects pumpkin and squash, but not cucumber, melon, or watermelon (12,27).

Phylogenetic analysis indicated that CuLCV is a distinct viral species sharing less than 85% (DNA A) and 71% (DNA B) nucleotide identity with all other begomoviral species described to date (31). These values are well below the threshold established by the International Committee for the Taxonomy of Viruses

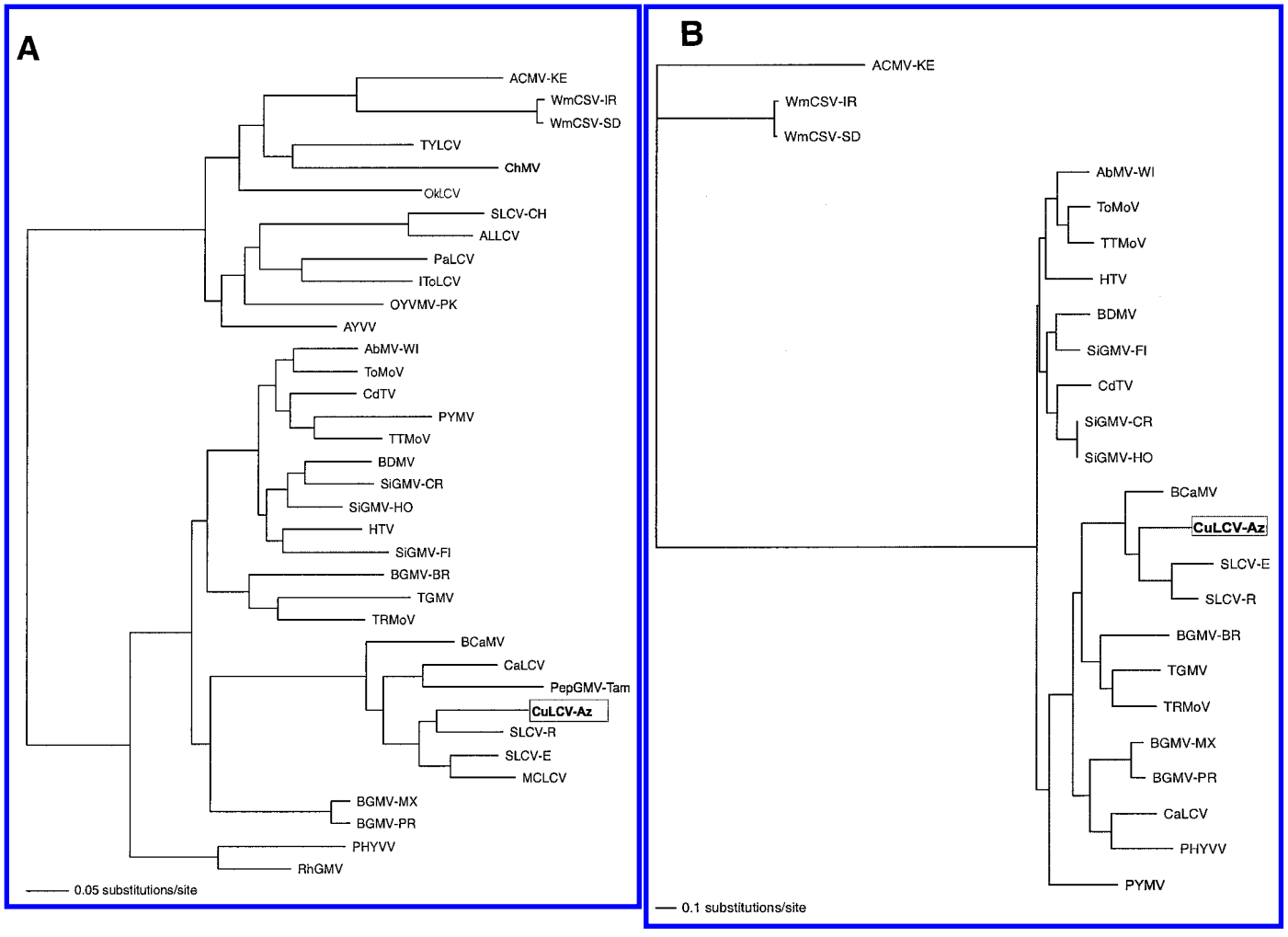

Fig. 3. Maximum likelihood tree showing predicted relationships for the **A,** DNA A or **B,** DNA B component nucleotide sequence with other well-studied geminiviruses. The trees presented are unrooted.

