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SHORT COMMUNICATION

Tomato leaf curl Guangxi virus is a distinct monopartite begomovirus species

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Abstract Three begomovirus isolates were obtained from tomato plants showing leaf curl symptoms in Guangxi province of China. Typical begomovirus DNA components representing the three isolates (GX-1, GX-2 and GX-3) were cloned and their full-length sequences were determined to be 2752 nucleotides. Nucleotide identities among the three viral sequences were 98.9–99.7%, but all shared <86.7% nucleotide sequence identity with other reported begomoviruses. The sequence data indicated that GX-1, GX-2 and GX-3 are isolates of a distinct begomovirus species for which the name Tomato leaf curl Guangxi virus (ToLCGXV) is proposed. Further analysis indicated that ToLCGXV probably originated through recombination among viruses related to Ageratum yellow vein virus, Tomato leaf curl China virus and Euphorbia leaf curl virus. PCR and Southern blot analyses demonstrated that isolates GX-1 and GX-2 were associated with DNAβ components, but not isolate GX-3. Sequence comparisons revealed that GX-1 and GX-2 DNAβ components shared the highest sequence identity (86.2%) with that of Tomato yellow leaf curl China virus (TYLCCNV). An infectious construct of ToLCGXV isolate GX-1 (ToLCGXV-GX) was produced and determined to be highly infectious in *Nicotiana benthamiana*, *N. glutinosa*, tobacco cvs. Samsun and Xanthi, tomato and *Petunia hybrida* plants inducing leaf curl and stunting symptoms. Co-inoculation of tomato plants with ToLCGXV-GX and TYLCCNV DNAβ resulted in disease symptoms similar to that caused by ToLCGXV-GX alone or that observed in infected field tomato plants.

Keywords Tomato · Tomato leaf curl Guangxi virus · Begomovirus · Satellite DNA · DNAβ · Recombinant

Geminiviruses are plant DNA viruses that are characterized by small circular single-stranded DNA (ssDNA) genomes and encapsidated in twinned icosahedral particles (Lazarowitz 1992). Geminiviruses are divided into four different genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*) according to their genome organizations, insect vectors and ranges of host plant (Fauquet et al. 2003). Most economically important geminiviruses belong to the genus *Begomovirus*, known to be transmitted exclusively through whiteflies (*Bemisia tabaci*) and infect only dicotyledonous plants. Most known begomoviruses comprise two similar-sized DNA components referred to as DNA-A and DNA-B.

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Other begomoviruses have only a single genomic component similar to the DNA-A and do not require a DNA-B component for infection of host plants (Dry et al. 1993; Navot et al. 1991; Xie and Zhou 2006). Several monopartite begomoviruses including Ageratum yellow vein virus (AYVV), Bhendi yellow vein mosaic virus (BYVMV), Cotton leaf curl Multan virus (CLCuMV) and Tomato yellow leaf curl China virus (TYLCCNV) were reported to contain a novel single-stranded DNA satellite known as DNAβ (Briddon et al. 2001, 2003; Cui et al. 2004a; Jose and Usha 2003; Mansoor et al. 2003; Saunders et al. 2000). These DNAβ components depend on their helper viruses for replication and movement in infected plants, transmission between plants, and play important roles in the induction of disease symptoms in infected plants.

Begomoviruses are important as emerging pathogens of various crops in several southern provinces (e.g., Yunnan, Guangdong, Guangxi and Hainan) of China (Cui et al. 2004b; Wang et al. 2004; Xie and Zhou 2003; Zhou et al. 2001, 2003). For example, TYLCCNV was identified in an infected tomato plant grown in the Guangxi province (Yin et al. 2001). Here, we describe a new distinct begomovirus isolated from an infected tomato grown in the Guangxi province.

