The Natural Occurrence of Two Distinct Begomoviruses Associated with DNAβ and a Recombinant DNA in a Tomato Plant from Indonesia

Tatsuya Kon, Sri H. Hidayat, Shu Hase, Hideki Takahashi, and Masato Ikegami

First, third, fourth, and fifth authors: Department of Life Science, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan; and second author: Department of Plant Pests and Diseases, Faculty of Agriculture, Bogor Agricultural University, Bogor 16680, Indonesia.
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ABSTRACT

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Two begomoviruses (Java virus-1 and Java virus-2), two satellite DNAs (DNAβ01 and DNAβ02), and a recombinant DNA (recDNA) were cloned from a single tomato plant from Indonesia with leaf curl symptoms, and the role of these satellite DNAs in the etiology of begomovirus disease was investigated. The genome organizations of the two viruses were similar to those of other Old World monopartite begomoviruses. Comparison of the sequences with other begomoviruses revealed that Java virus-1 was a newly described virus for which the name Tomato leaf curl Java virus (ToLCJAV) is proposed. Java virus-2 was a strain of *Ageratum yellow vein virus* (AYVV) (AYVV-[Java]). ToLCJAV or AYVV-[Java] alone did not induce leaf curl symptoms in tomato plants. However, in the presence of DNAβ02, both ToLCJAV and AYVV-[Java] induced leaf curl symptoms in tomato plants. In the presence of DNAβ01, these viruses induced mild leaf curl symptoms in tomato plants. The recDNA had a chimeric sequence, which arose from recombination among ToLCJAV, AYVV-[Java], DNAβ01, and DNAβ02; it was replicated only in the presence of AYVV-[Java] in tomato plants.

Geminiviruses have monopartite or bipartite circular single-stranded DNA (ssDNA) genomes, encapsidated in geminate particles. The family *Geminiviridae* consists of four genera, distinguished according to their host range, insect vector, and genome organization (11). The majority of the family *Geminiviridae* belong to the genus *Begomovirus*, which is transmitted by the whitefly (*Bemisia tabaci*). Tomato leaf curl diseases have caused losses in tomato production throughout the world (36), and many viruses causing leaf curl disease in tomato have been characterized (9,10,15,19,24,32). For example, *Tomato leaf curl New Delhi virus*-Severe (ToLCNDV-Svr) (24) has a bipartite genome consisting of two circular ssDNA molecules (DNA-A and DNA-B), whereas *Tomato leaf curl virus* (ToLCV) (9), *Tomato leaf curl Bangalore virus* (ToLCBV) (15), *Tobacco leaf curl Japan virus* (TbLCJV) (32), and *Tomato leaf curl Philippines virus* (ToLCPV) (19) are monopartite. Several distinct begomoviruses have been associated with tomato leaf curl in Indonesia (34).

Recently, satellite DNAs associated with several monopartite begomoviruses have been reported (3,5,16,27,28,37). Furthermore, infectious clones of the monopartite begomoviruses *Ageratum yellow vein virus* (AYVV) from Singapore, *Cotton leaf curl Multan virus* (CLCuMV) from Pakistan, *Bhendi yellow vein mosaic virus* (BYVMV) from India, *Tomato yellow leaf curl China virus*-[Tobacco Y10] (TYLCCNV-[Tb:Y10]), and *Eupatorium yellow vein virus* (EpYVV) were unable to induce typical symptoms. A novel group of satellite DNA molecules called DNAβ, which are associated with these viruses, is essential for inducing the characteristic disease symptoms (5,16,27,28,37). DNAβ is approximately half the size of the genomic DNA, and except for a conserved hairpin structure and a TAATATTAC loop sequence, has little sequence similarity with the genomic DNA of the associated begomovirus. DNAβ requires a helper begomovirus DNA for replication, encapsidation, insect transmission, and movement in plants (27).

In this paper, we identify and characterize two distinct begomoviruses, two satellite DNAβs, and a recombinant DNA from a single tomato plant with leaf curl symptoms from Indonesia. One begomovirus is newly described and is called Tomato leaf curl Java virus (ToLCJAV), whereas the other is a strain of AYVV-[Java]. Both satellite DNAβs (DNAβ01 and DNAβ02) replicated in the presence of either ToLCJAV or AYVV-[Java]. The recombinant DNA had a chimeric genome with sequences of begomovirus genomic DNA and satellite DNAβ.

Corresponding author: M. Ikegami; E-mail address: ikegami@bios.tohoku.ac.jp

The DDBJ, EMBL, and GenBank accession numbers of the sequences reported in this paper are AB100304 (ToLCJAV), AB100305 (AYVV-[Java]), AB100306 (DNAβ01), AB113651 (DNAβ02), and AB116230 (recombinant DNA).

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MATERIALS AND METHODS

Cloning of full-length viral genomic DNA. Tomato plants showing leaf curl symptoms were collected from Bandung, West Java, Indonesia. Nucleic acids were extracted from the leaf tissues of infected tomato plants, as described by Kon et al. (19). The polymerase chain reaction (PCR) was carried out as described by Sharma et al. (31).

Initially, a 2.7-kbp viral DNA PCR product was obtained using begomovirus DNA-specific degenerate primers UPV1 and UPC2 (6) and cloned into pGEM-T Easy vector (Promega, Madison, WI). Clones with two different sequences were obtained and designated Java virus-1 and Java virus-2. Two primers with an *Xba*I site (underlined), ToXba1 (5′-TCTAGACACGATGTTAG-TCACGTGGG-3′) and ToXba2 (5′-TCTAGACTCAAAAGA-CTGGACCTTAC-3′), were designed to obtain a full-length clone

of Java virus-1 (pToX1). Two primers with a *Bam*HI site (underlined), ToBam1 (5′-GGATCCTCTTTTGAACGAGTTTC-3′) and ToBam2 (5′-GGATCCCACATTTCTTTAATTTC-3′), were designed to obtain a full-length clone of Java virus-2 (pToB1).

Two methods were used in an attempt to detect a DNA-B component in virus-infected tomato plants. In the first method, PCR with CRv1 (5′-TAATATTACCGGATGGCCGC-3′) and CRc2 (5′-AAAAAATTATGCCA-3′) was designed to amplify near full-length DNA fragments representing the genome component(s) of the begomoviruses with an intergenic region (IR)/common region (CR) sequence with similarity to Java virus-1 and Java virus-2. The PCR-amplified viral genomic DNA was digested using restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, *Nco*I, and *Nde*I. In the second method, PCR detection of DNA-B was carried out using DNA-specific degenerate primers PCRc1 (5′-CTAGCTGCAGCATATTTACRARWATGCCA-3′; R = A, G; W = A, T) and PBL1v2040 (5′-GCCTCTGCAGCARTGRTCKATCTTCATACA-3′; K = G, T; R = A, G) (25).

Cloning of full-length satellite DNAβs, and satellite-like DNA. Initially, a 1.4-kbp DNAβ fragment was amplified using PCR and primers Beta01 and Beta02 designed for CLCuMV DNAβ (4). The PCR-amplified fragment was cloned in pGEM-T Easy vector and sequenced. Two different clones were obtained and designated DNAβ01 and DNAβ02. Primers StCla1 (5′-ATCGATGAATCTTTATACATGATCC-3′) and StCla2 (5′-ATCGATCGAGGAGATCAAAGCAGAAG-3′) were both designed with a *Cla*I site (underlined) to obtain full-length clones pToβ01 (DNAβ01) and pToβ02 (DNAβ02).

