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ORIGINAL ARTICLE

Evidence of local evolution of tomato-infecting begomovirus species in West Africa: characterization of tomato leaf curl Mali virus and tomato yellow leaf crumple virus from Mali

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Abstract Tomato yellow leaf curl (TYLC) and tomato leaf curl (ToLC) diseases are serious constraints to tomato production in Mali and other countries in West Africa. In 2003 and 2004, samples of tomato showing virus-like symptoms were collected during a survey of tomato virus diseases in Mali. Three predominant symptom phenotypes were observed: (1) TYLC/ToLC (stunted upright growth and upcurled leaves with interveinal yellowing and vein purpling), (2) yellow leaf crumple and (3) broccoli or bonsai (severe stunting and distorted growth). Squash blot (SB) hybridization with a general begomovirus probe and/or SB/PCR analyses revealed begomovirus infection in plants with each of these symptom phenotypes and no evidence of phytoplasma infection. Sequence analysis of PCR-amplified begomovirus fragments revealed two putative new begomovirus species associated with the TYLC/ToLC and yellow leaf crumple symptom phenotypes, respectively. Full-length clones of these begomoviruses were obtained using PCR and overlapping primers. When introduced into *N. benthamiana* and tomato plants, these clones induced upward leaf curling and crumpling (the TYLC/ToLC-associated begomovirus) or downward leaf curl/yellow mottle (yellow leaf crumple-associated begomovirus) symptoms. Thus, these begomoviruses were named tomato leaf curl Mali virus (ToLCMLV) and tomato yellow leaf crumple virus (ToYLCrV). The genome organization of both viruses was similar to those of other monopartite begomoviruses. ToLCMLV and ToYLCrV were most closely related to each other and to tobacco leaf curl Zimbabwe virus (TbLCZV-[ZW]) and tomato curly stunt virus from South Africa (ToCSV-ZA). Thus, these likely represent tomato-infecting begomoviruses that evolved from indigenous begomoviruses on the African continent. Mixed infections of ToLCMLV and ToYLCrV in *N. benthamiana* and tomato plants resulted in more severe symptoms than in plants infected with either virus alone, suggesting a synergistic interaction. Agroinoculation experiments indicated that both viruses induced symptomatic infections in tomato and tobacco, whereas neither virus induced disease symptoms in pepper, common bean, small sugar pumpkin, African eggplant, or *Arabidopsis*. Virus-specific PCR primers were developed for detection of ToLCMLV and ToYLCrV and will be used to further investigate the distribution and host range of these viruses.

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Introduction

Tomato yellow leaf curl (TYLC) and tomato leaf curl (ToLC) diseases are among the most devastating diseases of cultivated tomato (*Solanum lycopersicum*) in tropical and subtropical regions of the world [5, 34]. Infected plants are severely stunted and have a compact growth habit with bushy tops; severely infected plants are often referred to as broccoli or bonsai plants. Leaves show upward or downward curling with varying degrees of crumpling,

interveinal chlorosis and light green/yellow mottling. Infected plants may exhibit flower abortion and reduced fruit production, and yield losses can reach as high as 100%, particularly when plants are infected early in development.

The TYLC/ToLC diseases are caused by members of a complex of whitefly (*Bemisia tabaci* Genn.)-transmitted begomovirus species (genus *Begomovirus*, family *Geminiviridae*). Among the first of these to be described were tomato yellow leaf curl virus from Israel (TYLCV-[IL]; [21]) and tomato leaf curl virus from Australia (ToLCV-[AU]; [7]). Subsequently, molecular characterization of begomoviruses associated with TYLC/ToLC disease revealed that a complex of genetically distinct begomoviruses causes these disease symptoms in geographically distinct regions of the world [9, 13, 24, 34]. This is consistent with a scenario in which progenitor begomovirus(es), widely distributed in weed (or other) reservoirs, have independently evolved into tomato-infecting species whose members cause similar disease symptoms [28]. This process has been facilitated by the worldwide cultivation of the New World tomato host and the emergence of the whitefly vector. More recently, TYLCV-[IL] was introduced into the New World from the Old World, via long-distance transport of infected tomato transplants [20, 25, 30]. Finally, in some parts of the Old World, satellite DNA molecules, referred to as DNA-β, are associated with monopartite begomoviruses causing TYLC/ToLC and may influence symptom development [15, 19, 36].

Most of the begomoviruses causing TYLC/ToLC have a monopartite genome ($\sim$3.0 kb), with an organization analogous to that of the DNA-A component of the bipartite begomoviruses [12, 21, 28]. The genome organization is conserved, consistent with their evolution from a common ancestor. There are six genes: two overlapping on the virion sense (+) strand (V1 [precoat] and V2 [capsid protein; CP]), and four overlapping on the complementary sense (−) strand (C1 [replication-associated protein; Rep], C2, C3, and C4). For most of the tomato-infecting begomoviruses, tomato is the host plant to which these viruses are best adapted (i.e., replicates and spreads to the highest levels and induces characteristic disease symptoms). However, other plant species may also be infected, sometimes without symptoms [1, 4, 30]. In some regions, these reservoir hosts have been hypothesized to serve as "bridge" hosts in the absence of the tomato.

In Mali and other countries of West Africa, tomato is a very important cash vegetable crop, and TYLC/ToLC disease has become a major constraint, limiting tomato cultivation in certain areas. These diseases are particularly severe during the dry season (e.g., September–May in Mali), when whitefly vector populations are highest. The disease symptoms collectively referred to as TYLC/ToLC in West Africa, e.g., leaf curl/yellow leaf curl, yellow mottle and crumpling and severely stunted and distorted growth, are suggestive of the involvement of one or more begomoviruses. Evidence for this includes the detection of begomovirus infection in tomato with TYLC/ToLC symptoms in Burkina Faso by ELISA with monoclonal antibodies raised against the CP of African cassava mosaic virus [16].

This study was initiated to characterize begomoviruses associated with TYLC/ToLC symptoms in Mali and other countries in West Africa and to gain insight into the biology of these viruses, with the long-term goal of developing disease management strategies. In this report, we describe the cloning and characterization of two new begomoviruses involved in tomato virus disease epidemics in Mali: tomato leaf curl Mali virus (ToLCMLV) and tomato yellow leaf crumple virus (ToYLCrV). Agroinoculation systems were developed to facilitate host range determination and germplasm screening, and virus-specific PCR primers were developed for rapid detection of these viruses.

Materials and methods

Surveys of tomato-growing regions in Mali (Baguineda, Cinzana, Koulikoro, Sikasso, and Sotuba) were conducted in January 2003 and March 2004. Representative farmer fields and/or experimental plots were surveyed for symptoms of virus infection, and leaf samples were collected from tomato, pepper (*Capsicum annuum*) and other crop and weed plants that were showing symptoms of virus infection (stunted and distorted growth and leaf deformation and abnormal coloration). The majority of samples were collected from in or around farmer fields.

Squash blot (SB)-polymerase chain reaction (PCR) and cloning and sequencing of PCR-amplified DNA fragments

Leaf discs were taken from leaf samples and squashed in triplicate onto nylon membranes (Nytran) in Mali as previously described [10]. These membranes were returned to UC Davis where they were either hybridized with a general begomovirus probe or used for extraction of DNA. DNA was extracted from membranes using a modification of the Dellaporta method [6]. Here, the membrane piece with the tissue squash was thoroughly ground in 500 µl of grinding buffer, and then removed from the tube. The resulting

suspension was used for the DNA extraction procedure [6, 27]. The resulting pellet was suspended in 50 µl of ddH_2O, and a 5-µl aliquot of this DNA solution was used in the PCR.

For samples collected in 2003, PCR was performed with the primer pair PTYv787 and PTYc1121, which was designed from the TYLCV CP sequence and directs the amplification of an ~350-bp DNA fragment [30]. For samples collected in 2004, PCR was performed with the degenerate primer pair AV494 and AC1048, which targets the core region of the CP gene and directs the amplification of an ~550-bp DNA fragment [35]. To detect phytoplasma infection, PCR was conducted with the degenerate primer pair P1/Tint, which was designed from a conserved phytoplasma rDNA sequence and directs the amplification of an ~1.6-kb DNA fragment [33]. A DNA extract prepared from tomato leaves with big bud disease, collected in California, was used as the positive control (i.e., a known phytoplasma-infected plant).

PCR-amplified DNA fragments were cloned and sequenced as previously described [32]. The BLAST program was used to compare sequences with those in the GenBank, and sequence comparisons and alignments were performed with the Invitrogen Vector NTI (10.0.1) software.

Generating full-length begomovirus clones and particle bombardment inoculation

Full-length geminivirus clones were generated by PCR with overlapping primers [23]. Two pairs of overlapping primers were designed from partial begomovirus sequences obtained from PCR-amplified DNA fragments: B10 (B10V1, 5′-ATG TG**GGATCC**ATTATTGAATGAA-3′ and B10C1, 5′-ATA AT**GGATCC**CACATAGTGCAAC-3′) and K9 (K9V1, 5′-ATGTG**GGATCC**ACTTTTGAACGAG-3′ and K9C2, 5′-AAAGT**GGATCC**CACATTATGGACA-3′). The *Bam*HI site used for cloning is shown in bold. Recombinant plasmids having the expected size insert (~2.8 kb) were identified by restriction enzyme digestion analysis and sequencing.

