Characterization of a New Monopartite Begomovirus with a Betasatellite Associated with Leaf Curl, Yellow Vein, and Vein Enation in *Hibiscus rosa-sinensis*

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Abstract

A new begomovirus, tentatively named hibiscus yellow vein leaf curl virus (HYVLCV), was identified in *Hibiscus rosa-sinensis* plants showing symptoms of leaf curl, yellow vein, and vein enation on the undersides of the leaf in Taiwan. Sequence analysis of the full-length HYVLCV genome from the rolling cycle amplicon revealed a genome of 2,740 nucleotides that contains six open reading frames and a conserved sequence (5'-TAATATTAC-3') commonly found in geminiviral genomes. HYVLCV shares the highest nucleotide identity (88.8%) with cotton leaf curl Multan virus (CLCuMuV) genome, which is lower than the criteria (91%) set for species demarcation in the genus *Begomovirus*. No begomoviral DNA-B was detected; however, a begomovirus-associated DNA betasatellite (DNA-β) was detected. The DNA-β (1,355 nucleotides) shares the highest nucleotide identity (78.6%) with malvastrum yellow vein betasatellite (MaYVB). Because the identity is slightly higher than the criteria (78%) set for the species

demarcation threshold for a distinct DNA-β species, the DNA-β of HYVLCV reported in this study is considered the same species of MaYVB and tentatively named MaYVB-Hib. An expected 1,498-bp fragment was amplified with two HYVLCV-specific primers from 10 of 11 field-collected samples. Four independent amplicons were sequenced, revealing 100% nucleotide identity with the HYVLCV genome. Agroinoculation of a dimer of the infectious monopartite genome alone to *Nicotiana benthamiana* resulted in mild symptoms at 28 days postinoculation (dpi); coagroinoculation with the DNA-β satellite resulted in severe symptoms at 12 dpi. HYVLCV could be transmitted to healthy *H. rosa-sinensis* by grafting, resulting in yellow vein symptoms at 30 dpi.

Keywords: begomovirus, enation, *Hibiscus rosa-sinensis*, leaf curl, new virus, yellow vein

Hibiscus rosa-sinensis L., also known as Chinese hibiscus, is a popular malvaceous ornamental shrub commonly planted in the tropics and subtropics. *H. rosa-sinensis* is also known to have aesthetic and medicinal values (Ross 2003). Several RNA viruses have been reported to infect *H. rosa-sinensis*. These include hibiscus chlorotic ringspot virus (HCRSV) (genus *Carmovirus*) (Waterworth 1976), hibiscus latent ringspot virus (HLRSV) (genus *Nepovirus*) (Brunt et al. 1980), eggplant mottled dwarf virus (genus *Nucleorhabdovirus*) (De Stradis et al. 2008), okra mosaic virus (genus *Tymovirus*) (Stephan et al. 2008), and three viruses in the genus *Tobamovirus*, including tomato mosaic virus (Huang et al. 2004), hibiscus latent Singapore virus (HLSV) (Srinivasan and Wong 2006; Srinivasan et al. 2002), and hibiscus latent Fort Pierce virus (HLFPV) (Adkins et al. 2003, 2006).

Two DNA viruses, cotton leaf curl Multan virus (CLCuMuV), first reported in India, and cotton leaf curl Kokhran virus-Burewala

(CLCuKoV-Bu, formerly cotton leaf curl Burewala virus), first reported in Pakistan (both belonging to the genus *Begomovirus*), also could infect *H. rosa-sinensis* (Akhtar et al. 2014; Rajeshwari et al. 2005). Begomoviruses, usually transmitted by whiteflies, can infect several dicotyledonous plants. The begomoviral genome contains circular single-strand DNA, which can be either bipartite (composed of both DNA-A and DNA-B components) or monopartite (containing a single DNA-A like component) (Hanley-Bowdoin et al. 1999). The open reading frames (ORFs) of the DNA-A component encode six genes. The virion-sense strand encodes a coat protein (ORF AV1/V1) and a movement protein (ORF AV2/V2). The virion-complementary strand encodes a replication-associated protein (ORF AC1/C1), a transcriptional activator (ORF AC2/C2), a replication enhancer (ORF AC3/C3), and an AC4/C4 protein (ORF AC4/C4). (Hanley-Bowdoin et al. 1999; Zerbini et al. 2017). The virion-sense strand of DNA-B encodes a nuclear shuttle protein (ORF BV1) and the complementary strand encodes a movement protein (ORF BC1), implicated in viral movement (Zerbini et al. 2017). Some monopartite begomoviruses are associated with both DNA alphasatellite (family *Alphasatellitidae*) and DNA betasatellite (DNA-β) (genus *Betasatellite*; family *Tolecusatellitidae*) molecules. The alphasatellite is an autonomously replicating satellite that encodes a replication associated protein (Rep); however, its role in the virus infection cycle remains unknown. DNA-β depends on the helper begomovirus for its replication (Briddon et al. 2003; Zerbini et al. 2017). DNA-β encodes a βC1 protein (ORF βC1) in the complementary strand that functions as a gene-silencing suppressor (Cui et al. 2005). The βC1 protein can enhance the virulence of the helper begomovirus and the severity of symptoms (Briddon et al. 2001, 2003; Yang et al. 2008). The criteria used to define a new species in the genus *Begomovirus* are based on the nucleotide sequence identity of less than 91% for the full-length

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genome of monopartite or full-length DNA-A of bipartite begomoviruses (Brown et al. 2015). The species demarcation criteria for betasatellites are based on the full-length nucleotide sequence identity set at less than 78% (Briddon et al. 2008).