Initially, a 0.8-kbp satellite-like DNA fragment was amplified using primers V3518 and 3996 designed for AYVV recDNA (27). The PCR-amplified fragment was cloned in pGEM-T Easy vector and sequenced. Primers StBamH1 (5′-GGATCCTCTTTTGAACGAGTTTCCTG-3′) and StBamH2 (5′-GGATCCCACATGTTAAAATAATACTTGG-3′) were both designed with a *Bam*HI site (underlined) to obtain a full-length clone of satellite-like DNA (pToR1).

Sequence analysis. Sequencing was performed as described by Kon et al. (18) and the complete nucleotide sequences were compared with those of other monopartite begomoviruses or the DNA-A of bipartite begomoviruses. Phylogenetic analyses comparing Java virus-1, Java virus-2, open reading frame (ORF) V1 (coat protein [CP]), ORF C1 (replication associated protein: Rep), DNAβ01, or DNAβ02 sequences to other begomovirus sequences available from GenBank were computed from a distance matrix made using Kimura's two-parameter method. Multiple sequence alignments were performed using the optimal alignment method of DNAsis software. Phylogenetic trees were generated using the neighbor-joining method of MEGA version 3.0 with 1,000 bootstrap replications (20).

The detection of potential sequences, identification of likely parent sequences, and localization of possible recombination break points were carried out using RDP version 2.0 (22). To search for

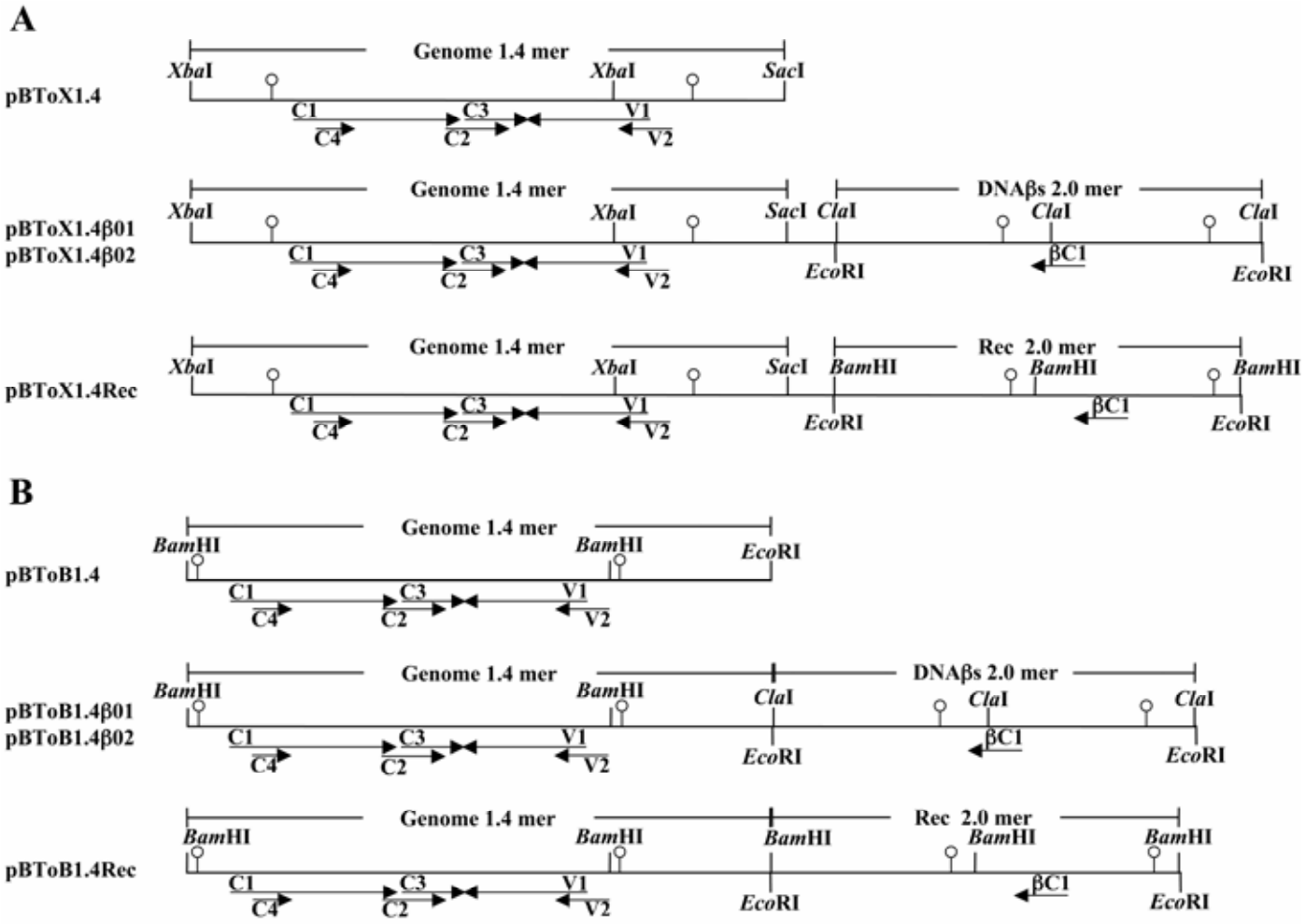


Fig. 1. Construction of infectious clones of Java virus-1, Java virus-2, DNAβs (DNAβ01 and DNAβ02), and satellite-like DNA. The open circles indicate the stem loop-forming region. The arrows represent predicted open reading frames in both orientations ("C" for complementary and "V" for viral sense). The restriction sites used for cloning are as follows: **A,** *Xba*I (nt 528) and *Sac*I (nt 2144) on Java virus-1 genome DNA, *Cla*I (nt 314) on DNAβ01 and 02, and *Bam*HI (nt 136) on satellite-like (rec) DNA; **B,** *Bam*HI (nt 139) and *Eco*RI (nt 1783) on Java virus-2 genome DNA, *Cla*I (nt 314) on DNAβ01 and 02, and *Bam*HI (nt 136) on satellite-like (rec) DNA.

recombination events, the RDP settings used were multiple comparison correction off, internal reference selection, highest acceptable probability 0.0001, and a window size of 10.

Constructing infectious clones and agroinoculation. Standard methods were used to produce the following clones containing partial and tandem repeats in pBI121 (Clontech, Palo Alto, CA) (Fig. 1): 1.4-mer of Java virus-1 (pBToX1.4), 1.4-mer of Java virus-2 (pBToB1.4), 1.4-mer of Java virus-1 + 2.0-mer of DNAβ01 (pBToX1.4β01), 1.4-mer of Java virus-1 + 2.0-mer of DNAβ02 (pBToX1.4β02), 1.4-mer of Java virus-2 + 2.0-mer of DNAβ01 (pBToB1.4β01), 1.4-mer of Java virus-2 + 2.0-mer of DNAβ02 (pBToB1.4β02), 1.4-mer of Java virus-1 + 2.0-mer of satellite-like DNA (pBToX1.4Rec), and 1.4-mer of Java virus-2 + 2.0-mer of satellite-like DNA (pBToX1.4Rec).

These recombinant plasmids were mobilized from *Escherichia coli* DH5α cells into *Agrobacterium tumefaciens* LBA4404 (14) by triparental mating using *E. coli* HB101 containing the helper plasmid pRK2013 (8). Tomato (*Lycopersicon esculentum* cv. Hausumomotaro) and *Nicotiana benthamiana* were used for agroinoculation experiments. Agroinoculation was performed as described by Kon et al. (18). Viral DNA was detected by PCR using viral genomic DNA-, DNAβ-, and satellite-like DNA-specific primers.