Infectivity of full-length begomovirus clones was determined by particle bombardment of excised linear double-stranded monomers into *N. benthamiana* (four to six-leaf stage), tomato (cv. Glamour, two-true-leaf stage) and pepper (cv. Golden Cal Wonder, two-true-leaf stage) seedlings as previously described [22]. Gold particles alone were used as the negative control. After bombardment, seedlings were planted in soil and maintained in a locked growth chamber. Symptom development was recorded 14–28 days post-bombardment (dpb). In selected plants, the presence of viral DNA was confirmed by PCR.

Production of multimeric clones

Recombinant plasmids containing multimeric copies (1.5-mers) of infectious clones were generated as described by Paplomatas et al. [22]. Here, an ~1.5-kb DNA fragment containing the intergenic region (IR) was released from a recombinant plasmid with a monomeric clone by double-digestion with *Bam*HI and either *Xba*I or *Nco*I. These fragments were purified and individually cloned into pBluescript SKII digested with *Bam*HI and *Xba*I, or pSL1180 digested with *Bam*HI and *Nco*I, to generate 0.5-mer clones. The full-length monomer, released by digestion with *Bam*HI, was purified and ligated into the appropriate *Bam*HI-digested 0.5-mer to generate a 1.5-mer construct.

Development of agroinoculation systems and host range determination

Multimeric (1.5-mer) inserts were released from recombinant plasmids by digestion with *Pvu*II, and individually cloned into the binary vector, pGA482, digested with *Hpa*I. *Agrobacterium tumefaciens* (strain C58) was transformed by electroporation with the resulting recombinant plasmids. Transformants were identified based on growth on media supplemented with kanamycin (50 µg/ml) and PCR analysis. *Agrobacterium* strains containing binary plasmids with begomovirus clones were used for agroinoculation experiments, and a strain containing the 'empty' vector pGA482 was used as the negative control.

Host range determination was performed using the agroinoculation system with tomato (cvs. Glamour and Roma), pepper (cvs. Cayenne Long and Carolina), common bean (*Phaseolus vulgaris* cv. Topcrop), small sugar pumpkin (*Cucurbita pepo*), eggplant (*Solanum melongena* cv. Black beauty), African eggplant (*Solanum aethiopicum* cv. Soxna), *Arabidopsis thaliana* (ecotype Columbia), tobacco (*Nicotiana tabacum* cv. Havana) and *N. benthamiana*. Seedlings were inoculated with *Agrobacterium* cell suspensions (OD_{600} = ~1.0) by needle puncture as described by Hou et al. [14]. For co-inoculations, *Agrobacterium* cultures were mixed in equal volumes. Agroinoculated plants were maintained in a locked growth chamber, and symptoms were recorded 21–28 days after inoculation. In plants that did not develop symptoms, PCR was used to detect the presence of viral DNA.

DNA sequencing and phylogenetic analysis

Sequencing of full-length infectious clones and DNA sequence comparisons were preformed as previously

described [32]. Nucleotide sequence identities and amino acid sequence identities and similarities were determined using Invitrogen Vector NTI (10.0.1) software. Total nucleotide sequences and those of individual open reading frames (ORFs) and the IR were aligned with those of other begomoviruses with CLUSTAL W (1.83). Phylogenetic trees were generated using the neighbor-joining method of MEGA version 3.0 with 1,000 bootstrap replications [17].

Generation of ToLCMLV- and ToYLCrV-specific primers

Divergent regions of the viral genome were identified based on results of sequence alignments. A virus-specific virion-sense primer was designed, which was paired with a common complementary primer designed from the sequence of the conserved 3′ region of the *CP* gene (1000c; 5′-ATGMGTACATGCCATATACA-3′). Gradient PCR analyses were used to optimize the PCR conditions for each primer pair.

Results

Survey for virus-like symptoms in tomato and pepper and detection of begomovirus and phytoplasma DNA

In January 2003 and March 2004, surveys for virus-like symptoms in tomato, pepper and other crops and weeds plants were conducted in five locations in Mali: Baguineda, Cinzana, Koulikoro, Sikasso, and Sotuba. All fields surveyed had plants with virus-like symptoms, and disease incidence was low moderate (20–30%) in Koulikoro; moderate (30–50%) in Cinzana, Sikasso, and Sotuba; and high (50–100%) in Baguineda. Tomato plants showed three predominant symptom phenotypes: (1) typical TYLC/ToLC symptoms (stunted and upright growth, and upcurled leaves with crumpling, interveinal light green/yellowing and vein clearing and purpling), (2) distorted growth and yellow leaf mottling and crumpling, and (3) broccoli or bonsai symptoms (severe stunting and distortion, with extensive shoot proliferation and extremely stunted, yellowed and underdeveloped leaves). The TYLC/ToLC symptom type was most common and was observed during both surveys in most fields from all five locations. The yellow mottle leaf crumple symptom type was observed in 2004 in Baguineda, Koulikoro, Sikasso, and Sotuba, but at lower incidences than TYLC/ToLC symptoms. The broccoli phenotype was observed at all locations in both surveys, but at lower incidences than TYLC/ToLC symptoms.

For the 2003 and 2004 surveys, leaf samples were collected from plants with representative disease symptoms. A total of 143 samples were squashed onto nylon membranes in Mali, and these included 97 tomato, 22 African eggplant, 16 pepper, 4 common bean, 2 weeds and one each of cassava and eggplant. Squash blot hybridization analysis with the general begomovirus DNA probe revealed begomovirus infection in tomatoes with TYLC/ToLC (51/53 samples), yellow mottle leaf crumple symptoms (13/13 samples), and the broccoli symptom phenotype (14/14 samples); the other 17 tomato samples had questionable symptoms and were negative. Begomovirus infection also was detected in peppers with leaf curl and yellow vein symptoms from Koulikoro (2003) and Baguineda (2004), but not in peppers from Koulikoro (2004) or Sikasso (2004). In addition, begomovirus infection was detected in symptomatic African eggplant from Baguineda (2004) and in the single cassava sample with mosaic. Begomovirus infection was not detected in most African eggplant samples (18/22 from Baguineda, Koulikoro, Sikasso and Cinzana), or in the common bean, eggplant or weed samples. These results indicated that begomovirus infection was associated with the three predominant symptom phenotypes in tomato and, to a lesser extent, to the virus-like symptoms observed in pepper.

Ten representative squash-blot-positive samples, representing the symptom diversity from the 2003 survey, were tested with SB/PCR and the TYLCV CP primer pair. These included tomato samples with TYLC/ToLC from Baguineda (2 samples), Cinzana (1 sample), Koulikoro (2 samples), and Sotuba (2 samples); tomatoes with the broccoli phenotype from Cinzana (1 sample); and peppers with upcurling and yellow vein symptoms from Koulikoro (2 samples). The expected-size ~350 bp DNA fragment was amplified from extracts prepared from all ten samples, and not from an extract from uninfected tomato leaves. No DNA-B component was detected in these samples based upon PCR with degenerate DNA-B primers (2040v and 970c; [27], whereas the expected-size 1.6 kb fragment was amplified from a DNA extract prepared from *N. benthamiana* leaves infected with the bipartite begomovirus bean dwarf mosaic virus (BDMV). These results suggest that these tomato and pepper plants were infected with a monopartite begomovirus(es).

Because of the similarity of the distorted growth and chlorosis and vein purpling symptoms in some samples with phytoplasma infection, SB/PCR was also conducted with degenerate phytoplasma primers. No DNA fragments were amplified from the DNA extracts from the ten tomato/pepper samples from Mali, nor from an extract from uninfected tomato leaves. The expected-size ~1.6-kb fragment was amplified from a DNA extract prepared from leaves of tomatoes with big bud symptoms. These results

suggest that phytoplasma infection was not associated with these virus-like disease symptoms in Mali.

For the 2004 survey, 14 squash-blot-positive tomato samples were selected to represent the observed symptom diversity and tested with SB-PCR and the degenerate CP primer pair [35]. These samples included TYLC/ToLC symptoms from Baguineda (2 samples), Cinzana, Koulikoro, Sikasso, and Sotuba (2 samples); yellow mottle leaf crumple symptoms from Baguineda, Koulikoro, Sikasso, and Sotuba; and broccoli symptoms from Baguineda (2 samples) and Cinzana. The expected-size ~550-bp DNA fragment was amplified from extracts prepared from all 14 samples (data not shown), consistent with begomovirus infection in all of these samples.