In 2013, two *H. rosa-sinensis* plants showing leaf curling, yellow vein, and vein enation on the undersides of the leaf were collected from Changhua County in central Taiwan. Whiteflies (*Bemisia tabaci* cryptic species complex) (De Barro et al. 2011; Xu et al. 2010) were observed to feed on symptomatic plants. We suspected that the disease may be caused by a begomovirus based on symptomatology and the presence of whiteflies in the field. In this report, we characterized a new monopartite begomovirus, tentatively named as hibiscus yellow vein leaf curl virus (HYVLCV), from the symptomatic *H. rosa-sinensis* plants. We present biological and molecular data to support the etiology of this new disease of *H. rosa-sinensis*.

Materials and Methods

Source and identification of the virus. Two *H. rosa-sinensis* L., plants showing leaf curling, yellow vein, and vein enation symptoms on the undersides of the leaf, which resembled those induced by begomoviruses, were collected from Tianwei Township, Changhua County (23°54'17.9"N 120°31'56.5"E) in central Taiwan in 2013. Total DNA was extracted from symptomatic hibiscus leaves as previously described (Chang et al. 2010) and used for PCR amplification with degenerate primers targeting the begomoviral DNA-A-like or DNA-B genome. Begomoviral DNA-A-like fragments were amplified by PCR using two degenerate primers, PAL1v1978 and PAR1c715 (Rojas et al. 1993). Two degenerate primers, DNABLC1 and DNABLV2 (Green et al. 2001), were used to amplify DNA-B. Two universal primers, β 01 and β 02 (Briddon et al. 2001), were used to amplify the begomovirus-associated DNA- β . Two pairs of universal primers, DNA101/DNA102 and UN101/UN102 (Bull et al. 2003; Xie et al. 2010), were used to amplify an alphasatellite molecule. Viral DNA fragments were amplified using *Taq* DNA polymerase (Protech Technology Enterprise, Taipei, Taiwan) in a thermal cycler (PTC-200; MJ Research). All oligonucleotide primers associated with begomovirus and DNA- β used in this study are listed in Table 1.

RNA was purified from symptomatic *H. rosa-sinensis* plants using the method described by Napoli et al. (1990) and used for cDNA synthesis according to the method described by Jan et al. (2000). The resultant cDNA was used to examine the presence of HCRSV (Li and Chang 2002) and HLSV (Y. K. Chen et al. 2006) commonly infecting *H. rosa-sinensis* in Taiwan by real-time (RT)-PCR using degenerate or specific primers. Other RNA viruses, including carmoviruses, nepoviruses, potyviruses, tospoviruses, tobamoviruses, and cucumber mosaic virus (CMV), commonly occurring in Taiwan were also examined by RT-PCR using genus-

specific primers (C. C. Chen et al. 2006; Colinet and Kummert 1993; Chu et al. 2001; Letschert et al. 2002; Zheng et al. 2010).

Cloning and sequencing of viral DNAs. The partial viral DNA fragment was amplified by PCR with begomovirus degenerate primers PAR1c715 and PAL1v1978, cloned into the TOPO-pCRII vector using a TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.), and sequenced. The rolling circle amplification (RCA) method (Inoue-Nagata et al. 2004; Wu et al. 2008) was performed to amplify full-length viral DNA concatemers with the primer HYVLCV-Rep-F using a RepliPhi Phi29 DNA Polymerase (Epicentre, Madison, WI, U.S.A.). The amplified viral DNA concatemers containing a single *NcoI* recognition site were digested with *NcoI* (New England Biolabs, Beverly, MA, U.S.A.) to release a 2.7-kb viral genome fragment, which was then cloned into the pCambia0380 plasmid (Cambia, Canberra, ACT, Australia) to yield a recombinant plasmid-HibA-1 (containing the complete viral DNA-A like sequence). HibA-1 was sequenced using four primers: HYVLCV-F, HYVLCV-R, 0380-L, and 0380-R (Table 1). A 1.3-kb DNA- β was amplified by PCR from total DNA using the universal primers β 01 and β 02 and also cloned to the TOPO-pCRII vector to produce Hibbeta-22 for sequence analysis. Sequencing was performed using an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Hammon, NJ, U.S.A.) available at the Biotechnology Centre, National Chung-Hsing University (Taichung, Taiwan).

Sequence and phylogenetic analysis. Database searches and comparisons of viral nucleotide sequences were performed using BLASTn and the Lasergene software package (DNASTAR, Madison, WI, U.S.A.). Sequence alignments were conducted using the ClustalW tool (Thompson et al. 1994). All pairwise comparisons of the monopartite begomoviruses and betasatellites were performed using the MUSCLE algorithm option implemented in the Species Demarcation Tool version 1.2 (SDT) (Muhire et al. 2014) and MegAlign program available in Lasergene software. Distance matrices of viral sequences and phylogenetic relationships were analyzed using the neighbor-joining method available in Molecular Evolutionary Genetics Analysis (MEGA) software MEGA (Kumar et al. 2018) and evaluated with 1,000 bootstrap replicates. Sequences of the closely related begomoviruses and associated DNA- β satellites were retrieved from GenBank and used for comparison and phylogenetic analysis in this study. Monopartite genome sequences of wheat dwarf virus and tomato leaf curl Taiwan virus (ToLCTWV) were used as outgroups. Recombination was predicted using the Recombination Detection Program, version 4 (RDP4) (Martin et al. 2015).