RESULTS

Genome organization of Java virus-1, Java virus-2, satellite DNAβs, and satellite-like DNA. The complete Java virus-1 and Java virus-2 genomes consisted of 2,752 and 2,735 nucleotides, respectively (accession nos. AB100304 and AB100305). The genome organizations of these viruses were similar to those of other monopartite begomoviruses originating from the Old World (Fig. 2). A 33-base potential stem loop-forming region was identical in the IRs of both viruses. In contrast, the iteron or Rep high-affinity binding site differed in Java virus-1 and Java virus-2. The Java virus-1 iteron was GGTCTCAA (nucleotides 2617 to 2624 and 2651 to 2659), whereas that of Java virus-2 was GGAGACA (nucleotides 2615 to 2621, 2642 to 2648, and 2649 to 2655). Efforts to detect DNA-B components associated with Java virus-1 and Java virus-2 based on restriction fragment length polymorphism analysis or PCR using primers PCRc1 and PBL1v2040 were unsuccessful. From these results, we concluded that Java virus-1 and Java virus-2 are monopartite begomoviruses.

DNAβ01 and DNAβ02 consisted of 1,346 and 1,354 nucleotides, respectively (accession nos. AB100306 and AB113651, respectively). Each DNAβ contained one complementary-sense ORF (βC1) (Fig. 2). DNAβ01 and DNAβ02 had no obvious sequence homology with the Java virus-1 or Java virus-2 genomic DNA, except for the nonanucleotide sequence TAATATT/AC. The sequences of DNAβ01 and DNAβ02 contained an A-rich region (≈65%) between nucleotides 736 to 941 and 721 to 950, respectively. The putative replication associated protein-binding motifs present in Java virus-1 (GGTCTCAA) and Java virus-2 (GGAGACA) were not found in DNAβ01 or DNAβ02, but similar sequences were located upstream from the stem loop structure: GGTGTGTA (nucleotides 1260 to 1267, DNAβ01: nucleotides 1268 to 1275, DNAβ02), GGTAGAAA (nucleotides 1278 to 1285, DNAβ01: nucleotides 1286 to 1293, DNAβ02), and GGAGAAG (nucleotides 1210 to 1216, DNAβ01: nucleotides 1218 to 1224, DNAβ02).

The nucleotide sequence of satellite-like DNA comprised 1,299 nucleotides (accession no. AB116230) and contained one complementary-sense ORF (βC1) (Fig. 2). The region from nucleotides 1112 to 233 of satellite-like DNA had the highest sequence identity with the corresponding sequences of Java virus-1 (nucleotides 2560 to 233: 82%) and Java virus-2 (nucleotides 2560 to 236: 84%). The satellite-like DNA sequence from nucleotides 367 to 1110 had the highest sequence identity with nucleotides 135 to 877 of DNAβ01 (86%) and nucleotides 135 to 879 of DNAβ02 (94%) (Fig. 2). The iteron (GGTCTCAA) present in Java virus-1 was not found in satellite-like DNA, whereas the iteron (GGAGACA) in Java virus-2 was found in satellite-like DNA (virus sense nucleotides 1167 to 1173, 1194 to 1200, and 1201 to 1207).

Sequence analysis of viral genomic DNA, DNAβ, and satellite-like DNA. The complete nucleotide sequences of Java virus-1 and Java virus-2 were compared with those of other begomoviruses. Java virus-1 had nucleotide sequence identities with AYVV of 87% and Java virus-2 of 85%. For ORF V1 (CP), Java virus-1 had the highest amino acid sequence identities with its counterparts from AYVV (87%), *Tomato leaf curl Laos virus* (ToLCLV) (87%), and Java virus-2 (87%). For ORF C1 (Rep), Java virus-1 had the highest amino acid sequence identities with its counterparts from AYVV (90%) and Java virus-2 (86%). Java virus-1 had nucleotide sequence IR identities with AYVV of 82% and Java virus-2 of 73%.

Java virus-2 had the highest total nucleotide sequence identity with AYVV (92%). When the deduced amino acid sequence of the CP was compared, Java virus-2 had high amino acid sequence identity with its counterpart from AYVV (97%). For Rep, Java virus-2 had the highest amino acid sequence identity with its counterparts from AYVV (93%). Java virus-2 had a nucleotide sequence IR identity with AYVV of 75%.

The phylogenetic tree based on comparison of the complete nucleotide sequence (DNA-A) showed that Java virus-2 clustered with *Ageratum yellow vein Taiwan virus*-[Taiwan] (AYVTV-[TW]), *Ageratum yellow vein China virus*-[Hainan 2] (AYVCNV-[Hn2]), and Java virus-1 (Fig. 3A). A similar relationship held when the CP sequence of Java virus-2 was analyzed (Fig. 4A). The phylogenetic analysis of the CP sequence revealed that Java virus-1 was distantly related to the ageratum-infecting begomovirus group (Fig. 4A). The phylogenetic tree based on comparison of the Rep sequences showed that Java virus-2 was similar to AYVV and clustered with ToLCPV, ToLCLV, and Java virus-1 (Fig. 4B). Fauquet and Stanley (11) proposed that a virus with less than 90%

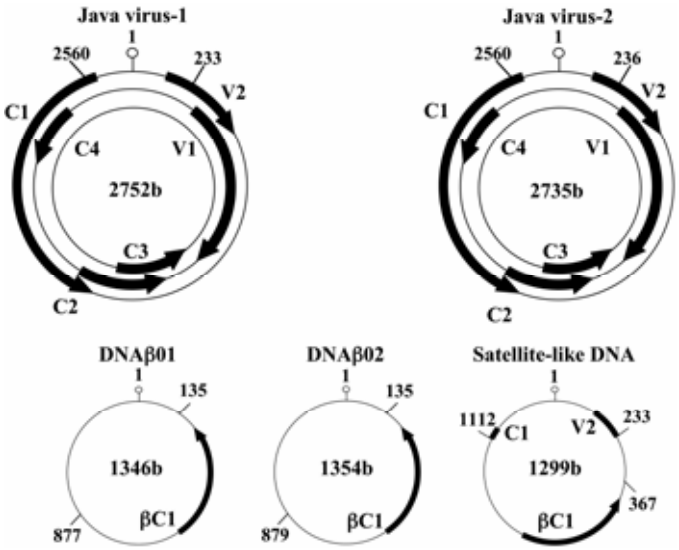


Fig. 2. The genome organizations of Java virus-1, Java virus-2, DNAβs (DNAβ01 and DNAβ02), and satellite-like DNA. Open circles indicate the stem loop-forming region containing the conserved nonanucleotide TAATATT↓AC. Nucleotide numbering starts from the nick site (↓) within the conserved nonanucleotide. Arrows represent predicted open reading frames in both orientations ("C" for complementary and "V" for viral sense) and potentially encoded proteins are shown as black arrows. The region of satellite-like DNA from nt 1112 to 233 originates from Java virus-1 (nt 2560 to 233) and Java virus-2 (nt 2560 to 236) genomic DNAs. The sequences of the satellite-like DNA (nt 367 to 1112), DNAβ01 (nt 135 to 877), and DNAβ02 (nt 135 to 879) have high sequence identity.

identity for the complete genome and CP sequences should be considered a distinct species. Therefore, Java virus-1 is proposed to be a new species: Tomato leaf curl Java virus (ToLCJAV), whereas Java virus-2 is a strain of AYVV, AYVV-[Java].