Cloning and sequencing of PCR-amplified DNA fragments

To determine the nature of the begomovirus(es) infecting the 10 samples from the 2003 survey, the ~350-bp DNA fragments were sequenced. Sequence analyses revealed that: (1) these were begomovirus CP DNA fragments, (2) the sequences were only ~80% identical to the corresponding sequence of TYLCV-[IL] and (3) the sequences were not identical (data not shown). These CP sequences were then aligned, the conserved regions were identified, and a primer pair was designed to direct the amplification of the remainder of the viral DNA component. When used in the SB-PCR, these primers (174v [5′-GTTAGTCTGT ATATGGCATGTA-3′] and 71c [5′-ACTAATGCCTGT CTCTCTTCA-3′]) directed the amplification of an ~2.5-kb DNA fragment from all ten samples. The ends of each of these fragments were sequenced, and sequence comparisons revealed the presence of two distinct begomoviruses (data not shown). One of these viruses (sequences > 95% identical) was detected in seven of the eight samples with TYLC/ToLC symptoms and in the sample with the broccoli symptoms. The sequence of this begomovirus also was nearly identical (98%) to a partial sequence of a begomovirus associated with leaf curl disease of tomato in Senegal. This begomovirus will be referred to as TYLC/ToLC-associated begomovirus.

The other begomovirus was detected in the two samples of peppers with leaf curl and yellow vein symptoms and the other tomato sample with TYLC/ToLC; all of these samples were from Koulikoro. The sequence of this begomovirus was most similar (79%) to that of the TYLC/ToLC-associated begomovirus, and it will be referred to as the pepper-associated begomovirus.

Similarly, the ~550-bp fragments amplified from the 2004 samples were sequenced. The sequences from four TYLC/ToLC samples and one sample with broccoli symptoms were 95–98% identical to that of the TYLC/ToLC-associated begomovirus; the sequences of three of the yellow mottle leaf crumple, two TYLC/ToLC and two samples with broccoli symptoms were 96–98% identical to the pepper-associated begomovirus; and sequences of one yellow mottle leaf crumple and one TYLC/ToLC sample were 98 and 96% identical, respectively, to TYLCV-IL. Together, the results for the 2003 and 2004 surveys indicate that at least three begomoviruses are associated with these disease symptoms in tomato in Mali.

Generation of full-length infectious clones of members of two putative new begomovirus species from West Africa

The partial sequences of the TYLC/ToLC-associated and pepper-associated begomoviruses were used to design PCR primers overlapping a *Bam*HI site in the C1 ORF for amplification of the full-length DNA component of these new monopartite begomoviruses. When used in the SB/PCR with the DNA extract of a TYLC/ToLC sample from Baguineda (sample B10), the TYLC/ToLC-associated begomovirus primers (B10V1 and B10C1) directed the amplification of an ~2.8-kb DNA fragment. Similarly, the pepper-associated begomovirus primers (K9V1/K9C2) directed the amplication of an ~2.8-kb DNA fragment from a DNA extract of pepper leaves with leaf curl and yellow vein symptoms from Koulikoro (sample K9). These fragments were digested with *Bam*HI, and each was cloned to generate the recombinant plasmids pTYToCV-1 and pPep-1, which have putative full-length clones of these monopartite begomoviruses.

The TYLC/ToLC-associated begomovirus DNA component is 2,773 nucleotides (Genbank accession No. AY502936), whereas that of the pepper-associated begomovirus is 2,786 nucleotides (Genbank accession No. AY502935). An analysis of these sequences for ORFs revealed that both begomoviruses have a genome organization similar to previously characterized monopartite begomoviruses, with six ORFs corresponding to the V1, V2, C1, C2, C3, and C4 [12, 21, 28]. The IR sequences (i.e., the sequence between the start codons of the C1 and V1 ORFs) of the TYLC/ToLC-associated and pepper-associated begomoviruses were 299 and 312 nucleotides, respectively, and both contained the characteristic inverted repeat capable of forming a stem-loop structure and the highly conserved nanonucleotide sequence (TAATAT TAC) within the loop sequence. The IR sequence of both viruses had the TATA box for the C1 ORF (encoding the replication-associated protein [Rep]) and Rep protein high-affinity binding sites. The high-affinity binding sites of the TYLC/ToLC-associated begomovirus are GGGGA and GGGGG, which differ from those typically associated with

TYLCV and ToLCV isolates (GGTGT and GGTGT). Those of the pepper-associated begomovirus were GGTGT and GGGGT, which are identical to those of another tomato-infecting begomovirus from Mali (tomato yellow leaf curl Mali virus [TYLCMLV]; [3] and more similar to those of TYLCV and ToLCV isolates.

Infectivity of the full-length clones of members of the two begomovirus species from West Africa

N. benthamiana plants bombarded with the full-length monomer or undigested multimeric clone of the TYLC/ToLC-associated begomovirus developed symptoms of stunted growth, upward leaf curling and swollen veins in 25% (6/24) and 31% (8/26) of inoculated plants, respectively. Plants inoculated with the monomer or multimeric clone of the pepper-associated begomovirus developed symptoms of stunted growth and downward leaf curling, mottling, and chlorosis in 40% (8/20) and 53% (18/34) of inoculated plants, respectively. Plants bombarded with gold particles only did not develop symptoms. Infection of selected symptomatic plants was confirmed by PCR and DNA sequencing. These results established the infectivity of these clones and, thus, the monopartite nature of these two begomoviruses.

Similar inoculations were performed with tomato and pepper seedlings. A small proportion ($\sim$10%) of tomato seedlings bombarded with the monomer (2/21) or multimer (2/20) of the TYLC/ToLC-associated begomovirus developed symptoms of mild leaf curling approximately 4 weeks post-bombardment. Pepper plants bombarded with these clones did not develop symptoms, nor was viral DNA detected in newly emerged leaves of these plants. Tomato and pepper seedlings bombarded with the monomer or multimer of the pepper-associated begomoviruses did not develop obvious disease symptoms; however, infections were detected in $\sim$10% of bombarded tomato (monomer, 2/24 and multimer, 2/20 plants) and pepper (monomer, 2/25 and multimer, 2/24 plants) based upon PCR analyses.

Agroinoculation systems for members of the two begomovirus species from West Africa

The multimeric clones of these two begomoviruses were cloned into the binary vector pGA482 to generate recombinant plasmids pTYToCbin and pPepbin, respectively.

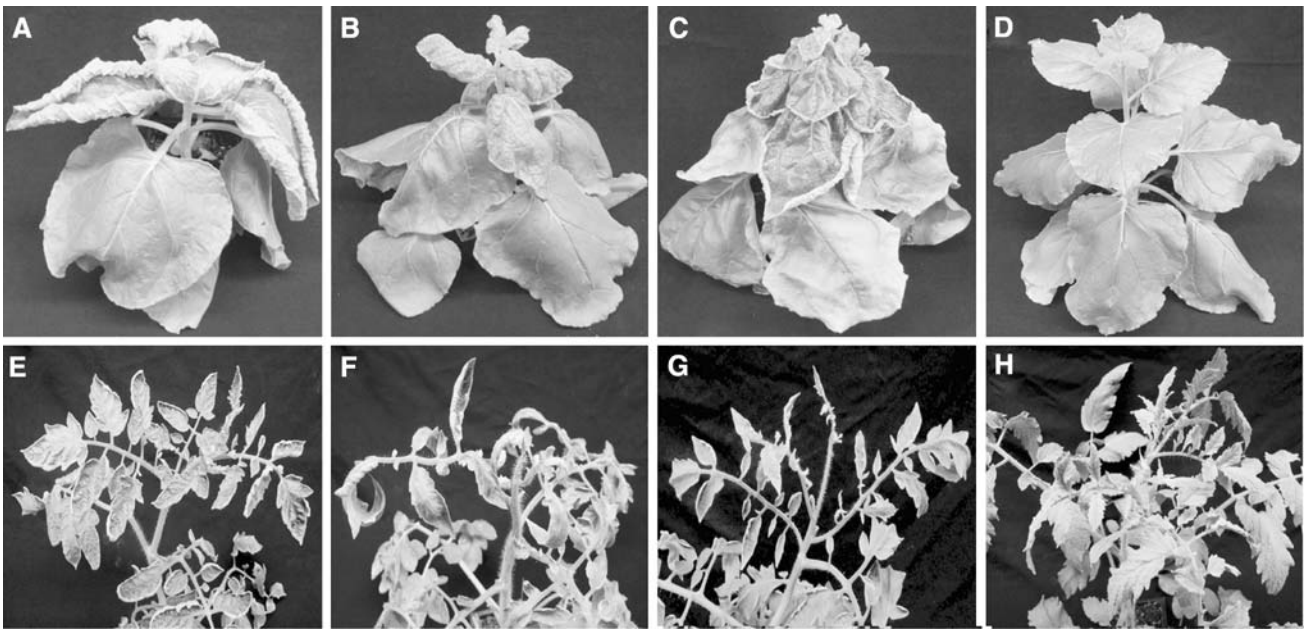


Fig. 1 Symptoms induced in *N. benthamiana* and tomato (*S. lycopersicum*, cv. Roma) plants 3–4 weeks after agroinoculation with begomoviruses from Mali associated with tomato yellow leaf curl (TYLC)/tomato leaf curl (ToLC) symptoms (=tomato leaf curl Mali virus [ToLCMLV]) or with pepper leaf curl and yellow vein and tomato yellow leaf crumple symptoms (=tomato yellow leaf crumple virus [ToYLCrV]). **a** Stunted growth and upward curling and vein swelling in leaves of an *N. benthamiana* plant infected with ToLCMLV. **b** Stunted growth and downward curling (epinasty) and crumpling in leaves of an *N. benthamiana* plant infected with ToYLCrV. **c** Severely stunted and distorted growth, and epinasty, upward leaf curl and vein swelling in an *N. benthamiana* plant co-infected with ToLCMLV and ToYLCrV. **d** A mock-inoculated *N. benthamiana* plant. **e** Upward leaf curling and crumple in leaves of a tomato plant (cv. Roma) infected with ToLCMLV. **f** Distorted growth and leaf curl, crumple and chlorosis in a tomato plant infected with ToYLCrV. **g** Severely stunted and distorted growth and upcurling, epinasty and crumpling (in the newly emerging leaves) in a tomato plant co-infected with ToLCMLV and ToYLCrV. **h** A mock-inoculated tomato plant