Virus isolates GX-1, GX-2 and GX-3 showing leaf curl symptoms were collected in infected *Lycopersicon esculentum* plants (cv. Hongbaoshi) in the same field in Tianyang city, Guangxi province, China. Total DNA was extracted from leaves of naturally infected symptomatic plants as described previously by Cui et al. (2004a). The degenerate primer pair (PA and PB) utilized in this study was described previously for amplification of a fragment covering part of the intergenic region (IR) and the AV2 gene of a begomovirus DNA-A-like component (Xie et al. 2002). PCR products amplified with the PA and PB primers were cloned and sequenced as described previously (Zhou et al. 2001). Based on the cloned sequences, overlapping primers GX-1F (5′-GTCC TCGTCACAAACAAAAGG-3′) and GX-1R (5′-CT GGTATAAGGGCTGTCGAA-3′) were then designed and used to further amplify the full-length viral DNA genome of the three virus isolates. The potential full-length DNAβ components were amplified from the collected samples using a set of abutting primers, beta0l (5′-GTAGGTACCACTACGCTACG CAGCAGCC-3′) and beta02 (5′-AGTGGTACCTA CCCTCCCAGGGGTACAC-3′) described by Briddon et al. (2002). PCR products with expected size were recovered individually and cloned into the pGEM-T Easy as instructed by the manufacturer (Promega, Madison, WI, USA). The resulting clones were sequenced using the MegaBACE 1000 DNA Analysis System (Amersharm Pharmacia, Piscataway, NJ, USA). Sequence data were assembled and analyzed using the DNAStar (DNAStar Inc., Madison, WI, USA) and DNAMAN Version 4 (Lynnon Biosoft, Quebec, Canada) programmes. Published begomovirus sequences used for comparisons in this study were all obtained through GenBank and are: Ageratum leaf curl virus (ALCuV, GenBank accession number AJ851005), Ageratum yellow vein China virus (AYVCNV, AJ495813), Ageratum yellow vein Sri Lanka virus (AYVSLV, AF314144), AYVV (AYVV, AJ849916), Cotton leaf curl Kokhran virus (CLCuKV, AJ002448), Euphorbia leaf curl virus (EuLCV, AJ558121), Ludwigia yellow vein virus (LuYVV, AJ965539), Malvastrum yellow vein virus (MYVV, AJ744881), Papaya leaf curl China virus (PaLCuCNV, AJ558125), Pepper leaf curl virus (PepLCV, AF414287), Senecio yellow mosaic virus (SeYMV, AJ876550), Squash leaf curl China virus (SLCCNV, AB027465), Stachytarpheta leaf curl virus (StaLCV, AJ564743), Tobacco curly shoot virus (TbCSV, AJ457986), Tobacco leaf curl Yunnan virus (TbLCYNV, AJ971267), Tomato leaf curl China virus (ToLCCNV, AJ704603), Tomato leaf curl Malaysia virus (ToLCMV, AF327436), TYLCCNV (AF311734) and Tomato yellow leaf curl Thailand virus (TYLCTHV, X63015).

To construct an infectious clone representing the GX-1 genome, we designed primers GX/KpnIF (5′-GGTACCTGAGTACAATGGGCTGTT-3′) and GX/KpnIR (5′-GGTACCAATCAAAGTGCAACA-CAG-3′). These two primers overlap at the unique *Kpn*I site of the GX-1 genome. The PCR product representing the full-length GX-1 genomic DNA (approximately 2.7 kb) was amplified from the extracted DNA of the GX-1 infected plant using primers GX/KpnIF and GX/KpnIR, and cloned into the pGEM-T vector (Promega) to produce pGEM-GX. After sequence determination, a *Kpn*I/*EcoR*I fragment was released from the pGEM-GX and placed into a binary vector (pBinPLUS, van Engelen et al. 1995) predigested with the *Kpn*I and *EcoR*I

restriction enzymes to produce pBinPLUS-GX-0.9A. We then released another fragment from the pGEM-GX construct using the *Kpn*I restriction enzyme and inserted the fragment into the *Kpn*I site within the pBinPLUS-GX-0.9A construct to produce pBinPLUS-GX-1.9A. This construct contained a 1.9-mer tandem repeat of the GX-1 genomic DNA and was electroporated into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) using a Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA). To determine the infectivity of the cloned GX-1, the transformed *A. tumefaciens* EHA105 was grown in a Luria broth at 28°C for 48 h (OD_{550} = 1). *Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum*, tobacco cvs. Samsun and Xanthi, *Lycopersicon esculentum* cv. Hongbaoshi and *Petunia hybrida* plants at 4–6 leaf stages were inoculated with *A. tumefaciens* EHA105 containing GX-1 genome sequence or co-inoculated together with *A. tumefaciens* EHA105 containing TYLCCNV-Y10 DNAβ (Zhou et al. 2003). Agro-inoculation was done by injecting approximately 0.2 ml of *Agrobecterium* culture into a stem or petiole per plant using 2 ml syringes attached to 21-gauge needles. The inoculated plants were grown in an insect-free cabinet with supplementary lighting to give a 16 h photoperiod.