A phylogenetic tree was constructed from the alignment of the complete nucleotide sequences of DNAβ01, DNAβ02, and selected DNAβs associated with other begomoviruses. DNAβ01 and DNAβ02 were similar to AYVV DNAβ, but distantly related to other DNAβs associated with other begomoviruses (Fig. 3B). The total nucleotide sequence of DNAβ01 had the highest identity (93%) with that of DNAβ02, followed by AYVV DNAβ (81%). The amino acid sequence of the βC1 product of DNAβ01

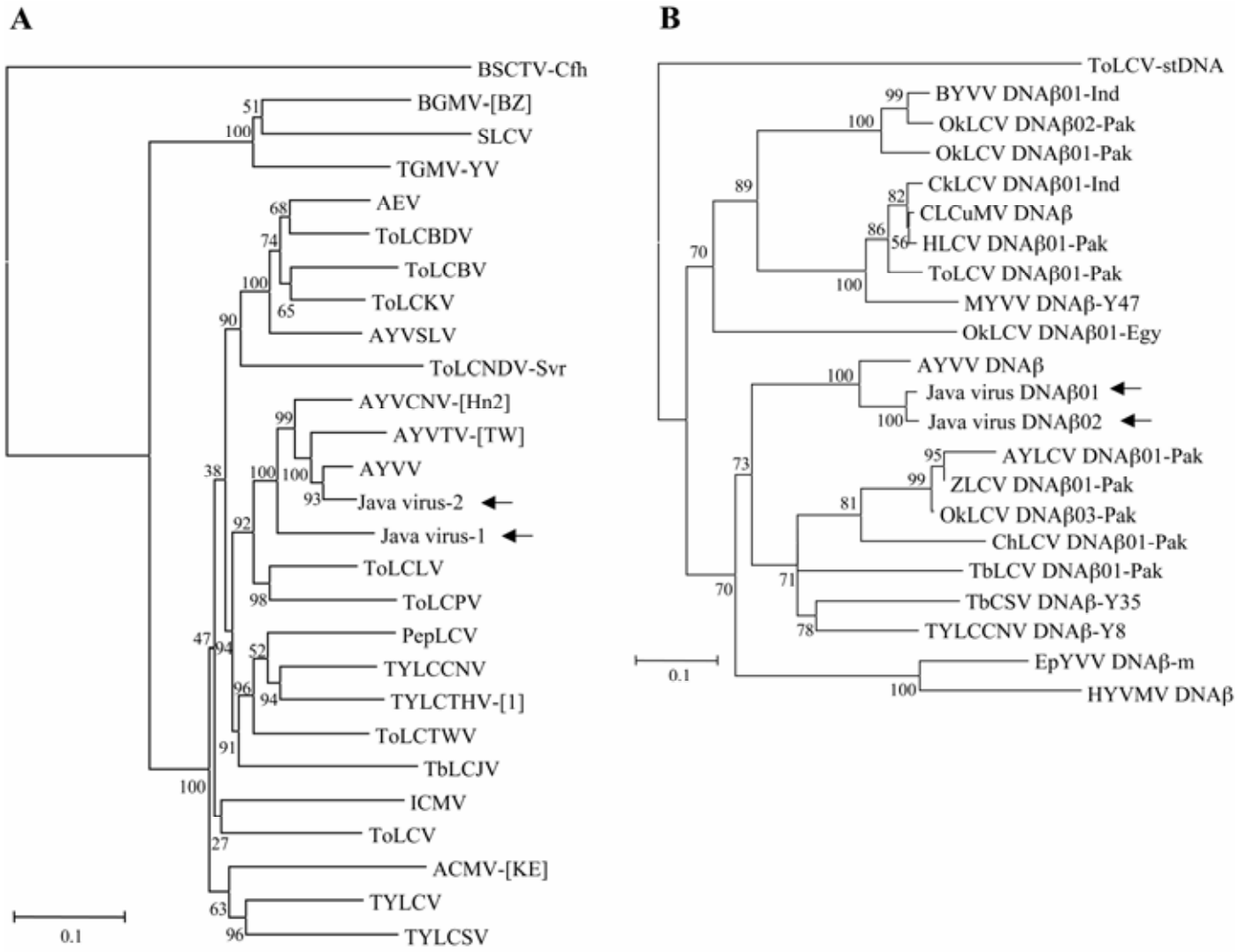


Fig. 3. A, Neighbor-joining phylogenetic tree obtained from the complete nucleotide sequences of Java virus-1, Java virus-2, and other begomovirus DNA-A components. The vertical distances are arbitrary and the horizontal distances are proportional to the calculated mutation distances. The tree was rooted on the sequence of *Beet severe curly top virus*-Cfh (BSCTV-Cfh: accession no. U02311), belonging to the distinct genus *Curtovirus*. The following sequences were obtained from GenBank and used for comparisons and phylogenetic analysis: *African cassava mosaic virus*-Kenya (ACMV-[KE]: J02057, DNA-A), *Ageratum enation virus* (AEV: AJ437618), *Ageratum yellow vein China virus*-[Hainan 2] (AYVCNV-[Hn2]: AJ495813), *Ageratum yellow vein SriLanka virus* (AYVSLV: AF314144), *Ageratum yellow vein Taiwan virus*-[Taiwan] (AYVTV-[TW]: AF307861), *Ageratum yellow vein virus* (AYVV: X74516), *Bean golden mosaic virus*-[Brazil] (BGMV-[BZ]: M88686, DNA-A), *Indian cassava mosaic virus* (ICMV: Z24758, DNA-A), *Pepper leaf curl virus* (PepLCV: AF134484), *Squash leaf curl virus* (SLCV: M38182, DNA-A), *Tobacco leaf curl Japan virus* (TbLCJV: AB028604), *Tomato golden mosaic virus*-Yellow vein (TGMV-YV: K02029, DNA-A), *Tomato leaf curl Bangalore virus* (ToLCBV: Z48182), *Tomato leaf curl Bangladesh virus* (ToLCBDV: AF188481), *Tomato leaf curl Karnataka virus* (ToLCKV: U38239), *Tomato leaf curl Laos virus* (ToLCLV: AF195482), *Tomato leaf curl New Delhi virus*-Severe (ToLCNDV-Svr: U15015, DNA-A), *Tomato leaf curl Philippines virus* (ToLCPV: AB050597), *Tomato leaf curl Taiwan virus* (ToLCTWV: U88692), *Tomato leaf curl virus* (ToLCV: S53251), *Tomato yellow leaf curl China virus* (TYLCCNV: AF311734), *Tomato yellow leaf curl Sardinia virus* (TYLCSV: X61153), *Tomato yellow leaf curl Thailand virus*-[1] (TYLCTHV-[1]: X63015, DNA-A), and *Tomato yellow leaf curl virus* (TYLCV: X15656). **B,** Neighbor-joining phylogenetic tree obtained for DNAβ01, DNAβ02, and other begomovirus DNAβ molecules. The tree was rooted on the sequence of *Tomato leaf curl virus* satellite DNA (ToLCV stDNA: accession no. U74627), a distinct sequence in the DNAβ of other begomoviruses. The vertical distances are arbitrary and the horizontal distances are proportional to the calculated mutation distances. The following sequences were obtained from the GenBank or EMBL databases and used for comparisons and phylogenetic analysis: *Ageratum yellow leaf curl virus* DNAβ01-Pakistan (AYLCV DNAβ01-Pak: AJ316026), AYVV DNAβ (AJ252072), *Bhendi yellow vein mosaic virus* DNAβ (BYVMV DNAβ: AJ308425), *Chili leaf curl virus* DNAβ01-Pak (ChLCV DNAβ01-Pak: AJ316032), *Cotton leaf curl Multan virus* DNAβ (CLCuMV DNAβ: AJ292769), *Cotton leaf curl virus* DNAβ01-India (CLCuVβ01-Ind: AJ316038), *Eupatorium yellow vein virus* DNAβ (EpYVV DNAβ-m: AJ438938), *Hibiscus leaf curl virus* DNAβ01-Pakistan (HLCV DNAβ01-Pak: AJ297908), *Honeysuckle yellow vein virus* DNAβ (HYVMV DNAβ: AJ316040), *Malvastrum yellow vein virus* DNAβ (MYVV DNAβ-Y47: AJ421482), *Okra leaf curl virus* DNAβ01-Egypt (OkLCV DNAβ01-Egy: AF397217), *Okra leaf curl virus* DNAβ01-Pakistan (OkLCV DNAβ01-Pak: AJ316029), *Okra leaf curl virus* DNAβ02-Pakistan (OkLCV DNAβ02-Pak: AJ316030), *Okra leaf curl virus* DNAβ03-Pakistan (OkLCV DNAβ03-Pak: AJ316031), *Tobacco curly shoot virus*-Y35 DNAβ (TbCSV DNAβ-Y35: AJ421484), *Tobacco leaf curl virus* DNAβ01-Pakistan (TbLCV DNAβ01-Pakistan: AJ316033), *Tomato leaf curl virus* DNAβ01-Pakistan (ToLCV DNAβ01-Pak: AJ316035), *Tomato yellow leaf curl China virus* DNAβ-Y8 (AJ421622), and *Zinnia leaf curl virus* DNAβ01-Pakistan (ZLCV DNAβ01-Pak: AJ316041).