Agrobacterium tumefaciens C58 was transformed with these plasmids, and selected transformants were characterized and used in agroinoculation experiments. *N. benthamiana* plants agroinoculated with the TYLC/ToLC-associated begomovirus developed symptoms of stunted growth, upward leaf curling and severe vein swelling (Fig. 1a), and these symptoms were indistinguishable from those observed in plants following particle bombardment inoculation. *N. benthamiana* plants agroinoculated with the pepper-associated begomovirus developed symptoms of stunted growth and downward leaf curling, mottling and chlorosis (Fig. 1b); these symptoms also were indistinguishable from those observed in plants following particle bombardment inoculation. For both viruses, symptoms developed ~3 to 4 weeks post-inoculation. In contrast to the moderate level of infectivity (~50%) achieved with particle bombardment, all 15 *N. benthamiana* seedlings agroinoculated with the TYLC/ToLC-associated or the pepper-associated begomoviruses became infected. Plants agroinoculated with the empty vector control did not develop symptoms (Fig. 1d).

Tomato plants agroinoculated with the TYLC/ToLC-associated begomovirus developed stunted growth and upward leaf curling, mottling, and interveinal chlorosis (Fig. 1e). Plants agroinoculated with the pepper-associated begomovirus developed stunted and distorted growth and downward leaf cupping, crumpling, chlorosis and purple veins (Fig. 1f). Tomato plants developed symptoms ~3 to 4 weeks postinoculation, whereas seedlings inoculated with the empty vector control did not develop symptoms (Fig. 1h). These results confirmed the infectivity of the TYLC/ToLC- and pepper-associated begomovirus clones in *N. benthamiana* and tomato and that these begomoviruses induce leaf curl and yellow leaf crumple symptoms, respectively, in tomato. These results also established that agroinoculation is an efficient inoculation method for both viruses.

N. benthamiana seedlings co-agroinoculated with these begomoviruses developed severely stunted growth and leaf epinasty, upcurling, mottling, yellowing and vein swelling (Fig. 1c). These symptoms were a combination of those induced by both viruses and were clearly more severe than those induced by either virus alone (compare Fig. 1a–c). Tomato seedlings co-inoculated with both viruses also developed severe symptoms of upright growth and severe stunting and distortion (particularly in the newly emerging growth ~3 to 4 weeks postinoculation) and leaf epinasty, upcurling, crumpling, yellowing and purple vein (Fig. 1g). Though not as striking as in *N. benthamiana*, the symptoms in plants co-infected with both viruses were also more severe than those induced by any single virus, and this was especially evident in newly emerged growth (compare newly emerged leaves of plants in Fig. 1e–g).

Agroinoculation was then used to determine the host range of the TYLC/ToLC-associated and pepper-associated begomoviruses (Table 1). Both viruses induced high rates of symptomatic infection in known susceptible

Table 1 Infectivity and partial host range of two tomato-infecting begomoviruses from Mali (tomato yellow leaf curl [TYLC]/tomato leaf curl [ToLC]-associated and pepper-associated begomoviruses) determined using agroinoculation

Begomovirus	Host	Symptoms	Viral DNA
TYLC/ToLC-associated	*S. lycopersicum* cv. Glamour	15/15***	15/15
	N. benthamiana	15/15***	15/15
	Nicotiana tabacum cv. Havana	7/15*	7/15
	Capsicum annuum cv. Cayenne Long	0/15	0/15
	Capsicum annuum cv. Carolina	0/15	0/15
	Solanum melongena cv. Black beauty	0/15	0/15
	Solanum aethiopicum cv. Soxna	0/10	0/10
	Arabidopsis thaliana	0/15	0/15
	Phaseolus vulgaris cv. Topcrop	1/20	1/20
	Cucurbita pepo cv. Small sugar	0/15	0/15
Pepper-associated	*S. lycopersicum* cv. Glamour	15/15***	15/15
	N. benthamiana	15/15***	15/15
	Nicotiana tabacum cv. Havana	10/15*	10/15
	Capsicum annuum cv. Cayenne Long	1/15	1/15
	Capsicum annuum cv. Carolina	0/15	0/15
	Solanum melongena cv. Black beauty	1/15	1/15
	Solanum aethiopicum cv. Soxna	1/10	1/10
	Arabidopsis thaliana	0/15	0/15
	Phaseolus vulgaris cv. Topcrop	0/20	0/20
	Cucurbita pepo cv. Small sugar	0/15	0/15

[a] Data represent totals of three independent experiments. Number of infected plants (presence of symptoms or detection of viral DNA by PCR)/total number of plants inoculated or tested. Symptom severity is indicated as follows: no asterisk = symptomless; *mild symptoms; ** moderate symptoms; and *** severe symptoms

tomato cultivars (cvs. Glamour and Roma). In addition, both viruses also induced symptomatic infections (downward leaf curling and crumpling) in ~50% of inoculated *N. tabacum* cv. Havana plants. Neither virus induced disease symptoms in the two pepper cultivars tested (cvs. Cayenne Long and Carolina). However, whereas the TYLC/ToLC-associated begomovirus was not infectious in pepper (i.e., viral DNA was not detected in newly emerged leaves by PCR), DNA of the pepper-associated begomovirus was detected in one cv. Cayenne Long plant (Table 1). Neither virus induced symptoms in agroinoculated *Arabidopsis*, common bean, eggplant, African eggplant, or pumpkin plants. No viral DNA was detected in inoculated *A. thaliana* and pumpkin plants, whereas TYLC/ToLC-associated begomovirus DNA was detected in newly emerged leaves of one common bean plant, and the pepper-associated begomovirus was detected in newly emerged leaves of one eggplant and African eggplant.

Comparison of the nucleotide and amino acid sequences of the TYLC/ToLC-associated and pepper-associated begomoviruses from West Africa with those of other begomoviruses

The results of comparisons of the nucleotide and amino acid sequences of the TYLC/ToLC-associated and pepper-associated begomoviruses with those of the most similar begomoviruses (only viruses having complete sequences available) are shown in Tables 2 and 3, respectively. The complete nucleotide sequence of the TYLC/ToLC-associated begomovirus was most similar to those of the pepper-associated begomovirus (79%), tomato curly stunt virus from South Africa (ToCSV-ZA) (78%), TYLCV-[SH2] (78%), and TYLCMLV and isolates of TYLCV (77%). Individual ORFs had nucleotide sequence identities ranging from 65 to 86% and amino acid identities/similarities ranging from 43 to 86 and 48 to 93%, respectively (Table 2). The most conserved ORFs were

Table 2 Percent nucleotide identities for the DNA component and intergenic region (IR) and nucleotide and derived amino acid identities and similarities for open reading frames of the TYLC/ToLC-associated begomovirus (tomato leaf curl Mali virus [ToLCMV]) and those of other begomoviruses

Begomovirus[a]	Total[b]	IR[c]	V1		V2 (CP)		C1		C2		C3		C4	
	nt	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
ToYLCrV	79	65	82	85 (91)[d]	83	86 (92)	78	80 (87)	81	67 (75)	80	72 (82)	86	67 (74)
ToCSV-[ZA]	78	61	80	78 (87)	78	87 (93)	80	79 (88)	80	68 (77)	81	79 (85)	88	70 (78)
TYLCV-[SH2]	78	62	84	85 (90)	84	85 (93)	77	77 (86)	80	69 (76)	79	70 (79)	68	49 (53)
TYLCV-Mld[PT]	77	59	84	85 (90)	83	85 (93)	77	79 (87)	80	70 (78)	79	70 (80)	69	48 (55)
TYLCV-[PR]	77	60	83	85 (91)	83	84 (93)	77	78 (86)	80	69 (76)	79	71 (81)	67	46 (52)
TYLCV-[CU]	77	62	83	85 (91)	83	84 (93)	77	78 (86)	79	69 (76)	78	69 (80)	67	46 (52)
TYLCV-[DO]	77	62	83	85 (91)	83	83 (93)	77	78 (87)	79	69 (76)	78	69 (79)	67	46 (52)
TYLCV-Mld[ES7297]	77	60	83	85 (90)	83	85 (93)	78	80 (87)	80	68 (76)	79	70 (81)	69	48 (55)
TYLCMLV	77	60	82	86 (91)	83	83 (92)	76	77 (86)	82	70 (74)	81	74 (84)	65	43 (48)
TYLCMLV-[ET]	77	60	82	86 (90)	84	83 (92)	77	79 (86)	82	69 (73)	79	72 (82)	67	44 (51)
TbLCZV-[ZW]	76	60	82	81 (90)	76	80 (89)	80	79 (88)	78	75 (65)	78	70 (82)	87	68 (77)
ToLCV-[AU]	73	62	73	60 (70)	76	76 (86)	76	78 (85)	71	62 (70)	73	65 (76)	68	48 (54)
TYLCV-[Tos]	70	62	84	85 (90)	84	85 (93)	77	78 (86)	80	70 (77)	79	72 (81)	68	49 (53)
TYLCV-Mld[Shi]	70	60	84	85 (90)	83	85 (93)	78	80 (87)	80	70 (77)	79	71 (81)	69	48 (55)
TYLCV-Mld[Aic]	70	60	84	85 (90)	83	85 (93)	78	80 (87)	80	70 (76)	79	70 (80)	69	48 (55)