Detection and sequencing of HYVLCV from field samples. Eleven *H. rosa-sinensis* plants displaying virus-like symptoms and two nonsymptomatic plants were collected from Changhua County in 2014. Total DNA was extracted and used for PCR amplification. Two primers (HYVLCV-F and HYVLCV-R) were designed to specifically amplify the HYVLCV partial DNA region composed of the 3' *C1* gene, *C2* gene, *C3* gene, and *V1* gene. The full-length

Table 1. Primers used in this study^a

Primer name	Primer sequence (5'–3')	Target, size	Reference
PAL1v1978	GCATCTGCAGGCCACATYGTCTTYCCNGT	DNA-A like, approximately 1,500 bp	Rojas et al. 1993
PAR1c715	GATTTCCTGCAGTTDATRTTYTCRTCCATCCA	...	...
DNABLC1	GTVAATGGRGTDCACTTCTG	DNA-B, approximately 2,500 bp	Green et al. 2001
DNABLV2	GAGTAGTAGTGBAKGTTGCA	...	...
β 01	GGTACCACTACGCTACGCAGCAGCC	DNA- β , approximately 1,300 bp	Briddon et al. 2001
β 02	GGTACCTACCCTCCCAGGGGTACAC	...	...
0380-L	GCGGACGTTTTAATGTACTGAAT	Vector pCambia-0380 (sequencing)	This study
0380-R	CTGTAATTCACACGTGGTGGTGGT	...	...
Actin-F	GGGATGTGAAGGAGAAGTTGGC	Actin, 79 bp	This study
Actin-R	ACGGATGAGCTGGTCTTTGTC	...	...
HYVLCV-F (nucleotide 594)	GGTAAGCGTTTCTGTGTTAAGT	HYVLCV, 1,498 bp	This study
HYVLCV-R (nucleotide 2,091)	GCAAACTAGACAGGATCTTTTC	...	...
HYVLCV-Rep-F (nucleotide 1,667)	TTGGGACCAGGATTGCAAAAG	HYVLCV Rep, 133 bp	This study
HYVLCV-Rep-R (nucleotide 1,799)	ACCCGCATTTCCTCAAACAC	...	...

^a HYVLCV = hibiscus yellow vein leaf curl virus. B = A or G, D = A or C or G, K = G or T, N = A or T or G or C, R = A or G, V = A or C or G, and Y = C or T.

HYVLCV genome was amplified from symptomatic leaves by RCA with the primer HYVLCV-Rep-F and digested with *Nco*I. The resultant 2.7-kb DNA fragment was cloned into pCambia0380 vector to yield four clones and sequenced. The begomovirus-associated DNA- β was detected by PCR with two primers (β 01 and β 02) (Table 1), cloned into the TOPO-pCRII vector using a TA cloning kit, and sequenced.

Pathogenicity tests via graft transmission. Viral transmission and pathogenicity were assessed by the cleft grafting method (Dijkstra and de Jager 1998). Symptomatic *H. rosa-sinensis* shoots were carefully cut, split into halves (2 to 3 cm deep), and used as stocks. Three *H. rosa-sinensis* shoots (presumably virus-free based on RT-PCR and PCR assays) were cut to form wedge-shaped clefts and inserted into the stocks. The grafted areas were sealed with parafilm. The plants were grown in an insect-proof greenhouse and observed for symptom development weekly. After 2 months, DNA was extracted from symptomatic and asymptomatic scions and examined by PCR using the primer pair HYVLCV-F/HYVLCV-R to amplify the monopartite genome and β 01 and β 02 to amplify DNA- β .

Construction of infectious clones. Full-length viral DNA concatemer obtained by RCA was partially digested with *Nco*I to produce a 5.5-kb tandem dimer, which was cloned into binary vector pCAMBIA1304 to generate the infectious clone pHib-2A, as described by Chang et al. (2010). An infectious clone of the DNA- β was constructed according to the method described by Bang et al. (2014). A partial DNA- β fragment (770 bp) containing the intergenic region (IR) was released from Hibbeta-22 after *Eco*RI and *Kpn*I digestion and cloned into the binary vector pCAMBIA1304 to generate pHib-0.6 β . After *Kpn*I digestion, the full-length DNA- β was head-to-tail cloned into *Kpn*I-digested pHib-0.6 β to generate the infectious clone pHib-1.6 β . The construct pHib-1.6 β was checked by *Eco*RI digestion. The pHib-2A and pHib-1.6 β constructs were independently transferred into *Rhizobium radiobacter* (EHA 105 strain) using a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA, U.S.A.).

Determination of host range. Agroinfection was performed as previously described (Chang et al. 2010). An *R. radiobacter* strain containing either a pHib-2A or a pHib-1.6 β construct was cultured separately in Luria-Bertani broth containing 10 mM morpholineethanesulfonic acid (pH 5.6), 40 μ M acetosyringone (AS), and kanamycin at 50 ppm for 16 h. Bacterial cells were harvested by centrifugation at 5,000 rpm (JA 25.5 Rotor; Beckman Coulter) for 10 min, resuspended in an agroinfiltration buffer (10 mM MgCl₂ and 150 μ M AS) and adjusted to an optical density with absorbance at 550 nm value equivalent to 1.0. Agrobacteria suspensions were infiltrated into indicator plants. Plants used for agroinoculation included the solanaceous plants *Nicotiana benthamiana* (as a positive control), *N. tabacum* 'White Burley', *N. tabacum* 'Xanthi', *N. edwardsonii*, *N. rustica*, *N. glutinosa*, *N. occidentalis*, *Capsicum annuum* (sweet pepper), and *Solanum lycopersicum*; the cucurbitaceous plants *Cucumis sativus* and *Citrullus lanatus*; the leguminosae plants *Vigna unguiculata* and *Pisum sativum*; and the Malvaceae family plants *Abelmoschus esculentus* and *H. rosa-sinensis*. Each plant species had four biological replicates. Leaves and stems of *H. rosa-sinensis* were infiltrated with agrobacterium once a week for 3 weeks. Agrobacteria carrying pHib-2A or pHib-1.6 β alone or in a combination were infiltrated. Plants infiltrated with *R. radiobacter* containing pCAMBIA1304 empty vector only were used as mock controls. The agroinoculated plants were maintained in an insect-proof greenhouse for at least 1 month for symptom development. Hibiscus plants treated with agroinfiltration were maintained for at least 2 months. Systemic and asymptomatic leaves were collected and total DNA was extracted from indicator plants after 1 month. PCR using primer pairs HYVLCV-F/HYVLCV-R and β 01/ β 02 was performed to detect HYVLCV and DNA- β , respectively.