had the highest identity (89%) with that of DNAβ02. The βC1 products of DNAβ01 and DNAβ02 also had relatively high amino acid sequence identities (79 and 85%, respectively) with that of AYVV DNAβ.

We tested whether satellite-like DNA had a chimeric genome that arose from viral genomic DNA and satellite DNA using RDP. RDP provided evidence for the occurrence of recombination among ToLCJAV, AYVV-[Java], DNAβ01, and DNAβ02 (Fig. 5). In these recombination events, a region (nucleotides 1155 to 39) of recDNA corresponds to nucleotides 2633 to 43 of AYVV-[Java] genomic DNA ($P = 2.870 \times 10^{-13}$), which included the IR. Two regions (nucleotides 77 to 123 and 190 to 216) of recDNA also correspond to nucleotides 77 to 123 and nucleotides 190 to 216 of ToLCJAV genomic DNA ($P = 2.580 \times 10^{-5}$ and 3.670×10^{-4}, respectively). An additional two regions (nucleotides 367 to 883 and 450 to 1110) of recDNA correspond to nucleotides 135 to 652 of DNAβ01 ($P = 9.981 \times 10^{-5}$) and nucleotides 219 to 879 of DNAβ02 ($P = 4.210 \times 10^{-9}$), respectively. From these results, we concluded that satellite-like DNA had a chimeric genome that arose from recombination involving ToLCJAV, AYVV-[Java], DNAβ01, and DNAβ02, and was given the name recombinant DNA (recDNA).

Infectivity and symptoms induced by viral genomic DNA, DNAβs, and recDNA. The infectivity of ToLCJAV, AYVV-[Java], DNAβ01, DNAβ02, and recDNA was examined in tomato and *N. benthamiana* (Table 1). Plants mock-agroinoculated with *Agrobacterium* cells with the empty binary vector did not develop symptoms (Fig. 6A). Tomato plants agroinoculated with ToLCJAV (clone pBToX1.4) were infected systemically, but displayed no symptoms (Fig. 6B). When tomato plants were co-agroinoculated with ToLCJAV and DNAβ01 (clone pBToX1.4β01), mild downward leaf curling symptoms were produced (Fig. 6G); whereas when tomato plants were co-agroinoculated with ToLCJAV and DNAβ02 (clone pBToX1.4β02), severe downward leaf curling symptoms developed (Fig. 6H). PCR detected ToLCJAV-, DNAβ01-, and DNAβ02-specific DNAs from agroinoculated tomato plants (Fig. 7A and B), and the PCR-amplified product was cloned and sequenced.

N. benthamiana plants agroinoculated with ToLCJAV developed mild downward leaf curling symptoms (Table 1). *N. benthamiana* plants co-agroinoculated with ToLCJAV and DNAβ01 developed mild downward leaf curling symptoms, whereas *N. benthamiana* plants co-agroinoculated with ToLCJAV and DNAβ02 developed severe downward leaf curling symptoms (Table 1).

Tomato plants agroinoculated with AYVV-[Java] (clone pBToB1.4) alone were infected systemically, but displayed no symptoms (Fig. 6C). Tomato plants co-agroinoculated with AYVV-[Java] and DNAβ01 (clone pBToB1.4β01) developed mild downward leaf curling (Fig. 6J), whereas tomato plants agroinoculated with AYVV-[Java] and DNAβ02 (clone pBToB1.4β02)