(ICTV) for distinct begomoviral species. We further note that SLCV-E and SLCV-R also share less than 90% nucleotide identity and may eventually be considered distinct species as well. Nonetheless, CuLCV, SLCV-E, and SLCV-R share a most recent common ancestor with each other and a fourth cucurbit-infecting begomovirus (MCLCV) recently described from Guatemala (5). Additional members of the SLCV cluster include three noncucurbit-infecting viruses: BCaMV, CaLCV, and PepGMV. The CuLCV-AZ A and B components occupy similar relative positions within the SLCV cluster on both trees, suggesting that both CuLCV-AZ genomic components share a congruent evolutionary background. In contrast, the CaLCV components cluster differently (8) (Fig. 3), suggesting that CaLCV may have originated as a reassortant.

Reassortment experiments indicated that the CuLCV-AZ A and B components form viable reassortants in pumpkin with the A and B components of SLCV-E. Additionally, one SLCV-R/CuLCV-AZ reassortant was viable, albeit less infectious on pumpkin. Reassortants among SLCV-E and SLCV-R also produce viable reassortants in some hosts (27). All three viruses are sympatric (i.e., they occur in the same geographic region and have overlapping host ranges). Thus, these three viruses have a reasonable chance of encountering one another in nature. In fact, a natural mixed infection of SLCV-E and SLCV-R was the original source material from which these two viruses were cloned (27). Collectively, these results indicate the potential for cucurbit-infecting viruses of the SLCV cluster to form reassortants with distinct biological properties. However, in our limited survey, CuLCV was not found in a mixed infection with SLCV-E or SLCV-R.

At the same time that we were examining CuLCV isolates from Arizona, Texas, and Mexico, a similar begomoviral genotype causing curling and crumpling of leaves was recovered from watermelon in the Imperial Valley of California (20). Guzman et al. (20) proposed the nomenclature *Cucurbit leaf crumple virus* (CuLCrV) for this California isolate. Because both virus isolates are clearly of the same species (Table 5), a single name must be designated. Isolates of the virus examined by us and the single isolate examined Guzman et al. (20) exhibited leaf curl symptoms in all cucurbit hosts observed under natural and experimental conditions. Leaf crumple symptoms were associated with the Californian isolate (20). We have never observed leaf crumple symptoms in plants inoculated with CuLCV-AZ or in natural infections of a variety of cucurbit species throughout Arizona, Texas, and northern Mexico. Thus, crumple symptoms are atypical of the CuLCV-AZ isolate. Therefore, we propose that the virus species in question be assigned the nomenclature *Cucurbit leaf curl virus* rather than *Cucurbit leaf crumple virus.* Ultimately, nomenclature for this begomovirus species will be determined by the ICTV.

Has recombination occurred among species of the SLCV cluster? Recombination among geminiviruses has contributed to the evolution of novel genomes. The clearest examples of ancient recombination among geminivirus species are *Horseradish curly top virus* (HrCTV) and *Tomato pseudo curly top virus* (TPCTV). In the case of HrCTV, it has been proposed that an ancestor of the SLCV cluster recombined with an ancestor of the curtoviruses (25). In the case of TPCTV, a begomovirus ancestor apparently recombined with an unknown treehopper-transmitted ancestor (1). An example of a recent recombination event may be found in *East African cassava mosaic virus* from Uganda, in which a portion of the *CP* gene has been exchanged among closely related cassava-infecting begomoviruses (45). Experimentally, recombination has been demonstrated to occur between two bipartite begomovirus species such that the CR of a noncognate DNA B is replaced with cognate sequences derived from the DNA A (21). We note that ORFs AC2 and AC3 of CuLCV-AZ share slightly greater nucleotide sequence identity with SLCV-E, whereas CuLCV-AZ ORFs AC1 and AV1 share somewhat greater sequence identity with SLCV-R (Table 5). This could be interpreted as evidence of recombination in which two of these viruses recombined to produce a hybrid DNA A of the third. The program developed by Martin and Rybicki (30) identifies the AC2-AC3 region as a site of possible recombination (data not shown). However, a manual examination of all polymorphic sites for which two of the three viruses are identical reveal no extended region of DNA A lacking shared polymorphisms of a particular pairing. This observation does not preclude recombination; however, it does indicate that, if recombination had occurred, many substitutions have subsequently accumulated. It also is possible that during divergence from a common ancestor, the three genomes were subjected to different selection pressures imposed, for example, by distinct wild hosts prior to emergence into cultivated cucurbits. Thus, we find the evidence for recombination inconclusive in this case.