[a] *ToYLCrV* tomato yellow leaf crumple virus from Mali, *ToCSV-[ZA]* tomato curly stunt virus from South Africa, *TYLCV-[SH2]* tomato yellow leaf curl virus (TYLCV) isolate SH2 from China, *TYLCV-Mld[PT]* TYLCV-Mild from Portugal, *TYLCV-[PR]* TYLCV from Puerto Rico, *TYLCV-[CU]* TYLCV from Cuba, *TYLCV-[DO]* TYLCV from Dominican Republic, *TYLCV-Mld[ES7297]* TYLCV-Mild isolate 7297 from Spain, *TYLCMLV* tomato yellow leaf curl Mali virus, *TYLCMLV-[ET]* TYLCMLV from Ethiopia, *TbLCZV-[ZW]* tobacco leaf curl Zimbabwe virus, *ToLCV-[AU]* tomato leaf curl virus (TLCV) from Australia, *TYLCV-[Tos]* TYLCV isolate Tosa from Japan, *TYLCV-Mld[Shi]* TYLCV-Mild from Shizuokua, *TYLCV-Mld[Aic]* TYLCV-Mild isolate Aichi from Japan

[b] Total comparison made with the complete nucleotide sequence

[c] IR sequence is between start codons of C1 and V1 ORFs

[d] Numbers in parentheses indicate percent amino acid similarity

the CP and V1, followed by C1, C3 and C2. The C4 ORF was the most divergent (Table 2). The results of sequence comparisons with individual ORFs were similar to those obtained for the complete sequence except that slightly higher nucleotide and amino acid sequence identities were identified for the C4 ORF of ToCSV-[ZA], tobacco leaf curl Zimbabwe virus (TbLCZV-[ZW]) and the pepper-associated begomovirus (Table 2). Based on the sequence comparisons, the infectivity studies, and the association with TYLC/ToLC symptoms, this begomovirus is named tomato leaf curl Mali virus (ToLCMLV).

The complete nucleotide sequence of the pepper-associated begomovirus was most similar to those of TbLCZV-[ZW] (82%), ToCSV-[ZA] (81%), TYLCMLV (80%), and ToLCMLV and various TYLCV isolates (79%) (Table 3). Similar results were obtained for comparisons made with most individual ORFs (Table 3), although higher identities were obtained for some ORFs. For example, the V1 and V2 (CP) ORFs had nucleotide sequence identities of ~90 and 84%, respectively, with those of various TYLCV isolates. The C1 (Rep) ORF had 87 and 85% nucleotide sequence identity and 93 and 91% amino acid similarity with those of TbLCZV-[ZW] and ToCSV-[ZA], respectively. The C4 ORF had 90, 89% and 86% nucleotide sequence identity with those of TbLCZV-[ZW], ToCSV-[ZA] and ToLCMLV, respectively (Table 3). Taken together with the infectivity results and the association with the yellow leaf crumple symptoms in tomatoes in the field, the pepper-associated begomovirus is named tomato yellow leaf crumple virus (ToYLCrV).

Phylogenetic analyses were performed with ToLCMLV and ToYLCrV nucleotide sequences, including the complete genome, the CP, C1, C4 ORFs, and the IR. The phylogenetic trees generated from these analyses revealed that ToLCMLV and ToYLCrV are not closely related to previously characterized isolates of TYLCV or ToLCV. Moreover, irrespective of the sequence examined, ToLCMLV, ToYLCrV, TbLCZV-[ZW] and ToCDV-[ZA] were most closely related and were placed in a clade of African tomato-infecting begomoviruses (Fig. 2). Neither the sequence comparisons nor the phylogenetic analyses indicated that ToLCMLV or ToYLCrV have recombinant

Table 3 Percent nucleotide identities for the DNA component and intergenic region (IR) and nucleotide and derived amino acid identities and similarities between open reading frames of the pepper-associated begomovirus (tomato yellow leaf crumple virus [ToYLCrV]) and those of other begomoviruses

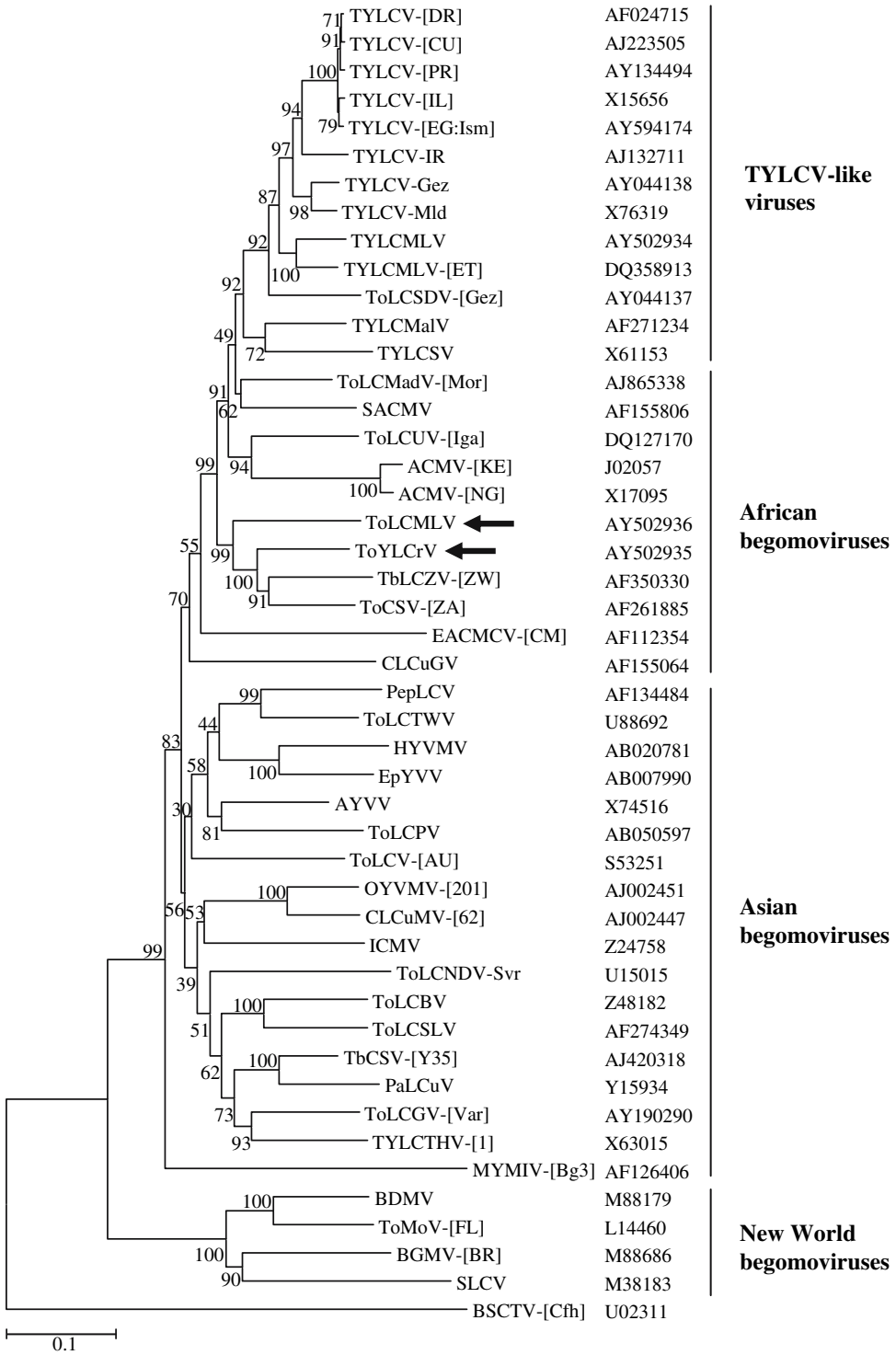
Begomovirus[a]	Total[b]	IR[c]	V1		V2 (CP)		C1		C2		C3		C4	
	nt	nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
TbLCZV-[ZW]	82	72	91	86 (91)[d]	78	84 (91)	87	88 (93)	84	75 (80)	80	75 (78)	90	79 (81)
ToCSV-[ZA]	81	65	80	85 (92)	82	87 (95)	85	84 (91)	83	73 (79)	84	79 (85)	89	77 (80)
TYLCMLV-[ET]	80	72	90	90 (92)	84	89 (95)	77	77 (86)	85	76 (82)	84	78 (87)	68	47 (53)
TYLCMLV	80	71	89	89 (93)	83	90 (97)	77	77 (86)	86	79 (84)	85	78 (87)	69	50 (52)
TYLCV-[DO]	79	67	89	89 (93)	84	89 (97)	75	74 (85)	83	73 (79)	82	75 (84)	66	43 (52)
TYLCV-Mld[PT]	79	67	89	88 (92)	84	90 (96)	77	87 (78)	83	73 (79)	82	76 (84)	66	47 (53)
TYLCV-[SH2]	79	64	89	88 (92)	84	90 (96)	75	74 (85)	83	73 (78)	80	78 (86)	67	45 (53)
TYLCV-Mld[ES7297]	79	65	89	87 (91)	84	90 (96)	77	78 (88)	82	73 (78)	82	76 (84)	66	47 (53)
ToLCMLV	79	65	82	85 (91)	83	86 (92)	78	80 (87)	81	67 (75)	80	72 (82)	86	67 (74)
TYLCV-[CU]	78	67	89	89 (93)	84	90 (97)	75	74 (85)	83	73 (79)	82	75 (84)	66	43 (52)
TYLCV-[PR]	78	66	88	89 (93)	84	89 (96)	75	74 (85)	83	73 (79)	82	76 (84)	66	43 (52)
ToLCV-[AU]	73	63	74	60 (71)	74	76 (85)	73	75 (83)	74	63 (70)	75	67 (75)	66	48 (53)
TYLCV-[Tos]	71	65	89	88 (92)	84	90 (96)	75	74 (85)	83	73 (79)	83	78 (85)	67	45 (53)
TYLCV-Mld[Aic]	71	67	89	88 (92)	84	90 (96)	77	88 (78)	83	73 (79)	83	76 (84)	66	47 (53)
TYLCV-Mld[Shi]	71	67	89	88 (92)	84	90 (96)	77	78 (88)	83	73 (79)	82	77 (84)	66	47 (53)