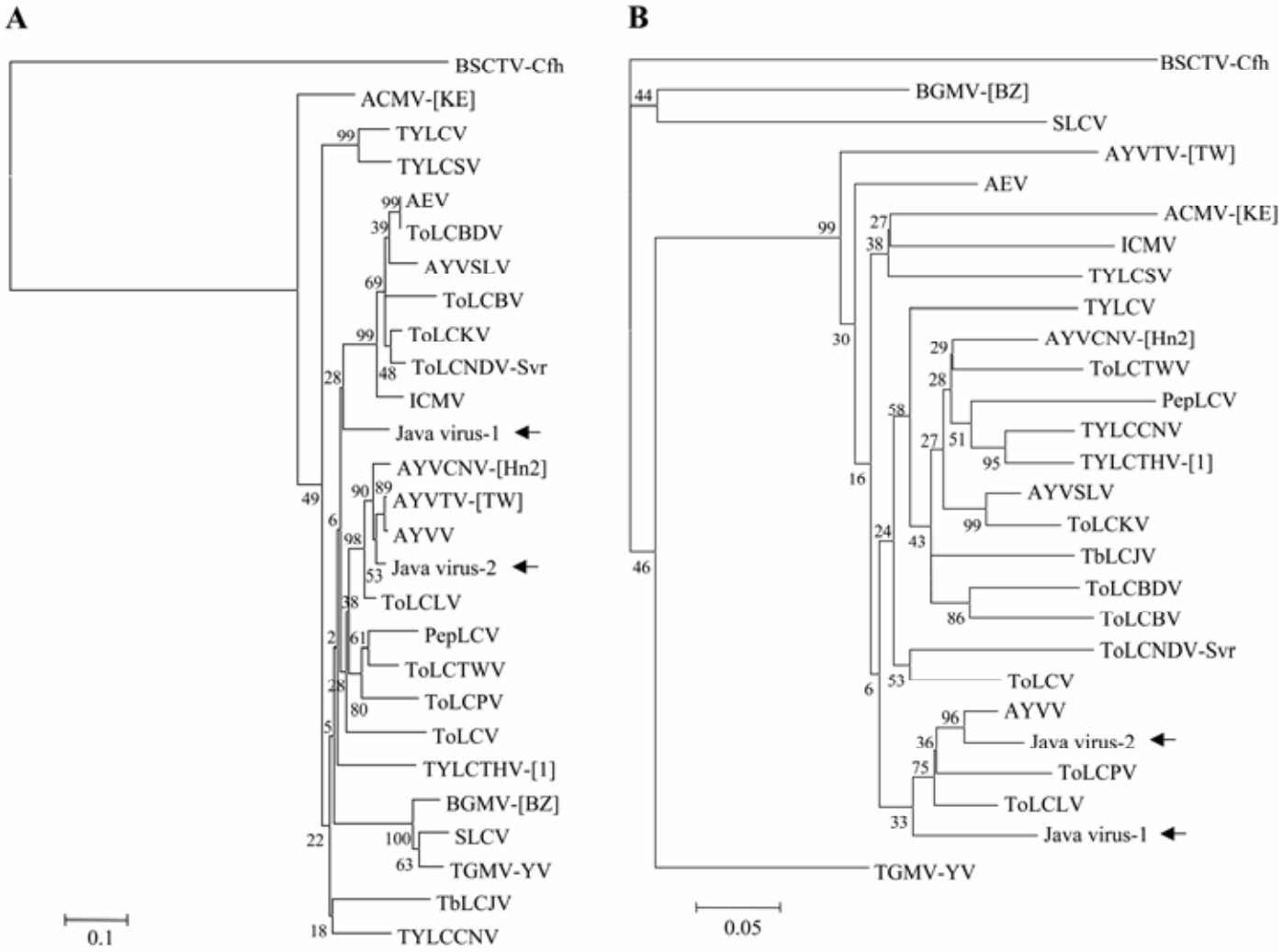


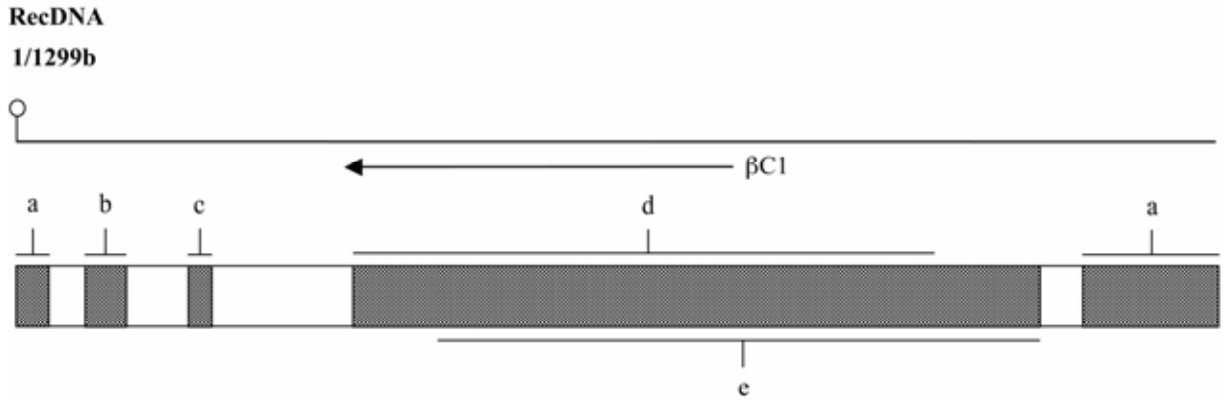
Fig. 4. A, Neighbor-joining phylogenetic tree obtained from the amino acid sequences of the coat proteins of Java virus-1, Java virus-2, and other begomoviruses. The vertical distances are arbitrary and the horizontal distances are proportional to the calculated mutation distances. **B,** Neighbor-joining phylogenetic tree obtained from the amino acid sequences of the replication associated proteins of Java virus-1, Java virus-2, and other begomoviruses. The vertical distances are arbitrary and the horizontal distances are proportional to the calculated mutation distances.

developed severe downward leaf curling (Fig. 6K). AYVV-[Java]-, DNAβ01-, and DNAβ02-specific DNAs were detected by PCR from agroinoculated tomato plants (Fig. 7A and B). No symptoms were observed on tomato plants agroinoculated with DNAβ01 or DNAβ02 alone (Fig. 6D and E).

N. benthamiana plants agroinoculated with AYVV-[Java] developed severe upward leaf curling and vein swelling symptoms (Table 1). *N. benthamiana* plants co-agroinoculated with AYVV-[Java] and DNAβ01 developed mild downward leaf curling symptoms, whereas *N. benthamiana* plants co-agroinoculated with AYVV-[Java] and DNAβ02 developed severe downward leaf curling symptoms (Table 1). Therefore, both DNAβ01 and DNAβ02 were replicated in the presence of either virus.

We tested whether recDNA is replicated by either ToLCJAV or AYVV-[Java]. Tomato plants co-agroinoculated with ToLCJAV and recDNA (clone pBToX1.4Rec) displayed no symptoms (Fig. 6I). ToLCJAV genomic DNA was detected in infected tomato plants, but not recDNA by PCR analysis (Fig. 7A and B). *N. benthamiana* plants co-agroinoculated with ToLCJAV and recDNA developed mild downward leaf curling symptoms (Table 1). Tomato plants co-agroinoculated with recDNA and AYVV-[Java] (clone pBToB1.4Rec) displayed no symptoms (Fig. 6L). However, both AYVV-[Java] genomic DNA and recDNA were detected in agroinoculated tomato plants by PCR analysis (Fig. 7A and B). No symptoms were observed in tomato plants agroinoculated with recDNA alone (Fig. 6F). *N. benthamiana* plants agroinoculated with AYVV-[Java] and recDNA developed severe upward leaf curling symptoms (Table 1).

When tomato plants were agroinoculated with ToLCJAV, AYVV-[Java], DNAβ01, DNAβ02, and recDNA, leaf curl symp-



Event	Region	Origin	*P*-value
a	nt. 1153-39	AYVV-[Java] (nt. 2633-43)	2.870×10^{-13}
b	nt. 77-123	ToLCJAV (nt. 81-129)	2.580×10^{-5}
c	nt. 190-216	ToLCJAV (nt. 218-246)	3.670×10^{-4}
d	nt. 367-883	DNAβ01 (nt. 135-652)	9.981×10^{-5}
e	nt. 450-1110	DNAβ02 (nt. 219-879)	4.210×10^{-9}

Fig. 5. A schematic representation of the recombinant regions in satellite-like DNA. Potential recombination break points and the probability that the indicated regions do not have a recombination origin are presented. The shading patterns indicate the viral genomic DNA or satellite DNAβ origins of the recombinant region. The open circle indicates the stem loop-forming region. The βC1 open reading frame is indicated with an arrow.

