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Serological and Molecular Characterizations of a Hibiscus-infecting Tobamovirus in Taiwan

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ABSTRACT

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A previously described hibiscus virus showing a tobamovirus profile was purified and used to produce polyclonal antisera in rabbit. The antiserum and viral RNA obtained in the study were serologically and molecularly characterized. The coat protein (CP) and movement protein (MP) genes of the virus were cloned and sequenced and the nucleic acid and the deduced amino acid sequences analyzed. The nucleic acid and amino acid sequences have high homology when compared with those of *Hibiscus latent Singapore virus* (HLSV, previously Hibiscus virus S, HVS) but less with those of *Hibiscus latent Fort Pierce virus* (HLFPV, previously Florida hibiscus virus, FHV). Results from current investigation indicate that the hibiscus virus cultures isolated in Taiwan are strains of HLSV.

Key words : hibiscus, *Hibiscus rosa-sinensis*, *Tobamovirus*

INTRODUCTION

Hibiscus (*Hibiscus rosa-sinensis* L.) is a popular woody ornamental commonly used as fence or potted plants in Taiwan. A tobamovirus-like viral agent was thought to be the cause of hibiscus showing vein-yellowing and mosaic [(6)]. The virus is not serologically related to *Tobacco mosaic virus* (TMV) and *Odontoglossum ringspot virus* (ORSV) in double diffusion test.

The virions of tobamoviruses are rigid-rods of approximately 300 x 18 nm. The genome consists of one single-stranded positive sense RNA of approximately 6.4-6.6 kb. There are methylguanosine cap and tRNA-like structure at the 5' and 3' termini, respectively. Four open reading frames (ORFs) are present within the tobamovirus genome. Two overlapping ORFs begin at the 5' proximal start codon. Termination at the first in-frame stop codon producing a 125-130 kDa protein and a 180-190 kDa protein is produced by readthrough of its termination codon. Both gene products are necessary for efficient replication. The remaining two ORFs encode movement protein (MP) and coat protein (CP) and were expressed from individual subgenomic mRNAs [(15)]. Tobamoviruses have been classified into two subgroups which have different genomic locations of their origin of virion assembly [(10)]. The origin of assembly is located within the ORF for the MP of subgroup I tobamoviruses which infect solanaceous plants, and within the CP ORF of subgroup II tobamoviruses that infect cucurbits. Nucleotide sequence comparisons and the different organization of MP and CP ORFs suggest a third subgroup (crucifer-pathogenic) distinct from two aforementioned subgroups of tobamoviruses [(3)].

Recently, two hibiscus-infecting tobamoviruses have been reported in Singapore and in Florida, USA. The viruses were originally designated as Hibiscus virus S (HVS) [(21)] and Florida hibiscus virus (FHV) [(1)] and have been officially renamed as *Hibiscus latent Singapore virus* (HLSV) and *Hibiscus latent Fort Pierce virus* (HLFPV), respectively [(2, 12, 23)]. The CP gene of HLFPV shares 68% nucleotide sequence identity and 73% deduced amino acid sequence identity with those of HLSV indicating that these viruses are two distinct species in the genus *Tobamovirus* [(1)]. In addition, the CP genes and deduced proteins of HLSV and HLFPV are only 45 to 53% and 37 to 57% identical at nucleotide and amino acid levels to all other

tobamoviruses [1, 21]. Based on this information, a fourth (malvaceous) tobamovirus subgroup has been proposed [1].

In this paper, we analyzed the serological relationships and compared the CP and MP sequences of the hibiscus-infecting tobamovirus occurring in Taiwan with those reported. Results indicate that the virus isolates occurring in Taiwan are strains of HLSV.

MATERIALS AND METHODS

Virus and plants

Leaves showing vein-yellowing and mosaic symptoms were collected from hibiscus plants growing on the campus of National Chung Hsing University, Taichung. Virus cultures were isolated from the diseased leaves and established by three successive local lesion isolations and maintained in *Chenopodium quinoa*. Some common indicator plants in *Solanaceae, Cucurbitaceae, Cruciferae* and *Malvaceae*, including cotton (*Gossypium hirsutum*), okra (*Abslmoschus esculentus*), roselle (*Hibiscus sabdariffa*), cotton rose (*Hibiscus mutabilis*), and *Abelmoschus moschatus*, were used in host range studies. Inoculation was carried out mechanically by inoculating leaves with crude sap of leaves from infected plants.