[a] *TbLCZV-[ZW]* tobacco leaf curl Zimbabwe virus, *ToCSV-[ZA]* tomato curly stunt virus from South Africa, *TYLCMLV* tomato yellow leaf curl Mali virus, *TYLCMLV-ET* TYLCMLV from Ethiopia, *TYLCV-[DO]* TYLCV from Dominican Republic, *TYLCV-Mld[PT]* TYLCV-Mild from Portugal, *TYLCV-[SH2]* TYLCV isolate SH2 from China, *TYLCV-Mld[ES7297]* TYLCV-Mild isolate 7297 from Spain, *ToLCMLV* tomato leaf curl Mali virus, *TYLCV-[CU]* TYLCV from Cuba, *TYLCV-[PR]* TYLCV from Puerto Rico, *ToLCV-[AU]* tomato leaf curl virus (TLCV) from Australia, *TYLCV-[Tos]* TYLCV isolate Tosa from Japan, *TYLCV-Mld[Aic]* TYLCV-Mild isolate Aichi from Japan, *TYLCV-Mld[Shi]* TYLCV-Mild from Shizuokua

[b] Total comparison made with the complete nucleotide sequence

[c] IR sequence is between start codons of C1 and V1 ORFs

[d] Numbers in parentheses indicate percent amino acid similarity



genomes (i.e., having sequences derived from members of different begomovirus species or placed in different phylogenetic clusters) (Tables 2, 3 and data not shown). Thus, these results are consistent with ToLCMLV and ToYLCrV representing members of new monopartite begomovirus species.

Development of ToLCMLV- and ToYLCrV-specific primers

The ToLCMLV and ToYLCrV sequences were aligned with other monopartite begomoviruses, including TYLCV. Divergent regions were identified in the C1 and C4 ORFs

◀ **Fig. 2** Phylogenetic consensus tree showing the relationship of tomato leaf curl Mali virus (ToLCMLV), tomato yellow leaf crumple virus (ToYLCrV), and other begomoviruses based on an alignment of complete nucleotide sequences. Phylogenetic analyses were performed with MEGA version 3.0 using the neighbor-joining method as described by Kumar et al. [17]. Branch strengths were evaluated by constructing 1000 trees in bootstrap analysis by step-wise addition at random. Bootstrap values are shown above or under the horizontal line. Horizontal lines are in proportion to the number of nucleotide differences between branch nodes. As out-group, an isolate of Beet severe curly top virus-[Cfh], (BSCTV-[Cfh]) belonging to the genus *Curtovirus*, was used. The following sequences were obtained from GenBank and used for comparisons and phylogenetic analysis: African cassava mosaic virus-[Nigeria] (ACMV-[NG]), African cassava mosaic virus-[Kenya] (ACMV-[KE]), Ageratum yellow vein virus (AYVV), bean dwarf mosaic virus (BDMV), bean golden mosaic virus-[Brazil] (BGMV-[BR]), cotton leaf curl Gezira virus (CLCuGV: AF155064), cotton leaf curl Multan virus-[62] (CLCuMV-[62]), East African cassava mosaic Cameroon virus-[Cameroon] (EACMCV-[CM]), Eupatorium yellow vein virus (EpYVV), honeysuckle yellow vein mosaic virus (HYVMV), Indian cassava mosaic virus (ICMV), mungbean yellow mosaic India virus-[Bg3], (MYMV-[Bg3]), okra yellow vein mosaic virus-[201] (OYVMV-[201]), papaya leaf curl virus (PaLCuV), pepper leaf curl virus (PepLCV), South African cassava mosaic virus (SACMV), squash leaf curl virus (SLCV), tobacco curly shoot virus-[Yunnan 35] (TbCSV-[Y35]), tobacco leaf curl Zimbabwe virus-[Zimbabwe] (TbLCZV-[ZW]), tomato curly stunt virus-[South Africa] (ToCSV-[SA]), tomato leaf curl Bangalore virus (ToLCBV), tomato leaf curl Gujarat virus-[Varanasi] (ToLCGV-[Var]), tomato leaf curl Madagascar virus-[Morondova] (ToLCMadV-[Mor]), tomato leaf curl Mali virus (ToLCMLV), tomato leaf curl New Delhi virus-Sever (ToLCNDV-Svr), tomato leaf curl Philippines virus (ToLCPV), tomato leaf curl Sri Lanka virus (ToLCSLV), tomato leaf curl Sudan virus-[Gezira] (ToLCSDV-[Gez]), tomato leaf curl Taiwan virus (ToLCTWV), tomato leaf curl Uganda virus-[Iganga] (ToLCUV-[Iga]), tomato leaf curl virus-[Australia] (ToLCV-[AU]), tomato mottle virus-[Florida] (ToMoV-[Flo]), tomato yellow leaf crumple virus (ToYLCrV), tomato yellow leaf curl Malaga virus (TYLCMalV), tomato yellow leaf curl Mali virus (TYLCMLV), tomato yellow leaf curl Mali virus-[Ethiopia] (TYLCMLV-[ET]), tomato yellow leaf curl Sardinia virus (TYLCSV), tomato yellow leaf curl Thailand virus-[1] (TYLCTHV-[1]), tomato yellow leaf curl virus-[Cuba] (TYLCV-[CU]), tomato yellow leaf curl virus-[Dominican Republic] (TYLCV-[DO]), tomato yellow leaf curl virus-[Egypt, Ismailia (TYLCV-[EG:Ism]), tomato yellow leaf curl virus-[Israel] (TYLCV-[IL]), tomato yellow leaf curl virus-[Puerto Rico] (TYLCV-[PR]), tomato yellow leaf curl virus-Gezira (TYLCV-Gez), tomato yellow leaf curl virus-Iran (TYLCV-IR), tomato yellow leaf curl virus-Mild (TYLCV-Mld). The positions of ToLCMLV and ToYLCrV are indicated with arrows

and the IR (data not shown). A specific primer for ToLCMLV was designed from the C1 ORF sequence (primer 1990v, 5′-CGCGCAGCGGAATCTCTTAT-3′), whereas one for ToYLCrV was designed from the IR sequence (primer 2635v, 5′-CAATTGACCGCTCTTGG-3′). These primers were each paired with a common primer (1000c, 5′-ATGMGTACATGCCATATACA-3′), designed from a conserved sequence in the *CP* gene (Fig. 3a). The ToLCMLV primer pair (1990v/1000c) directed the amplification of the expected ~1.8-kb fragment from DNA extracts from leaves of ToLCMLV-infected *N. benthamiana* or tomato plants, and no fragment from DNA extracts from leaves of ToYLCrV-infected or uninfected plants. The ToYLCrV primer pair (2635v/1000c) directed the amplification of the expected ~1.1-kb fragment from DNA extracts of leaves of ToYLCrV-infected *N. benthamiana* or tomato plants, and no fragment from DNA extracts of leaves of ToLCMLV-infected plants or uninfected plants (Fig. 3b). The following PCR conditions were optimized for specific amplification of the DNA fragments of ToLCMLV and ToYLCrV: 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, followed by 72°C for 10 min. In multiplex PCR experiments, these primers directed the target DNA fragments from DNA extracts from leaves from plants co-infected with both viruses (data not shown).