TABLE 1. Symptom phenotypes of Tomato leaf curl Java virus (ToLCJAV), *Ageratum yellow vein virus* (AYVV-[Java]), DNAβ01, DNAβ02, and recDNA

Plant	Inoculum[a]	Infectivity[b]	Symptoms
Tomato	pBToX1.4	19/24 (3)	No symptoms
(*Lycopersicon esculentum*)	pBToX1.4β01	20/24 (3)	Mild downward leaf curling
cv. Hausumomotaro	pBToX1.4β02	23/24 (3)	Severe downward leaf curling
	pBToX1.4Rec	20/24 (3)	No symptoms
	pBToB1.4	20/24 (3)	No symptoms
	pBToB1.4β01	20/24 (3)	Mild downward leaf curling
	pBToB1.4β02	23/24 (3)	Severe downward leaf curling
	pBToB1.4Rec	21/24 (3)	No symptoms
	pBToX1.4β01 + pBToB1.4β02 + pBToRec	25/32 (4)	Severe downward leaf curling
Nicotiana benthamiana	pBToX1.4	14/15 (3)	Mild downward leaf curling
	pBToX1.4β01	14/15 (3)	Mild downward leaf curling
	pBToX1.4β02	15/15 (3)	Severe downward leaf curling and stunting
	pBToX1.4Rec	15/15 (3)	Mild downward leaf curling
	pBToB1.4	15/15 (3)	Severe upward leaf curling and vein swelling
	pBToB1.4β01	14/15 (3)	Mild downward leaf curling
	pBToB1.4β02	15/15 (3)	Severe downward leaf curling and stunting
	pBToB1.4Rec	15/15 (3)	Severe upward leaf curling and vein swelling
	pBToX1.4β01 + pBToB1.4β02 + pBToRec	23/25 (4)	Severe downward leaf curling and stunting

[a] pBToX1.4, ToLCJAV; pBToX1.4β01, ToLCJAV + DNAβ01; pBToX1.4β02, ToLCJAV + DNAβ02; pBToX1.4Rec, ToLCJAV + recDNA; pBToB1.4, AYVV-[Java]; pBToB1.4β01, AYVV-[Java] + DNAβ01; pBToB1.4β02, AYVV-[Java] + DNAβ02; pBToB1.4Rec, AYVV-[Java] + recDNA; and pBToRec, recDNA.
[b] Number of infected plants/number of plants inoculated (number of experiments).

toms developed and resembled the symptoms in tomato plants infected with ToLCJAV and DNAβ02 or AYVV-[Java] and DNAβ02 (Fig. 6M). The presence of viral genomic DNA, DNAβs, and recDNA in agroinoculated plants was confirmed by PCR analysis (Fig. 7A and B).

DISCUSSION

In this study, we described the cloning, sequencing, phylogenetics, and biological activities of two distinct monopartite begomovirus species along with two associated satellite DNAβs

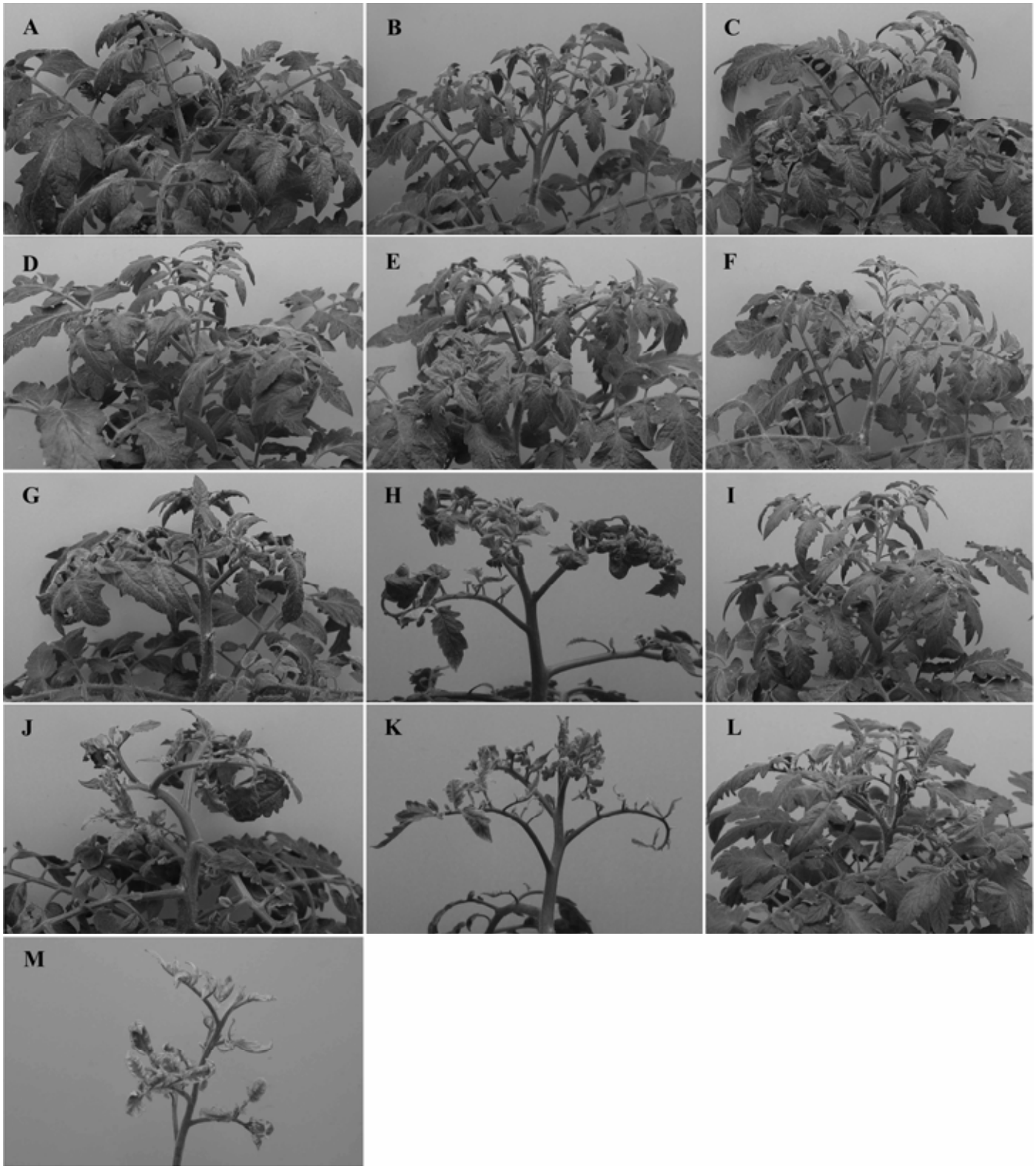


Fig. 6. Tomato plants inoculated with **A,** mock-agroinoculation, **B,** Tomato leaf curl Java virus (ToLCJAV) alone, **C,** *Ageratum yellow vein virus* (AYVV-[Java]) alone, **D,** DNAβ01 alone, **E,** DNAβ02 alone, **F,** recDNA alone, **G,** ToLCJAV + DNAβ01, **H,** ToLCJAV + DNAβ02, **I,** ToLCJAV + recDNA, **J,** AYVV-[Java] + DNAβ01, **K,** AYVV-[Java] + DNAβ02, **L,** AYVV-[Java] + recDNA, and **M,** ToLCJAV + AYVV-[Java] + DNAβ01 + DNAβ02 + recDNA. The plants were photographed 25 days postinoculation.

as well as a recombinant defective DNA which combined sequences of a parent begomovirus and a DNAβ from tomato plants with leaf curl in Java, Indonesia. This is the first detailed analysis for Indonesia, extending earlier partial investigations of the same authors (34). Comparisons of the complete nucleotide sequences with other begomoviruses revealed that Java virus-1 is a new species for which the name Tomato leaf curl Java virus (ToLCJAV) is proposed, whereas Java virus-2 was identified as a strain of AYVV, AYVV-[Java]. We previously reported that several begomoviruses are associated with tomato leaf curl disease in Java, Indonesia (34). Based on sequence comparisons and phylogenetic analyses, the viruses clustered into two groups. Group I was most closely related to AYVV-[Java] and group II had high sequence identity with ToLCJAV. The infectious clones of ToLCJAV or AYVV-[Java] alone did not induce leaf curl symptoms in tomato plants. By contrast, in the presence of DNAβ02, either ToLCJAV or AYVV-[Java] induced leaf curl symptoms in tomato plants. The leaf symptoms that DNAβ02 induced in agroinoculated tomato plants were similar to those observed in the field in Java, Indonesia. ToLCJAV/DNAβ02 and AYVV-[Java]/DNAβ02 may be tomato leaf curl disease complexes. Neither ToLCJAV nor AYVV-[Java] induced leaf curl symptoms in tomato plants in the presence of DNAβ01. Therefore, DNAβ01 appears to cause mild leaf curl symptoms that were observed in tomato plants, and may be defective, yet maintained in plants by ToLCJAV or AYVV-[Java].