TABLE 4. Percent nucleotide identities, inferred from maximum likelihood distance, of the *Cucurbit leaf curl virus* DNA A and B with those of well-studied begomoviruses

	Nucleotide identity (%)[a]	
Virus	DNA A	DNA B
Abutilon mosaic virus-West Indies	69.2	61.7
Beet curly top virus	53.5	NA[b]
Bean calico mosaic virus	80.2	70.6
Bean dwarf mosaic virus	69.2	63.9
Bean golden mosaic virus-Brazil	70.0	62.7
Bean golden mosaic virus-Puerto Rico	69.7	63.4
Cabbage leaf curl virus	78.9	62.6
Chino del tomate virus	70.2	62.2
Melon chlorotic leaf curl virus	74.1	NA
Pepper huasteco yellow vein virus	64.6	61.0
Potato yellow mosaic virus	70.3	59.7
Sida golden mosaic virus-Costa Rica	69.8	64.9
Squash leaf curl virus-China	58.9	NA
Squash leaf curl virus-E	82.8	66.7
Squash leaf curl virus-R	84.1	68.7
Tomato golden mosaic virus	70.6	64.1
Tomato mottle virus	69.4	62.3
Pepper golden mosaic virus-Tamaulipas	77.2	NA
Tomato yellow leaf curl virus-Israel[b]	59.5	NA
Watermelon chlorotic stunt virus-Iran	57.5	44.6
Watermelon chlorotic stunt virus-Sudan	57.5	43.8

[a] Distances were calculated by PAUP for all pairwise comparisons.
[b] DNA B not available (NA), or monopartite genomes lacking DNA B.

TABLE 5. Percent nucleotide and amino acid similarities, predicted by the Clustal option of MegAlign, for open reading frames of the Arizona isolate of *Cucurbit leaf curl virus* (CuLCV-AZ) compared with the most closely related begomoviruses or isolates described to date

	Nucleotide/amino acid[a]						
Virus	AV1	AC1	AC2	AC3	AC4	BV1	BC1
Bean calico mosaic virus	81.7/90.4	78.7/84.0	77.3/69.7	78.9/78.2	82.4/68.2	64.9/69.6	71.7/77.5
Cabbage leaf curl virus	82.3/91.2	75.7/79.1	71.3/67.7	70.7/66.9	82.4/66.4	63.0/65.4	66.1/73.8
Melon chlorotic leaf curl virus	84.4/91.2	78.7/79.0	77.0/69.7	78.7/76.7	83.1/85.2	NA	NA
Cucurbit leaf crumple virus	99.2/99.2	99.6/99.7	99.7/99.2	99.2/98.5	100/100	96.9/96.9	98.5/98.6
Squash leaf curl virus-E	85.1/90.8	80.4/81.9	76.5/71.2	78.9/76.7	84.4/69.7	63.2/61.5	75.784.0
Squash leaf curl virus-R	88.8/92.4	85.1/86.9	71.8/68.5	75.2/69.9	92.3/70.5	64.7/66.7	74.6/83.7

[a] NA = not available.

Emergence of begomoviruses in the Sonoran Desert agroecosystem. The rapid and widespread appearance of CuLCV in cucurbit cultivation represents the most recent example of begomovirus emergence in the American Southwest and northern Mexico. Previous examples of begomovirus emergence in the region include SLCV-E and SLCV-R (11,16,27) in cucurbits, BCaMV in bean (8), PepGMV and *Pepper huasteco yellow vein virus* in peppers (39,43), and *Chino del tomate virus* (9) and *Tomato leaf curl Sinaloa virus* (22) in tomato. Emergent begomoviruses become noticed primarily due to the new economic consequences of the diseases they cause in cultivated crops and to altered host range or virulence. During the last half century, irrigated agriculture has intensified in the Sonoran Desert and adjacent agroecosystems eastward to the Rio Grande Valley of Texas, and throughout northern and western Mexico. Important consequences are increased host plant density and extension of growing season, and a concomitant increase in whitefly population densities. The natural communities of the Sonoran Desert are sparse, such that both host and vector population densities are naturally low. Under these natural conditions, reduced virulence would likely be favored, because the probability of transmission from plant to plant is low. However, under the present conditions of intensive agriculture, virus–host combinations that result in severe disease are both possible and sustainable as the probability of plant-to-plant transmission is dramatically increased with present practices. Another factor probably involved in the emergence of new begomoviruses in the Sonoran agroecosystem is the displacement of the A biotype of *B. tabaci* by the polyphagous B biotype (13). Will there be future episodes of begomovirus emergence? Given the previous history of begomovirus emergence in the region, and the demonstrated ability of some of these viruses to produce viable reassortants, it seems quite likely that the next episode of emergence is only a matter of time.

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