Relative quantification of viral DNA in *N. benthamiana*. Total DNA (50 ng) was extracted from *N. benthamiana* 12 days postinoculation (dpi) and used in real-time PCR amplification with the iQ

SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.). Two primers (HYVLCV-Rep-F and HYVLCV-Rep-R) specifically targeting the HibA-1 C1 gene sequence were used to quantify HYVLCV. The *N. benthamiana actin* gene (accession number AY179605) was amplified with primers actin-F and actin-R as internal controls. Amplification was conducted using a CFX Connect real-time PCR detection system (Bio-Rad). HYVLCV viral DNA was normalized with respect to the *actin* gene using CFX Manager Software 3.1 (Bio-Rad). Standard errors were calculated from three independent experiments.

Results

Detection of a begomovirus from symptomatic *H. rosa-sinensis* plants. Hibiscus plants showing yellow vein, leaf curling, and vein enation (Fig. 1A to C), suspected to be caused by a virus, were collected in 2013. PCR was conducted to detect begomoviral DNA-A, DNA-B, alphasatellite, and DNA- β with degenerate or universal primers. An expected, a 1.5-kb DNA fragment of partial viral DNA was amplified from total DNA extracted from symptomatic hibiscus leaves using the degenerate primers targeting DNA-A-like molecules (Fig. 2A) and cloned. Sequence analysis revealed that the obtained 1,460 nucleotides (nt) shared 90.6 to 90.8% identity with the monopartite genome of six CLCuMuV isolates (GenBank accession numbers KF413618, KP762786, JX914662, JX286664, JQ317603, and KF766951). Two universal primers targeting begomovirus-associated DNA- β amplified an expected 1.3-kb DNA fragment (Fig. 2A). No product was amplified from symptomatic *H. rosa-sinensis* leaves with the alphasatellite universal primers (data not shown) and begomoviral DNA-B degenerate primers (Fig. 2A). DNA fragments with expected sizes were amplified with the primers targeting DNA-B of tomato yellow leaf curl Thailand virus and DNA- β of ToLCTWV associated DNA- β (positive controls) (Fig. 2A).

No product was amplified by RT-PCR with the primers targeting carmoviruses, nepoviruses, potyviruses, tospoviruses, tobamoviruses, CMV, HLSV, and HLFPPV (data not shown), thus ruling out the presence of these target RNA viruses in symptomatic hibiscus plants.

Sequence determination, organization, and phylogenetic analysis of HYVLCV genome. The full-length DNA A-like molecule was amplified, sequenced, and compared to determine the taxonomic status of the monopartite begomovirus present in symptomatic *H. rosa-sinensis* plants. A 2.7-kb DNA fragment was obtained from the *Nco*I-digested RCA product and cloned to generate the HibA-1 clone (HYVLCV genome). Sequence analysis revealed that HYVLCV contains 2,740 nt (GenBank accession number MF140451), which was within the size range for known begomoviral monopartite genome sequences. HYVLCV was found to contain six putative ORFs, including the V1 (771 nt) and V2 (348 nt) genes on the viral-sense strand and the CI (1092 nt), C2 (453 nt), C3 (405 nt), and C4 (303 nt) genes on the complementary strand (Fig. 2B). A geminiviral conserved sequence (5'-TAATATTAC-3') (Stenger et al. 1991) was also found in the 269-nt IR region. A comparison of the HYVLCV sequence with those of other begomoviruses available in GenBank using BLASTn and SDT revealed that HYVLCV was closely related to 47 begomoviruses (Table 2). The full-length HYVLCV sequence shared 85.9 to 88.8% maximum nucleotide identity with 13 isolates of CLCuMuV (GenBank accession number KY373212), a monopartite begomovirus known to infect hibiscus in India (Rajeshwari et al. 2005). According to the current criteria of the International Committee on Taxonomy of Viruses (ICTV), a new begomoviral species is determined by the full-length genome of a monopartite genome or the full-length of DNA-A sequence identity set on less than 91%. Thus, we proposed HYVLCV as a tentative new species in the genus *Begomovirus*. Phylogenetic analyses of HYVLCV and 47 closely related begomoviruses available in the GenBank database revealed two distinct clusters. One cluster consisted of malvastrum yellow vein virus (MaYVV), tomato leaf curl New Delhi virus 2 (ToLCNDV2), pea leaf distortion virus, cotton leaf curl Bangalore virus, kenaf leaf curl virus (KLCuV), hollyhock leaf curl virus, eclipta yellow vein virus, and malvastrum yellow

mosaic virus isolates. Another cluster consisted of CLCuMuV and CLCuKoV-Bu isolates (Fig. 3A). Isolates of HYVLCV formed a distinct clade from other begomoviruses and was close to the CLCuMuV clade (Fig. 3A).

Analysis of DNA- β associated with HYVLCV. The full-length DNA- β component was amplified by PCR (Fig. 2A) and cloned to generate the Hibbeta-22 clone. Sequence analysis revealed that Hibbeta-22 contains 1,355 nt (GenBank accession number MF140456), which encodes a putative β C1 (357 amino acid) in the complementary sense (Fig. 2B). Comparisons of the Hibbeta-22 sequence with those of other DNA- β available in GenBank using BLASTn and SDT revealed that Hibbeta-22 was closely related to 31 DNA- β of begomoviruses infecting the family Malvaceae (Table 3). Phylogenetic analysis showed that Hibbeta-22 was most close to the malvastrum yellow vein betasatellite (MaYVB) clade compared with other DNA- β sequences (Fig. 3B). Hibbeta-22 shared 78.4 to 78.6% nucleotide identity with five MaYVB isolates, which was slightly higher than the ICTV threshold (78%) set for DNA- β (Briddon et al. 2008). Therefore, this DNA- β associated with HYVLCV was considered a divergent isolate of MaYVB and tentatively named MaYVB-Hib.