Discussion

Tomato production in many countries of West Africa (e.g., Benin, Burkina Faso, Ghana, Mali, Niger, Senegal and Togo) has been negatively impacted by epidemics of TYLC/ToLC. Several lines of evidence have suggested a begomovirus etiology for these diseases: (1) characteristic disease symptoms, (2) the consistent association of high populations of whiteflies with these disease outbreaks, and (3) the detection of begomovirus CP in plants with these symptoms [16]. In this study, squash blot hybridization and SB-PCR were used to demonstrate begomovirus infection in tomatoes with three distinct symptom phenotypes (typical TYLC/ToLC, yellow mottle leaf crumple and the broccoli symptoms) from five tomato-growing areas of Mali. Although some symptoms resembled those induced by phytoplasma infection, SB-PCR failed to reveal phytoplasma DNA in any of the samples. This was also consistent with the lack of virescence or phyllody symptoms in these plants. Thus, our results indicated a begomovirus etiology for this diversity of tomato virus disease symptoms as well as for the leaf curling and yellow vein symptoms observed in pepper in Koulikoro and Baguineda.

Because of the characteristic yellow leaf curl symptoms commonly associated with viral disease epidemics in tomatoes in West Africa, it is commonly assumed that the causal agent is TYLCV. Indeed, the amplification of CP fragments, mediated by TYLCV CP primers, from all ten squash-blot-positive samples collected from four locations in Mali in 2003 was consistent with this hypothesis. However, the relatively low level of sequence identity (80%) of these fragments with the TYLCV CP sequence suggested that a genetically distinct begomovirus(es) may be involved. Sequence analyses of other regions of the viral

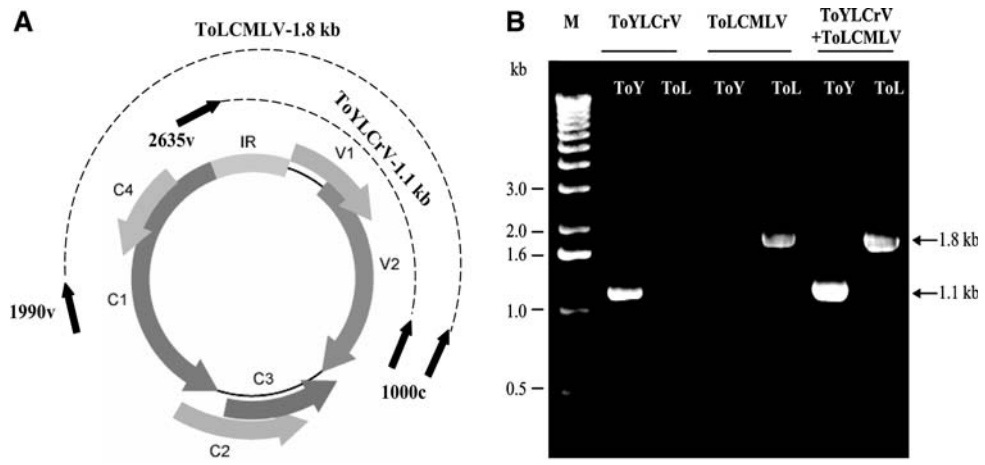


Fig. 3 Detection of tomato leaf curl Mali virus (ToLCMLV) and tomato yellow leaf crumple virus (ToYLCrV) by PCR with virus-specific primers. **a** Diagramatic illustration showing the location of the ToLCMLV- and ToYLCrV-specific primers 1990v and 2635v, respectively, on a generalized monopartite begomovirus genome map. When paired with the common primer (1000c), designed to anneal in the capsid protein gene, the ToLCMLV primer directs the amplification of an ~1.8-kb fragment, whereas the ToYLCrV primer directs the amplification of an ~1.1-kb fragment. **b** Ethidium bromide-stained gel showing ToLCMLV and ToYLCrV DNA fragments amplified by PCR with the ToLCMLV-specific primer pair (ToL) or the ToYLCrV-specific primer pair (ToY). M is the molecular weight marker (M, 1 kb ladder), ToYLCrV is a DNA extract from leaves of a tomato plant infected with ToYLCrV, ToLCMLV is a DNA extract from leaves of a tomato plant infected with ToLCMLV, and ToYLCrV+ToLCMLV is a DNA extract from leaves of a tomato plant co-infected with ToLCMLV and ToYLCrV

genome supported this hypothesis and revealed the presence of members of at least two new monopartite begomovirus species. Analysis of the complete genome of these begomoviruses, together with the failure to detect an associated DNA-B component, suggested that these were members of Old World monopartite begomovirus species. Definitive evidence of the monopartite nature of these viruses came from the infectivity of the cloned viral DNAs in *N. benthamiana* and tomato.

ToLCMLV was named based on the predominant symptom type (upright growth and upward leaf curling, interveinal light green-yellowing and swollen/purple veins) it was associated with and the finding that the infectious cloned DNA induced identical symptoms in tomato plants. The finding that the ToLCMLV sequence was 98% identical to a partial sequence of a begomovirus associated with tomato leaf curl disease in Senegal indicates that ToLCMLV causes TYLC/ToLC in other countries of West Africa. This has been confirmed by recent surveys using the ToLCMLV-specific primer pair, in which ToLCMLV was detected in tomatoes with TYLC/ToLC in Benin, Burkina Faso, Ghana, Niger, Senegal, and Togo (unpublished data).

The situation with the pepper-associated begomovirus was more complex. This begomovirus was originally cloned from pepper plants with upcurled leaves and yellow vein symptoms. Thus, it was initially named pepper yellow vein virus. However, infectivity studies indicated that the virus was poorly infectious in pepper (i.e., symptomless infections in a small number of plants). Moreover, this was most likely not a defect in the clone, as it was highly infectious in *N. benthamiana* and tomato. In the 2004 survey, this begomovirus was consistently associated with the yellow mottle leaf crumple symptom phenotype in tomato, and similar symptoms were induced in tomato and *N. benthamiana* by the infectious cloned DNA of this virus. Thus, the name tomato yellow leaf crumple virus (ToYLCrV) is proposed based upon the predominant disease symptoms it is associated with in the field and the high rate of infectivity and symptoms induced in tomato by the infectious cloned DNA. Surveys conducted in West Africa with SB-PCR and the ToYLCrV-specific primer pair revealed the association of ToYLCrV with the yellow mottle leaf crumpling symptoms in tomatoes in Burkina Faso, Benin, Ghana, Niger, Senegal, and Togo (unpublished data). Thus, ToYLCrV appears to be an important component of the begomovirus complex causing viral disease epidemics of tomatoes in West Africa. The role of ToYLCrV in the leaf curl and yellow vein disease of pepper is less clear. Though not infectious in pepper under the conditions used in this study, ToYLCrV has been commonly detected in field-collected peppers from Mali (this study and unpublished data) and Burkina Faso (unpublished data). Thus, disease development in pepper may involve a complex of ToYLCrV, another begomovirus(es) and/or a satellite DNA (e.g., a DNA-β). Alternatively, this may relate to the nature of the peppers grown in West Africa, though the cultivars used in our study are known to be susceptible to bipartite begomoviruses delivered via agroinoculation (unpublished data).

Additional experiments are needed to determine the etiology of this pepper disease and the role of ToYLCrV. These results also demonstrate that care should be taken when naming a begomovirus (or any other virus) based only on association with a disease symptom, and that definitive naming should await completion of Koch's postulates with an infectious clone.

Phylogenetic analyses and sequence comparisons indicated that ToLCMLV and ToYLCrV were most closely related to each other and two other African tomato-infecting begomoviruses, TbLCZV-[ZW] and ToCSV-[ZA]. Thus, ToLCMLV and ToYLCrV probably represent indigenous begomoviruses that evolved to infect tomato in West Africa. The finding of new genetically distinct begomoviruses associated with TYLC/ToLC in another region of the world is another example of local evolution of genetically distinct begomoviruses that induce similar disease symptoms in a crop plant grown over a wide geographic area. One of the first examples of this phenomenon was for Bean golden mosaic virus and Bean golden yellow mosaic virus, which are distinct bipartite begomovirus species whose members cause bean golden mosaic disease of common bean in Brazil and the Caribbean/Mexico, respectively [8, 11]. Cassava mosaic disease is also caused by members of a number of genetically distinct species and recombinants [18]. However, this phenomenon is perhaps now best demonstrated by the diversity of begomoviruses and recombinants that cause TYLC/ToLC in Africa, Asia, Europe, the Middle East, Australia and Central and South America [9, 13, 24, 34]. This likely reflects the genetic diversity and wide distribution of indigenous begomoviruses present in weeds or other plants in tropical and semi-tropical regions and the capacity of these viruses to evolve into tomato-infecting variants following introduction events mediated by high populations of *B. tabaci*. In contrast to the genetic diversity in the pathogen, the similar symptoms induced in the tomato host by these viruses reflect common mechanisms of pathogenicity.