To confirm GX-1 infected in the *Agrobacterium* inoculated plants, total nucleic acids were extracted from young leaves of each plant at 35 days post-inoculation (dpi) as previously described (Cui et al. 2004a). Nucleic acids were separated through electrophoresis in 1% agarose gels in TBE buffer (90 mM Tris-Borate, 2 mM EDTA, pH 8.3), blotted onto nylon membranes (Hybond-N^+, Amersham Biosciences, Buckinghamshire, England), and probed with digoxigenin-labelled probes. A 1200 bp fragment was PCR amplified from the pGEM-GX construct using primers GX/KpnIF and GX-1R. A second 115 bp fragment representing the satellite conserved region (SCR) of TYLCCNV-Y10 DNAβ was PCR amplified as previously described (Xie and Zhou 2006; Zhou et al. 2003). The two resulting PCR products were then used as templates to produce GX-1- and DNAβ-specific probes with a random primer DNA labelling kit as instructed by the manufacturer (Roche, Mannheim, Germany).

Results of our sequence analysis indicated that isolates GX-1, GX-2 and GX-3 (GenBank accession numbers AM236784-86) all contained 2752 nucleotides and had the same genome organization as begomovirus DNA-A, including two ORFs [AV2 and AV1 (CP)] in the virion-sense DNA sequence and four ORFs [AC1 (Rep) to AC4] in the complementary-sense DNA sequence separated by an IR. The IR sequence contained features characteristic of begomoviruses: a putative stem-loop structure with the nonanucleotide sequence TAATATTAC in the loop; a TATA motif at nucleotides 2674–2677; and a repeat sequence (CAATCGG) predicted to be the Rep binding site, occurring at nucleotides 2625–2631 and 2653–2659. The complete sequences of the three isolates shared 98.9–99.7% nucleotide sequence identity.

In order to compare the cloned sequences with the published begomovirus sequences, sequence identity searches were performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/). Extensive comparison of sequences indicated that GX-1 shared the highest nucleotide sequence identity (86.7%) with AYVV (Table 1). The IR of GX-1 had the highest sequence identity (76.9%) with EuLCV. Further comparison of individual predicted viral proteins showed that GX-1 shared the highest amino acid sequence identity with the CP and AV2 (96.1% and 97.4%, respectively) of ToLCCNV, AC1 (99.0%) of AYVV, AC2 and AC3 (97.8% and 98.5%, respectively) of AYVCNV, and AC4 (91.9%) of ALCuV. In addition, the predicted AC1, AC2, AC3 and AC4 proteins of GX-1 had high sequence identity (>90%) with AYVV. The ORF comparison data indicated that GX-1 is most closely related to AYVV and ToLCCNV (Table 1).

Alignments were made among DNA-A sequences of GX-1, AYVV and ToLCCNV. Closer inspection of the alignments revealed that the GX-1 DNA-A sequence can be divided into three regions which showed contrasting nucleotide identities with the cognate sequences of other begomoviruses. The first region included part of the IR (nucleotide position 15–144). The second region contained part of the IR, and *AV2* and *AV1* ORFs (nucleotide position 145–1071). The third region included part of the IR, and *AC1*, *AC2*, *AC3* and *AC4* ORFs (nucleotide position 1072–2752 and 1–14). The % identities of the three regions between GX-1 and AYVV were approximately 60, 75 and 99, respectively, and approximately 60, 93 and 77, respectively, when compared with that of ToLCCNV. Sequence searches of the first region of GX-1 DNA-A using the BLAST

Table 1 Percentage of nucleotide or predicted amino acid sequence identities between GX-1 and other begomoviruses