Virus detection of field samples. PCR analysis of 11 symptomatic hibiscus samples collected from Changhua County in 2014 revealed that two primers (HYVLCV-F and HYVLCV-R) (Table 1) were able to amplify an expected 1.5-kb DNA fragment from 10 of the 11 test samples and a positive control sample. An expected 1.3 kb of DNA- β was amplified from 8 of 11 test samples and the positive control sample. No DNA was amplified from nonsymptomatic plants using these primers (data not shown). Full-length DNA A-like fragments were amplified from four samples and cloned into pCAM-BIA0380 vector to yield Hib2-2 (accession number MF140452), Hib5-5 (MF140453), Hib6-2 (MF140454), and Hib8-8 (MF140455) and sequenced. Sequence analysis revealed that the four viral clones (Hib2-2, Hib5-5, Hib6-2, and Hib8-8) (2,740 nt) shared 99.9 to

100% nucleotide identity with HibA-1. The full-length DNA- β was amplified from two samples and cloned to generate Hibbeta-2 and Hibbeta-8. Sequence analysis revealed that Hibbeta-2 and Hibbeta-8 shared 99.3 to 99.6% nucleotide identity with MaYVB-Hib. Hibbeta-2 and Hibbeta-8 shared nucleotide identity ranging from 78.4 to 79% with five MaYVB isolates.

Analysis for recombination. Recombination was examined using the RDP4 analysis. Alignment of HYVLCV with monopartite begomoviral sequences retrieved from the GenBank sequence database (Fig. 3A) revealed that recombination likely occurred in all HYVLCV isolates. The RDP recombination analysis identified a putative recombination event in the HYVLCV with different *P* values depending on the methods used (Table 4). The putative recombination breakpoints were predicted to be located within the AC1 and IR region (1,729 to 2,728). The RDP analysis indicated that KLCuV-Y341 (isolate TH4872-9, accession number KY373212) was likely the major parent of HYVLCV and the minor parent was CLCuMuV-TH4872-9 (isolate Y341, accession number FN806778). HYVLCV nucleotides (1,729 to 2,728) showed 100% identity with the minor parent. The remaining nucleotides (1 to 1,728 and 2,729 to 2,740) showed 92.3% identity with the major parent. Similar analysis of MaYVB-Hib and β satellite sequences from the database (Fig. 3B) revealed no recombination within MaYVB-Hib.

Back inoculation via graft transmission. Assays for virus transmission were performed by cleft grafting. The three virus-free *H. rosa-sinensis* (Fig. 1D) scions grafted onto HYVLCV-positive stocks developed yellow vein symptom at 30 to 40 dpi (Fig. 1E) followed by vein enation and leaf curl symptoms at 60 dpi. The presence of HYVLCV and MaYVB-Hib in the symptomatic *H. rosa-sinensis* scions was confirmed by PCR (Fig. 1F).

Infectivity assays of HYVLCV and its associated β satellite MaYVB-Hib. Agroinfiltration of pHib-2A or pHib-1.6 β (Fig. 4A) alone into *N. benthamiana* plants resulted in no visible symptom at

Fig. 1. Symptoms of diseased *Hibiscus rosa-sinensis* plants. *H. rosa-sinensis* showing the symptoms of **A**, leaf curling; **B**, vein yellowing, and **C**, enation (indicated by arrows) on the abaxial surface of the leaf. **D**, Healthy leaf. **E**, Leaf vein yellowing was observed on *H. rosa-sinensis* scions 1 month after being grafted onto a diseased *H. rosa-sinensis* plant. **F**, Presence of hibiscus yellow vein leaf curl virus (HYVLCV) and malvastrum yellow vein betasatellite (MaYVB)-Hib in three grafting hibiscus was confirmed by PCR using primers specially targeting HYVLCV or MaYVB-Hib. Abbreviations: G = grafting hibiscus, P = HYVLCV-infected hibiscus collected from field, and N = healthy hibiscus.

12 dpi (Fig. 4B and C). However, plants coinfecting with pHib-2A and pHib-1.6 β developed severe mosaic, vein yellowing, and leaf curl symptoms at 12 dpi (Fig. 4D). Plants infiltrated with pHib-2A alone developed yellowing and leaf curling at 28 dpi (Fig. 4E). The presence of HYVLCV, MaYVB-Hib, or both in *N. benthamiana* systemic leaves was confirmed by PCR. HYVLCV was detected in plants infiltrated with pHib-2A alone and coinoculated with pHib-1.6 β . MaYVB-Hib was detected only in plants coinoculated with pHib-2A and pHib-1.6 β . Neither HYVLCV nor MaYVB-Hib was detected in plants treated with empty vector (Fig. 4F). Similar results were observed from analysis of real-time PCR. Plants coinoculated with pHib-2A and pHib-1.6 β had HYVLCV DNA levels twofold higher than those inoculated with pHib-2A alone (Fig. 5).

Determination of host range by agroinoculation. In total, 15 different plant species ($n = 4$ plants/species) were tested for susceptibility to HYVLCV by agroinoculation. *N. benthamiana*, *N. edwardsonii*, *N. tabacum* 'Xanthi', and *N. tabacum* 'White Burley' developed systemic symptoms after agroinoculation (Table 5). All plant species showed mosaic, vein yellowing, and leaf curl symptoms on the apical leaves. The presence of HYVLCV and MaYVB-Hib in symptomatic plants was confirmed by PCR assays. No visible symptom was observed on the Solanaceae plants *N. glutinosa*, *N. occidentalis*, *N. rustica*, *Capsicum annuum* (sweet pepper), and *S. lycopersicum*; the cucurbitaceous plants *Cucumis sativus* and *Citrullus lanatus*; the Leguminosae plants *V. unguiculata* and *P. sativum*; and the Malvaceae plants *A. esculentus* and *H. rosa-sinensis* at 30 dpi. No symptom was observed on *H. rosa-sinensis*

Fig. 2. Detection of hibiscus yellow vein leaf curl virus (HYVLCV) isolate HibA-1 by PCR. **A**, Amplification of begomoviral monopartite, DNA-A, DNA-B, and associated β satellite (beta-sat) by PCR using degenerate and universal primers. Lane M = marker, lane H = HYVLCV isolate HibA-1, lane TH = tomato yellow leaf curl Thailand virus, and lane TW β = tomato leaf curl Taiwan virus associated β satellite. **B**, Schematics showing genome organization of the HYVLCV genome and its associated β satellite malvastrum yellow vein betasatellite (MaYVB)-Hib; nt = nucleotides. Relative positions of the primers used in this study and the size of the amplified fragments are also indicated.

leaves at 60 dpi. PCR analysis of these plants also resulted in negative reactions.