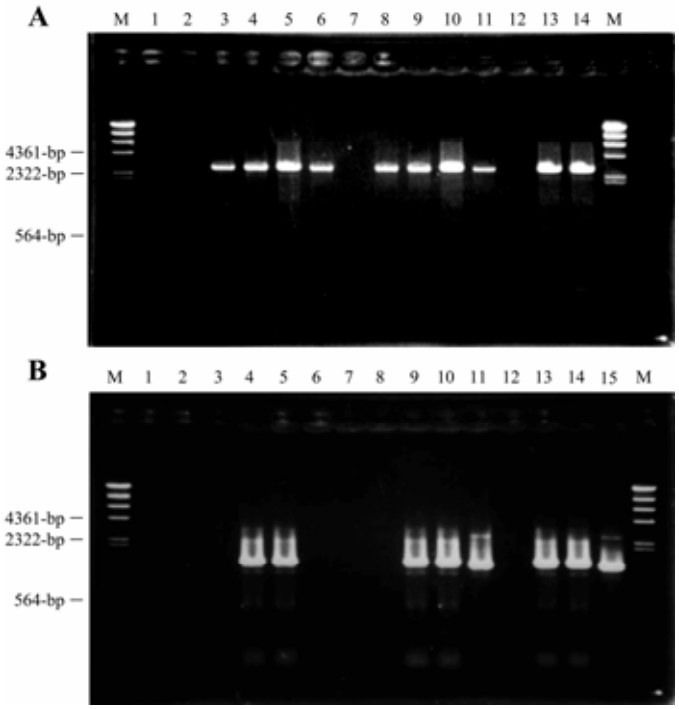


Fig. 7. Agarose gel electrophoresis of the polymerase chain reaction products obtained from agroinoculated tomato plants. Total DNA was extracted from the upper leaves of each tomato plant. M is a size marker consisting of λDNA digested with *Hin*dIII. **A,** Plant samples: mock-agroinoculation (lane 1), DNAβ01 alone (lane 2), Tomato leaf curl Java virus (ToLCJAV) alone (lane 3), ToLCJAV + DNAβ01 (lane 4), ToLCJAV + DNAβ02 (lane 5), ToLCJAV + recDNA (lane 6), DNAβ01 alone (lane 7), *Ageratum yellow vein virus* (AYVV-[Java]) alone (lane 8), AYVV-[Java] + DNAβ01 (lane 9), AYVV-[Java] + DNAβ02 (lane 10), AYVV-[Java] + recDNA (lane 11), recDNA alone (lane 12), and ToLCJAV + AYVV-[Java] + DNAβ01 + DNAβ02 + recDNA (lanes 13 and 14). The viral genomic full-length DNA products were amplified with the primer pairs ToXba1 and ToXba2 (lanes 1 to 6 and 13) and ToBam1 and ToBam2 (lanes 7 to 12 and 14). **B,** Plant samples: mock-agroinoculation (lane 1), DNAβ01 alone (lane 2), ToLCJAV alone (lane 3), ToLCJAV + DNAβ01 (lane 4), ToLCJAV + DNAβ02 (lane 5), ToLCJAV + recDNA (lane 6), DNAβ01 alone (lane 7), AYVV-[Java] alone (lane 8), AYVV-[Java] + DNAβ01 (lane 9), AYVV-[Java] + DNAβ02 (lane 10), AYVV-[Java] + recDNA (lane 11), recDNA alone (lane 12), and ToLCJAV + AYVV-[Java] + DNAβ01 + DNAβ02 + recDNA (lanes 13 to 15). The DNAβ01 and DNAβ02 full-length DNA fragments were amplified with the primer pairs StCla1 and StCla2 (lanes 1 to 10, 13, and 14) and the recDNA full-length DNA products were amplified with the primer pairs StBamH1 and StBamH2 (lanes 11, 12, and 15).

DNAβ relies on its helper begomovirus for replication, movement within plants, and transmission between plants (27). DNAβ01 and DNAβ02 each contained an A-rich sequence. The A-rich region may have originated via sequence duplication to satisfy a size requirement for encapsidation and virus systemic movement (27). We showed that two DNAβ molecules (DNAβ01 and DNAβ02) are replicated by two distinct monopartite begomoviruses (ToLCJAV and AYVV-[Java]). The iteron is the sequence-specific binding site for Rep (2,7,13). The putative Rep-binding motifs present in ToLCJAV and AYVV-[Java] were not found in DNAβ01 and DNAβ02, but similar sequences are located upstream from the stem loop structure in these molecules. DNAβ01 and DNAβ02 do not contain the iterons of their respective helper begomoviruses, suggesting a more relaxed specificity for begomovirus Rep binding during their replication. DNAβ appears to be capable of being replicated by the Rep proteins from a diverse range of begomoviruses (1,3,21,26,30).

The recDNA had the highest sequence identity with ToLCJAV, AYVV-[Java], DNAβ01, and DNAβ02. Therefore, recDNA has a chimeric genome, which arose via recombination between a begomovirus and DNAβ. Further evidence for recombination in recDNA involving ToLCJAV, AYVV-[Java], DNAβ01, and DNAβ02 was supported by the statistical analysis using RDP. The recDNA replicated in the presence of AYVV-[Java], but not in the presence of ToLCJAV. The iteron sequence of recDNA was identical with that of AYVV-[Java]. A similar recombinant DNA component was found in AYVV-infected *Ageratum conyzoides* plants and *Cotton leaf curl virus*-infected cotton plants (5,27). These results demonstrate the potential for the emergence of new satellite-like DNAs by recombination between previously existing begomoviruses and satellite DNAβs.

Tan et al. (35) showed that AYVV could systemically infect tomato plants; however, the role of AYVV in tomato leaf curl in the field remained to be established. In the presence of DNAβ02, AYVV-[Java] caused tomato leaf curl disease in tomato plants, suggesting a possible role for this virus in this disease. The natural host of AYVV is *A. conyzoides*, a common weed, which often grows near tomato fields in Southeast Asia, including Indonesia, and frequently exhibits yellow vein symptoms. Thus, this weed could be a reservoir for viruses that cause tomato leaf curl disease. AYVV-[Java] alone induced upward leaf curling and vein swelling in *N. benthamiana* plants, but the symptoms changed to downward leaf curling in the presence of DNAβ01 or DNAβ02. Consistent with our results, a change from upward to downward leaf curling in the presence of DNAβ has been demonstrated experimentally for AYVV in *N. benthamiana* plants (21, 29,33). These results further support the hypothesis that DNAβ plays an important role in symptom induction.

We isolated two distinct begomovirus species from a single tomato plant with leaf curl symptoms. When two distinct begomoviruses infect a single plant in the field, the viruses may replicate simultaneously in the same cell, which is a prerequisite for recombination (23). A mixed infection is one of the prerequisites for developing new recombinant viruses or strains. The emergence of new begomovirus species in nature via recombination between existing species has been demonstrated (12,17,30). We have isolated a new recombinant begomovirus that arose between ToLCJAV and AYVV-[Java] in *A. conyzoides* plants, which will be reported elsewhere.

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