Virus	DNA-A[a]	IR[a]	AV2[b]	AV1[b]	AC1[b]	AC2[b]	AC3[b]	AC4[b]
ALCuV	83.0	74.2	88.0	85.7	93.0	70.4	74.1	91.9
AYVCNV	78.7	52.9	59.0	81.3	89.8	97.8	98.5	44.3
AYVSLV	68.7	51.2	70.4	75.4	82.5	59.0	70.1	46.4
AYVV	86.7	72.9	72.6	80.6	99.0	96.3	97.0	90.9
CLCuKV	70.0	44.8	71.8	79.7	81.9	60.4	66.4	65.7
EuLCV	74.3	76.9	65.0	77.0	84.2	59.3	66.7	82.5
LuYVV	67.4	43.4	70.7	75.5	74.0	72.6	74.6	41.2
MYVV	67.7	55.6	68.7	78.1	79.3	58.8	63.4	46.5
PaLCuCNV	82.7	61.7	88.8	86.4	88.6	95.6	97.8	48.5
PepLCV	71.2	55.0	87.9	84.8	78.4	62.7	73.1	50.5
SeYMV	71.1	44.1	74.6	80.5	82.7	55.3	66.4	71.7
SLCCNV	62.6	42.8	61.5	77.7	71.2	47.8	58.5	20.2
StaLCV	74.9	46.3	83.6	81.7	85.3	65.7	73.1	83.3
TbCSV	71.9	65.8	71.3	78.9	80.6	57.5	67.9	45.5
TbLCYNV	85.0	58.4	70.9	91.0	90.9	92.6	94.8	86.5
ToLCCNV	78.3	62.3	97.4	96.1	78.2	61.0	65.2	48.0
ToLCMV	78.6	55.5	66.7	82.1	92.0	83.0	79.1	85.4
TYLCCNV	71.0	54.4	80.2	81.3	77.1	65.2	69.6	44.9
TYLCTHV	73.7	53.0	69.6	90.6	79.2	70.1	74.6	46.4

[a] Nucleotide sequence identity

[b] Predicted amino acid sequence identity

algorithm indicated that this region of GX-1 is 99% identical to that of EuLCV. Based on the above information, we propose that GX-1 has arisen by recombination among viruses related to AYVV, ToLCCNV and EuLCV.

To determine whether satellite DNA molecules were present in the originally collected tomato leaf samples, we analyzed these samples through PCR using universal primers specific for DNAβ. Results of the PCR experiments confirmed that DNAβ molecules were present in the GX-1 and GX-2-infected tomato samples, but not in the GX-3-infected samples (data not shown). These results were also confirmed by Southern blot analysis (data not shown). The full length GX-1 and GX-2 DNAβ (accession numbers AJ704578–79) contained 1347 and 1346 nucleotides respectively. The sequence identity between the two isolates was 99.5%. Both GX-1 and GX-2 DNAβ shared 86.2% sequence identity with the TYLCCNV-Y10 DNAβ.

To investigate the infectivity of GX-1 in a plant, an infectious construct (pBinPLUS-GX-1-1.9A) was made and its infectivity was tested in several host plants (Table 2). Agro-inoculation of pBinPLUS-GX-1-1.9A to *N. benthamiana*, *N. glutinosa*, tobacco, tomato and *P. hybrida* resulted in upward curling of young developing leaves of *N. benthamiana*, tobacco, and *P. hybrida* by 8–10 dpi followed by severe leaf curling, vein swelling on the underside of the leaves and stunting of the plant (Fig. 1A, C and D). In inoculated *N. glutinosa* plants, downward leaf curling symptoms were observed in young leaves by 9 dpi followed by stunting of the plant (Fig. 1B). Tomato plants inoculated with the virus did not develop virus symptoms until 20 dpi. Virus symptoms in GX-1-infected tomato plants appeared as mild distortion of young leaves and puckering and crinkling in developed leaves (Fig. 1E). Symptoms in tomato plants inoculated with pBinPLUS-GX-1-1.9A were similar to those observed in infected field tomato plants.