Discussion

In this study, we characterized a new begomovirus named HYVLCV from diseased *H. rosa-sinensis* plants showing leaf curling, yellow vein, and vein enation symptoms. The causal agent contains a monopartite begomovirus and a helper DNA-β satellite. Sequence analysis reveals that HYVLCV shares 85.9 to 88.8%

nucleotide identity with 13 CLCuMuV isolates and 86.0 to 88.7% nucleotide identity with seven MaYVV isolates, which are also monopartite begomoviruses. CLCuMuV has been reported to infect hibiscus in India (Rajeshwari et al. 2005). MaYVV has been reported to infect *Sida acuta* (isolate C [CN-Yn340-Sid-08]) (Li and Zhou 2010) and *Malvastrum coromandelianum* (isolates A[CN-Yn278-04] and A[Y281]) in China. Based on the demarcation of begomovirus species set at <91% nucleotide identity for monopartite genome (Brown et al. 2015), our results indicate that HYVLCV is a putative

Table 2. Pairwise comparisons of full-length genome nucleotide and nucleotide/deduced amino acid sequences of open reading frames (ORFs) of hibiscus yellow vein leaf curl virus (GenBank accession number MF140451) with closely related monopartite begomoviruses available in GenBank

Virus ^a	Nucleotides (%) ^b	Nucleotides/amino acids					
		VI (%)	V2 (%)	CI (%)	C2 (%)	C3 (%)	C4 (%)
CLCuMuV (13)	85.9–88.8	79.8–81.6/90.3–91.1	79.6–80.2/63.8–74.1	90.6–97.6/89.8–98.4	88.2–91.8/66.9–86.8	84.1–87.9/69.6–87.3	93.4–100/85.1–100
MaYVV (7)	86.0–88.7	91.2–92.7/95.7–97.7	93.4–95.1/90.3–95.7	81.2–86.4/83.5–89.3	93.4–94.5/88.7–90.7	91.9–94.6/88.1–92.6	74.9–85.9/48.0–71.1
ToLCNDV2 (1)	88.3	88.8/95.3	91.7/86.2	90.2/91.2	91.6/86.1	90.3/83.2	92.4/85.1
PeLDV (4)	88.3–88.4	91.2–91.8/94.2–94.9	89.4–90.8/89.7–92.2	87.7–88.0/91.2–91.7	92.5/88.7	92.1/89.6	88.4/74.3
CLCuBaV (2)	85.8–87.6	87.9–88.2/93.4–93.8	89.4–89.7/87.1–87.9	85.7–90.4/87.6–91.7	90.3–90.9/84.1–87.4	88.1–89.4/83.7–85.2	88.4–94.1/75.2–90.1
KLCuV (3)	86.7–87.2	90.7–91.7/95.7–96.1	92.0–95.4/89.7–94.0	83.6–83.9/86.0–86.3	94.0–94.3/88.1–90.1	92.6–95.1/89.6–93.3	75.3–77.0/48.0–50.0
HoLCV (3)	84.9–87.1	87.4–87.8/94.9–95.3	89.1–90.8/88.8–89.7	83.9–87.9/87.9–90.7	89.4–90.3/81.5–82.8	88.4–90.8/85.2–85.9	86.8–91.1/71.3–78.2
EcYVV (3)	85.8–86.5	86.9–88.7/93.4–95.3	86.9–88.7/87.9–91.4	84.5–84.6/87.3–87.8	89.2–91.2/82.1–85.4	89.6–89.9/85.9–88.1	88.1–88.4/76.2–77.2
CLCuKoV-Bu (9)	85.4–86.3	79.4–80.8/91.4–92.6	75.6–76.4/66.4–67.2	94.6–95.3/94.5–96.2	NA ^c	76.8–86.9/71.9–81.5	97.0–98.5/92.1–97.0
MaYMV (2)	85.3–85.4	80.3–81.2/85.6	96.3/96.6	85.0–85.5/88.7–89.0	93.6/90.7	93.8/91.1	86.9–88.4/70.1–72.3

^a Abbreviations: malvastrum yellow vein virus (MaYVV), cotton leaf curl Multan virus (CLCuMuV), tomato leaf curl New Delhi virus 2 (ToLCNDV2), pea leaf distortion virus (PeLDV), cotton leaf curl Bangalore virus (CLCuBaV), kenaf leaf curl virus (KLCuV), hollyhock leaf curl virus (HoLCV), eclipta yellow vein virus (EcYVV), cotton leaf curl Kokhran virus-Burewala (CLCuKoV-Bu), and malvastrum yellow mosaic virus (MaYMV). Numbers within parentheses represent the number of viral sequences used for comparison.

^b Full-length nucleotides.

^c The CLCuKoV-Bu ORF C2 is currently not available for pairwise comparison.