The range of viral disease symptoms observed in tomatoes in the field in West Africa can not be explained by infections by ToLCMLV or ToYLCrV alone. Indeed, several lines of evidence support the hypothesis that this diversity of symptoms in the field reflects a disease complex composed of mixed infections of multiple begomoviruses and satellite DNAs (e.g., DNA-β). First, the diversity of symptoms observed in tomato was greater than could be explained based upon the results of our infectivity studies with ToLCMLV and ToYLCrV. Second, synergism between ToLCMLV and ToYLCrV, in which increased symptom severity occurred in mixed infections, was observed and likely adds to the complexity of symptoms in the field. Indeed, mixed infections of ToLCMLV and ToYLCrV are commonly detected in field-collected samples, including plants showing the broccoli phenotype. Third, other begomoviruses have been associated with the TYLC/ToLC complex in West Africa, including the recombinant virus, TYLCMLV [3]. Finally, a DNA-β has been associated with TYLCMLV, which results in the development of more severe and diverse symptoms in tomato and other host plants [3]. DNA-βs are required for induction of characteristic disease symptoms by other monopartite begomoviruses, such as cotton leaf curl Multan virus, Ageratum yellow vein virus and tomato leaf curl Java virus [2, 15, 31]. Although we did not find satellite DNAs associated with ToLCMLV or ToYLCrV in the samples collected in these surveys, we cannot rule out a role for these entities in the broccoli disease phenotype. Thus, it appears likely that the diversity and severity of virus disease symptoms in tomatoes in Mali and elsewhere in West Africa is a result of complex synergistic interactions between begomoviruses and DNA-β. Of course, these results do not rule out involvement of other viruses in these epidemics (e.g., RNA viruses such as potyviruses).

Tools developed in this study will facilitate the development of management strategies for these begomoviruses. Particle bombardment was not an efficient means of infecting tomatoes with the cloned DNAs of ToLCMLV and ToYLCrV, which may reflect phloem-limitation of these monopartite tomato-infecting begomoviruses [26, 29]. However, agroinoculation provided high rates of infectivity in *N. benthamiana* and tomato, presumably reflecting delivery of viral DNA to phloem or phloem-associated cells [14]. Thus, agroinoculation can be used to identify sources of resistance to these begomoviruses and to investigate viral host range. Virus-specific primers can be used to investigate the geographical distribution and host range of these viruses. For example, results of host plant surveys with SB-PCR and these primers have indicated that tomato is the primary host of these viruses (unpublished data), which is consistent with the host range data obtained in the agroinoculation experiments. This suggests that a tomato-host-free period could be used as part of an integrated disease management for these begomovirus diseases, similar to the successful strategy used to manage TYLC in the Dominican Republic [30].

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References

1. Bedford ID, Kelly A, Banks GK, Briddon RW, Cenis JL, Markham PG (1999) *Solanum nigrum:* an indigenous weed

reservoir for a tomato yellow leaf curl geminivirus in Southern Spain. Eur J Plant Pathol 104:221–222
2. Briddon RW, Mansoor S, Bedford ID, Pinner MS, Saunders K, Stanley J, Zafar Y, Malik KA, Markham PG (2001) Identification of DNA components required for induction of cotton leaf curl disease. Virology 285:234–243
3. Chen LF, Hagen C, Zhou Y, Noussourou M, Kon T, Rojas M, Gilbertson RL (2007) Emergence of tomato leaf curl and yellow leaf curl diseases in West Africa: identification of a novel begomovirus-satellite DNA complex. Phytopathology 97:S154
4. Cohen S, Kern J, Harpaz I, Ben-Joseph R (1988) Epidemiological studies of the tomato yellow leaf curl virus (TYLCV) in the Jordan Valley, Israel. Phytoparasitica 16:259–270
5. Czosnek H, Laterrot H (1997) A worldwide survey of tomato yellow leaf curl viruses. Arch Virol 142:1391–1406
6. Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21
7. Dry IB, Rigden JE, Krake LR, Mullineaux PM, Rezaian MA (1993) Nucleotide sequence and genome organization of tomato leaf curl geminivirus. J Gen Virol 74:147–151
8. Faria JC, Maxwell DP (1999) Variability in geminivirus isolates associated with *Phaseolus* spp. in Brazil. Phytopathology 84:321–329
9. Fauquet C, Stanley J (2003) Geminivirus classification and nomenclature: progress and problems. Ann Appl Biol 142:165–189
10. Gilbertson RL, Faria JC, Hanson SF, Morales F, Ahlquist P, Maxwell DP, Russell DR (1991) Cloning of the complete DNA genomes of four bean-infecting geminiviruses and determining their infectivity by electric discharge particle acceleration. Phytopathology 81:980–985
11. Gilbertson RL, Hidayat SH, Paplomatas EJ, Rojas MR, Hou YM, Maxwell DP (1993) Pseudorecombination between infectious cloned DNA components of tomato mottle and bean dwarf mosaic geminiviruses. J Gen Virol 74:23–31
12. Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D (1999) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Plant Sci 18:71–106
13. Hong YG, Harrison BD (1995) Nucleotide sequences from tomato leaf curl viruses from different countries: evidence for three geographically separate branches in evolution of the coat protein of whitefly transmitted geminiviruses. J Gen Virol 76:2043–2049
14. Hou YM, Paplomatas EJ, Gilbertson RL (1998) Host adaptation and replication properties of two bipartite geminiviruses and their pseudorecombinants. Mol Plant-Microbe Interact 11:208–217
15. Kon T, Hidayat SH, Hase S, Takahashi H, Ikegami M (2006) The natural occurrence of two distinct begomoviruses associated with DNAβ and a recombinant DNA in a tomato plant from Indonesia. Phytopathology 96:517–525
16. Konate G, Fargette D, Swanson MM, Harrison BD (1995) Occurrence of whitefly-transmitted geminiviruses in crops in Burkina Faso, and their serological detection and differentiation. Ann Appl Biol 126:121–129
17. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
18. Legg JP, Fauquet CM (2004) Cassava mosaic geminiviruses in Africa. Plant Mol Biol 56:585–599
19. Mansoor S, Briddon RW, Zafar Y, Stanley J (2003) Geminivirus disease complexes: an emerging threat. Trends Plant Sci 8:128–134
20. Nakhla MK, Maxwell DP, Carvalho MG, Gilbertson RL (1994) Widespread occurrence of the eastern Mediterranean strain of tomato yellow leaf curl geminivirus in tomatoes in the Dominican Republic. Plant Dis 78:926
21. Navot N, Picherski E, Zeidan M, Zamir D, Czosnek H (1991) Tomato yellow leaf curl virus: a whitefly-transmitted geminivirus with a single genomic molecule. Virology 185:151–161
22. Paplomatas EJ, Patel VP, Hou YM, Noueiry AO, Gilbertson RL (1994) Molecular characterization of a new sap-transmissible bipartite genome geminivirus infecting tomatoes in Mexico. Phytopathology 84:1215–1224
23. Patel VP, Rojas MR, Paplomatas EJ, Gilbertson RL (1993) Cloning biologically active geminivirus DNA using PCR and overlapping primers. Nucleic Acids Res 21:1325–1326
24. Polston JE, Anderson P (1997) The emergence of whitefly-transmitted geminiviruses in tomato in the Western Hemisphere. Plant Dis 81:1358–1369
25. Polston J, McGovern RJ, Brown LG (1999) Introduction of tomato yellow leaf curl virus in Florida and implications for the spread of this and other geminiviruses of tomato. Plant Dis 83:984–988
26. Rasheed MS, Selth LA, Koltunow AM, Randles JW, Rezaian MA (2006) Single-stranded DNA of Tomato leaf curl virus accumulates in the cytoplasm of phloem cells. Virology 348:120–132
27. Rojas MR, Gilbertson RL, Russell DR, Maxwell DP (1993) Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis 77:340–347
28. Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu Rev Phytopathol 43:361–394
29. Rojas MR, Jiang H, Salati R, Xoconostle-Cazares B, Sudarshana M R, Lucas WJ, Gilbertson RL (2001) Functional analysis of proteins involved in movement of the monopartite begomovirus, Tomato yellow leaf curl virus. Virology 291:110–125
30. Salati R, Nahkla MK, Rojas MR, Guzman P, Jaquez J, Maxwell DP, Gilbertson RL (2002) *Tomato yellow leaf curl virus* in the Dominican Republic: Characterization of an infectious clone, virus monitoring in whiteflies, and identification of reservoir hosts. Phytopathology 92:487–496
31. Saunders K, Norman A, Gucciardo S, Stanley J (2004) The DNA beta satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity protein (betaC1). Virology 324:37–47
32. Seo Y-S, Zhou Y-C, Turini TA, Cook CG, Gilbertson RL, Natwick ET (2006) Evaluation of cotton germ plasm for resistance to the whitefly and cotton leaf crumple (CLCr) disease and etiology of CLCr in California's Imperial Valley. Plant Dis 90:877–884
33. Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, Ahrens U, Lorenz KH, Seemuller E, Kirkpatrick BC (1996) Phytoplasma-specific primers based on sequences of the 16S–23S rRNA spacer region. Appl Environ Microbiol 62:2988–2993
34. Varma A, Malathi VG (2003) Emerging geminivirus problems: a serious threat to crop production. Ann Appl Biol 142:145–164
35. Wyatt SD, Brown JK (1996) Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. Phytopathology 86:1288–1293
36. Zhou X, Xie Y, Tao X, Zhang Z, Li Z, Fauquet CM (2003) Characterization of DNAβ associated with begomoviruses in China and evidence for co-evolution with their cognate viral DNA-A. J Gen Virol 84:237–247