Because DNAβ was detected in samples infected with GX-1 and GX-2, and DNAβ of GX-1 shared a high sequence identity with TYLCCNV-Y10 DNAβ (86.2%). Therefore the infectious construct of

Table 2 Infectivity and disease symptoms induced by GX-1 in the presence or absence of TYLCCNV DNAβ

Plant species	Inoculum[a]	Infectivity[b]	Onset of symptoms	Symptoms
N. benthamiana	GX-1	28/28	9 dpi	Upward leaf curling, vein thickening and stunting
	GX-1 + Y10β	26/26	9 dpi	Upward leaf curling, vein thickening and stunting
N. glutinosa	GX-1	30/30	9 dpi	Downward leaf curling, vein thickening and stunting
	GX-1 + Y10β	31/31	9 dpi	Severe downward leaf curling, vein thickening, enations, and stunting
N. tabacum	GX-1	21/23	10 dpi	Upward leaf curling, vein thickening and stunting
	GX-1 + Y10β	24/25	10 dpi	Severe upward leaf curling, vein thickening, enations and stunting
N. tabacum cv. Samsun	GX-1	13/16	10 dpi	Upward leaf curling, vein thickening and stunting
	GX-1 + Y10β	17/18	10 dpi	Severe upward leaf curling, vein thickening, enations and stunting
N. tabacum cv. Xanthi	GX-1	20/21	10 dpi	Upward leaf curling, vein thickening and stunting
	GX-1 + Y10β	22/22	10 dpi	Severe upward leaf curling, vein thickening, enations and plant stunting
L. esculentum	GX-1	29/38	20 dpi	Leaf distortion, leaf puckering and plant stunting
	GX-1 + Y10β	30/38	20 dpi	Leaf distortion, leaf puckering and plant stunting
P. hybrida	GX-1	25/25	8 dpi	Upward leaf curling and plant stunting
	GX-1 + Y10β	27/27	8 dpi	Upward leaf curling and plant stunting

[a] Y10β: TYLCCNV-Y10 DNAβ

[b] Number of plants infected/inoculated

TYLCCNV DNAβ was co-inoculated with GX-1 DNA-A by agroinoculation to determine the function of DNAβ in disease symptom induction in different host plants. In GX-1 DNA-A and TYLCCNV DNAβ co-infected tomato plants, virus symptoms were similar to those induced by GX-1 DNA-A alone suggesting that the presence of TYLCCNV DNAβ in infected tomato plants did not affect the symptom development (Fig. 1E). However, co-infected tobacco plants produced slightly more severe leaf curling symptoms than those induced by GX-1 DNA-A alone, and both co-infected *N. glutinosa* and tobacco plants showed enations on the undersides of their leaves (Table 2). These symptom observations indicated that the DNAβ in infected plants could enhance disease symptoms but in a host-dependent manner.

We then decided to determine the accumulation level of DNAβ in co-infected *N. benthamiana*, tobacco cv. Samsun and tomato plants using Southern blot analysis. Our results showed that DNAβ of TYLCCNV did accumulate to high levels in symptomatic leaves of all co-inoculated plants, suggesting that TYLCCNV DNAβ was able to replicate and spread systemically in infected plants in association with GX-1 (Fig. 2). The accumulation level of GX-1 DNA-A in pBinPLUS-GX-1-1.9A inoculated plants was similar to that in plants co-inoculated with pBinPLUS-GX-1-1.9A and TYLCCNV DNAβ, indicating that GX-1 DNA-A accumulation in infected plants was not affected by the presence or absence of DNAβ.

Although the nucleotide sequences of GX-1, GX-2 and GX-3 DNA-A shared high sequence identity among themselves (98.9–99.7%), they shared relatively lower sequence identity (<86.7%) with other reported begomoviruses. Begomoviruses are classified on the basis of genome sequence. Isolates of begomoviruses sharing <89% sequence identity are generally considered to be distinct species (Fauquet and Stanley 2005). Thus we propose GX-1, GX-2 and GX-3 as three isolates of a distinct begomovirus species and

Fig. 1 Symptoms induced by GX-1 DNA-A alone ($\beta-$) or GX-1 DNA-A together with TYLCCNV-Y10 DNAβ ($\beta+$). Plant species used for inoculation study include *N. benthamiana* (**A**), *N. glutinosa* (**B**), *N. tabacum* cv. Samsun (**C**), *N. tabacum* (**D**) and tomato (**E**). Photographs were taken at 30 dpi. Uninfected plants were used as control (CK)