Fig. 3. Phylogenetic relationships of **A**, hibiscus yellow vein leaf curl virus (HYVLCV) genome and **B**, its associated β satellite malvastrum yellow vein betasatellite (MaYVB)-Hib with those of closely related begomoviruses. The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates available in MEGAX. Abbreviations: malvastrum yellow vein virus (MaYVV), cotton leaf curl Multan virus (CLCuMuV), tomato leaf curl New Delhi virus 2 (ToLCNDV2), pea leaf distortion virus (PeLDV), cotton leaf curl Bangalore virus (CLCuBaV), kenaf leaf curl virus (KLCuV), hollyhock leaf curl virus (HoLCV), eclipta yellow vein virus (EcYVV), cotton leaf curl Kokhran virus-Burewala (CLCuKoV-Bu), malvastrum yellow mosaic virus (MaYMV), malvastrum yellow vein betasatellite (MaYVB), luffa leaf distortion betasatellite (LuLDB), cotton leaf curl Multan betasatellite (CLCuMuB), kenaf leaf curl betasatellite (KLCuB), sida yellow vein betasatellite (SiYVB), and cotton leaf curl betasatellite (CLCuB). DNA sequences of wheat dwarf virus (WDV) and tomato leaf curl Taiwan virus (ToLCTWV) were used as outgroups.

Table 3. Pairwise comparisons of full-length genome nucleotide sequence of malvastrum yellow vein betasatellite (MaYVB)-Hib (GenBank accession number MF140456) with closely related begomovirus-associated β satellites available in GenBank

Virus name ^a	β satellite DNA nucleotides (%)	β C1 nucleotides (%)	β C1 amino acids (%)
MaYVB (5)	78.4–78.6	84.6–86.0	80.7–84.0
MaYVYnB (5)	77.2–78.3	81.0–83.2	80.7–82.4
LuLDB (5)	73.2–78.4	81.5–81.8	79.8
CLCuMuB (5)	74.5–76.1	80.7–81.0	77.3–78.2
KLCuB (4)	72.7–76.5	79.8–80.1	75.6–78.2
SiYVB (1)	76.3	78.4	76.5
CLCuB (6)	73.4–74.3	76.2–79.3	71.4–76.5

^a Abbreviations: malvastrum yellow vein betasatellite (MaYVB), malvastrum yellow vein Yunnan betasatellite (MaYVYnB), luffa leaf distortion betasatellite (LuLDB), cotton leaf curl Multan betasatellite (CLCuMuB), kenaf leaf curl betasatellite (KLCuB), sida yellow vein betasatellite (SiYVB), and cotton leaf curl betasatellite (CLCuB). Numbers within parentheses represent the number of viral sequences used for comparison.

Table 4. Detection of a putative recombination event in hibiscus yellow vein leaf curl virus (HYVLCV)^a

Event	Region	Major parent	Minor parent	Detection method, average <i>P</i> value					
				RDP	GENCONV	BootScan	MaxChi	Chimaera	3Seq
HYVLCV	1,729–2,728	KLCuV-Y341 (92.3%)	CLCuMuV-TH4872-9 (100%)	4.863×10^{-62}	7.007×10^{-52}	2.642×10^{-46}	2.485×10^{-7}	8.347×10^{-31}	9.365×10^{-13}

^a Abbreviations: kenaf leaf curl virus (KLCuV), cotton leaf curl Multan virus (CLCuMuV), and RDP = Recombination Detection Program.

Fig. 4. Agroinoculation assays of infectious hibiscus yellow vein leaf curl virus (HYVLCV) viral DNA and associated betasatellite malvastrum yellow vein betasatellite (MaYVB)-Hib. **A**, Construction map of infectious clones, tandem-repeated HYVLCV genome (pHib-2A), and partial-repeated associated betasatellite DNA (pHib-1.6 β). *Nicotiana benthamiana* inoculated with **B**, HYVLCV or **C**, MaYVB-Hib alone shows no symptoms at 12 days postinoculation (dpi), whereas **D**, coinoculation results in mosaic, vein yellowing, and leaf curl symptoms at 12 dpi. **E**, *N. benthamiana* inoculated with HYVLCV alone shows yellowing and leaf curling symptoms at 28 dpi. **F**, The presence of HYVLCV and MaYVB-Hib was confirmed (12 dpi) by PCR using primers specially targeting HYVLCV or MaYVB-Hib. Abbreviations: H = healthy *N. benthamiana*, N = agroinfiltration with empty vector as a mock control, A = inoculation of pHib-2A, β = inoculation of pHib-1.6 β , A+ β = coinoculation of pHib-2A and pHib-1.6 β , and P = HYVLCV-infected *Hibiscus rosa-sinensis* collected from field.

new begomovirus. RDP4 analysis of the complete sequence of HYVLCV with other monopartite begomoviral sequences in the database reveals that HYVLCV is likely a result of viral recombination between CLCuMuV (minor parent) and KLCuV (major parent). Both CLCuMuV and KLCuV have been reported to infect *Hibiscus* plants (Paul et al. 2009; Rajeshwari et al. 2005).

Plants infected by begomoviruses carrying DNA- β satellites often show enation symptoms (Briddon et al. 2003; Tahir and Mansoor 2011). Similar vein enation was observed on the abaxial surface of diseased *H. rosa-sinensis* leaves (Fig. 1C). It has also been reported that transgenic *Arabidopsis* plants expressing β C1 of DNA- β satellite alone exhibit vein enation (Yang et al. 2008). A begomoviral DNA- β satellite (Hibbata-22) designated MaYVB-Hib was detected, cloned, and sequenced. Pairwise examination of nucleotide sequences of β satellites shares higher than 78% nucleotide identity with those of MaYVB, malvastrum yellow vein Yunnan betasatellite (MaYVYNB), and luffa leaf distortion betasatellite (LuLDB). SDT and pairwise comparisons analyses

also reveal that MaYVB-Hib shows low nucleotide identity with MaYVYNB (78.3%) and LuLDB (78.4%). MaYVB-Hib shares the highest nucleotide identity with MaYVB (78.6%). Two field plants infected with the HYVLCV without DNA- β display milder vein yellowing and leaf curl symptoms compared with the HYVLCV with DNA- β . The β C1 gene in the DNA- β has been demonstrated to be required for virulence (Briddon et al. 2001; Mansoor et al. 2003; Yang et al. 2008). HYVLCV was not detected by DNA-A-specific primers in one hibiscus plant showing virus-like symptoms, which indicates that the symptoms were caused by another virus rather than HYVLCV.