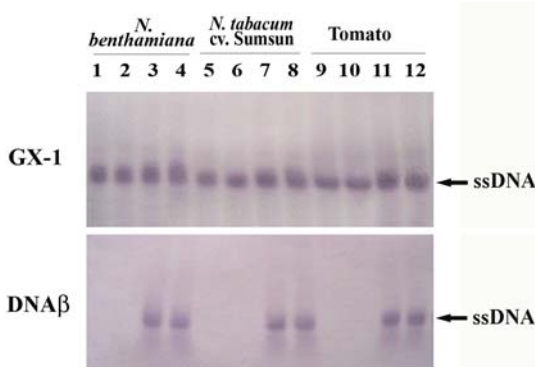

Fig. 2 Southern blot analysis of viral DNA-A and DNAβ components in infected plant tissues. Nucleic acids were extracted from *N. benthamiana* (lanes 1–4), tobacco cv. Samsun (lanes 5–8) and tomato (lanes 9–12) plants agroinoculated with GX-1 alone (lanes 1–2, 5–6, and 9–10), or together with TYLCCNV DNAβ (lanes 3–4, 7–8, and 11–12) at 35 dpi. The blots were probed with either GX-1 specific (upper panel) or TYLCCNV DNAβ specific probe (lower panel). Approximately equal amounts (20 µg) of nucleic acids were loaded in each lane. The positions of single-stranded (ss) DNA forms are indicated

name them as isolates of Tomato leaf curl Guangxi virus (ToLCGXV), in accordance with the current species demarcation criteria (Stanley et al. 2005).

Recombination among begomoviruses has been reported between strains and between species, and also possibly at the genus level (Harrison and Robinson 1999; Stanley et al. 1986; Zhou et al. 1997). We previously isolated and identified several new begomovirus species in plants grown in China and considered them as natural recombinants (Xie et al. 2002; Xie and Zhou 2003; Ma et al. 2004). Genomic data presented in this paper indicate that the *AC1*, *AC2*, *AC3* and *AC4* ORFs and part of IR of ToLCGXV probably originated from a begomovirus related to AYVV. The *AV1* and *AV2* ORFs of ToLCGXV originated from a begomovirus related to ToLCCNV, and part of IR of ToLCGXV from one related to EuLCV. These findings are consistent with several previous studies and support the idea that recombination among begomovirus strains or species is an important driving force in the evolution of begomoviruses (Padidam et al. 1999).

Recently, an increasing number of monopartite begomoviruses have been reported to be associated with DNAβ molecules. DNAβ molecules of the same begomovirus species share 72–99% sequence identity, while 36–57% sequence identity is found

between DNAβ molecules belonging to distinct begomoviruses (Zhou et al. 2003). DNAβ of ToLCGXV isolates GX-1 and GX-2 shared the highest sequence identity (86.2%) with that of TYLCCNV, indicating that the DNAβs associated with GX-1 and GX-2 DNA-A are the same type of component associated with TYLCCNV. Our bioassays showed that ToLCGXV DNA-A alone is capable of causing infection in its host plants. Our results also showed that symptoms in tomato plants infected with ToLCGXV DNA-A alone were similar to those observed in the original infected tomato plants. Similar results were noticed in other tested solanaceous species. Co-inoculation of tomato plants with ToLCGXV DNA-A and TYLCCNV DNAβ did not yield enhanced disease symptoms in the infected plants, demonstrating that DNAβ may not have played a role in disease symptom development in this host plant. Because ToLCGXV isolate GX-3 was not associated with DNAβ, it is possible that DNAβ is not required for ToLCGXV infection in tomato in the field. A similar report by Xie and Zhou (2006) showed that among the samples infected with TbLCYNV, only a small number of them contained DNAβ molecules, and DNAβ did not yield enhanced disease symptoms in the infected plants. As TYLCCNV isolates were previously shown to be associated with DNAβ molecules in tomato grown in the Guangxi province, China (Xu and Zhou 2006), we speculate that some ToLCGXV isolates acquired a DNAβ component during a mixed infection with viruses related to TYLCCNV.

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