Experiments have demonstrated that HYVLCV alone could infect *N. benthamiana* and induce yellowing and mild leaf curling symptoms at 28 dpi but the timing for symptoms was considerably longer and symptom development was less severe than for the plants coinfecting with HYVLCV and MaYVB-Hib, which developed severe mosaic, vein yellowing, and leaf curl symptoms at 12 dpi. Also, qPCR analysis showed that the presence of MaYVB-Hib enhances the titer of HYVLCV in the *N. benthamiana*. This result is similar to those described for interaction between tomato leaf curl China virus and tomato leaf curl China virus β satellites (Yang et al. 2011). However, agroinfiltration assays coupled with PCR examination revealed that MaYVB-Hib alone fails to live in host plants without its helper virus. Although both HYVLCV and MaYVB-Hib, after being coinoculated, are biologically active and capable of causing symptoms on *N. benthamiana*, the agroinoculation of infectious clones of both molecules fails to infect *H. rosa-sinensis* after several attempts (data not shown). Studies in okra yellow crinkle virus and cotton leaf curl Gezira virus revealed that infectious clones of either virus, after being introduced by agroinoculation, fail to infect malvaceous okra plants, presumably due to the incompatibility with *Agrobacterium tumefaciens* (Kon et al. 2009). Studies in jatropha mosaic virus also revealed that infectious clones are unable to establish infectivity in jatropha by agroinoculation (Melgarejo et al. 2015). Moreover, infectious clones of CLCuMuV have been shown to exhibit less infectivity using either *Agrobacterium*-mediated inoculation or the biolistic system (Briddon et al. 2001). The mechanisms attributable to the failure of agroinoculation remain elusive; however, it could be simply due to limited host ranges of *A. tumefaciens* (Saeed 2008). This problem can be resolved by grafting or whitefly transmission of the virus. Two *H. rosa-sinensis*-infecting begomoviruses, CLCuMuV and CLCuKoV-Bu, have been shown to be pathogenic in back-inoculation

Fig. 5. Semiquantitative assessment of hibiscus yellow vein leaf curl virus (HYVLCV) in *Nicotiana benthamiana* (12 days postinoculation) by real-time PCR. *N. benthamiana* was infiltrated with empty vector (Mock), pHib-2A (A), pHib-1.6 β (β), or a combination of pHib-2A and pHib-1.6 β (A+ β). Relative viral DNA accumulation was quantified and normalized with respect to the *N. benthamiana actin* gene.

Table 5. Plant species agroinfiltration with infectious hibiscus yellow vein leaf curl virus HYVLCV viral DNA and associated betasatellite

Family, species	Infected/inoculated	Latent period (days)	Virus detection (PCR)-A/ β ^a	Symptoms ^b
Cucurbitaceae				
<i>Cucumis sativus</i>	0/4	...	—	NS
<i>Citrullus lanatus</i>	0/4	...	—	NS
Legumisoae				
<i>Vigna unguiculata</i>	0/4	...	—	NS
<i>Pisum sativum</i>	0/4	...	—	NS
Malvaceae				
<i>Abelmoschus esculentus</i>	0/4	...	—	NS
<i>Hibiscus rosa-sinensis</i>	0/4	...	—	NS
Solanaceae				
<i>Nicotiana benthamiana</i>	4/4	7–9	+/+	Mo, YV, LC
<i>N. tabacum</i> ‘White Burley’	1/4	18	+/+	Mo, YV, LC, VE
<i>N. tabacum</i> ‘Xanthi’	1/4	18	+/+	Mo, YV, LC, VE
<i>N. glutinosa</i>	0/4	...	—	NS
<i>N. occidentalis</i>	0/4	...	—	NS
<i>N. edwardsonii</i>	4/4	7–9	+/+	Mo, YV, LC
<i>N. rustica</i>	0/4	...	—	NS
<i>Capsicum annuum</i>	0/4	...	—	NS
<i>Solanum lycopersicum</i>	0/4	...	—	NS

^a Symbols: + indicates the presence of HYVLCV and associated betasatellite was confirmed (12 days postinoculation) by PCR using primers specially targeting HYVLCV or associated betasatellite and — indicates that no viral DNA was detected by PCR.

^b NS = no symptoms, Mo = mosaic, LC = leaf curl, YV = yellow vein, and VE = vein enation.

assays using grafting or whitefly transmission (Akhtar et al. 2014; Rajeshwari et al. 2005). In the present study, we have also demonstrated that HYVLCV can be transmitted via grafting. Three HYVLCV-free *H. rosa-sinensis* scions developed leaf yellow vein symptoms 30 to 40 days after grafting onto the HYVLCV-infected stocks and developed vein enation and leaf curl symptoms at 60 dpi. HYVLCV and MaYVB-Hib could be detected in the symptomatic scions by PCR (Fig. 1F). The results confirm the infectivity of HYVLCV in *H. rosa-sinensis*.

H. rosa-sinensis is often propagated via vegetative cuttings in Taiwan. Sequence analysis of four HYVLCV clones (Hib2-2, Hib5-5, Hib6-2, and Hib8-8) revealed 100% nucleotide identity of genomic DNA sequences with HYVLCV. Future studies should investigate the ability of whiteflies to transmit the newly identified begomovirus, which may contribute to its rapid spread. Although agroinoculation has revealed that HYVLCV can propagate in certain *Nicotiana* spp., the exact host range of HYVLCV remains unknown. Primers specifically targeting the HYVLCV monopartite genome have been tested for detection of the virus from field-collected samples, which will be useful for fast and reliable diagnosis of HYVLCV. The primers also could facilitate determination of the host range of HYVLCV in nature.

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