Purifications of virions and viral RNA

Virions and viral RNA were purified according to the method described by Chapman [5]. Leaves of infected *C. quinoa* were homogenized with phosphate buffer (0.2 M, pH 7.2) and virions were concentrated with 8% polyethyl glycol (PEG) 6000 and purified by differential centrifugation followed by 10-40% sucrose density gradient centrifugation. Viral RNA was extracted from purified virions by using phenol-chloroform extraction and precipitation by ethanol.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of CP subunit was determined on 12% gels by SDS-PAGE as previously described [13].

Antiserum and immunoassays

Antisera were prepared in New Zealand white rabbits by intramuscular injections, once with complete adjuvant-emulsified purified virus (0.5 mg) followed by three injections with incomplete adjuvant-emulsified virions (0.5 mg) at 7-day intervals. HLFPV and HLSV antisera were kindly provided by Dr. S. Adkins (USHRL-ARS-USDA, Fort Pierce, Florida, USA) and Dr. S.M. Wong (National Singapore University, Singapore, Republic of Singapore), respectively, and, TMV, ORSV and *Cucumber green mottle mosaic virus* (CGMMV), by Dr. C.A. Chang (TARI, Wufeng, Taichung, Taiwan).

The SDS-PAGE gels were electro-blotted onto a nitrocellulose membrane and the protein blots were immunostained with tobamovirus-specific antisera or IgG at 1/2000 dilution and developed with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoylphosphate ρ-toluidine salts (BCIP) as described [8].

RT-PCR

The primer pairs for RT-PCR for amplification of CP and MP genes were designed using sequences of HLSV (Acce. No. AF395898 and AF395899, respectively)[21]. The forward and reverse sequences of primer set are 5'-CCATGCCTTACCTTAA-3' / 5'-GGTTACGTTGTAGTAG-3' for CP gene and 5'-CCATGAGTCTTAATCTGGC-3' / 5'-CCTTAAGGTAAGGCATCGC-3' for MP gene. The predicted length of the amplified DNA fragments of CP and MP are 492 bp and 849 bp, respectively. Viral RNA was used to synthesize first strand cDNA using MMLV-reverse transcriptase (Epicentre, Madison, WI, USA) with reverse primers. PCR amplifications of cDNA were carried out under the following conditions: denaturation for 3 min at 94℃ and then 32 cycles of denaturation for 50 sec at 94℃, annealing for 50 sec at 55℃, and extension for 1 min at 72℃. The final extension was a 7-min incubation at 72℃. PCR products were analyzed by electrophoresis on a 1% agarose gel and the DNA bands were visualized by ethidium bromide staining and by UV transilluminator.

Cloning and sequencing of CP and MP genes

PCR products of expected size were cloned into the pCR® 2.1-TOPO® vector (Invitrogen, Carlsbad, CA, USA) according to the instruction of the manufacturer. The plasmid DNAs of the transformants were purified by the mini-prep method [4, 20] and the inserts were confirmed by the automatic DNA sequencing system (ABI3730; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Amino acid sequences were translated by the software of SeqWeb Version2.02, Web-based Sequence Analysis (GCG® Wisconsin Package®, Accelrys Inc. CA, USA). Nucleotide and amino acid sequences were analyzed by the BestFit and the PileUp programs of the SeqWeb version 2.02.

Phylogenetic analysis

The phylogenetic analysis of the CP and MP genes among 19 tobamoviruses was conducted by the Neighbor-Joining program of PHYLIP version 3.5c [9]. Genetic distances were calculated using the program PRODIST. The phylogenetic trees were constructed by a distance method (NEIGHBOR) using the original data set and 1000 bootstrap data set generated by the program SEQBOOT from the original set. The consensus tree was generated using the program CONSENSE.

RESULTS

The virus and host range

Localized infections were found in *C. quinoa* and *C. amaranticolor* when plants were inoculated with leaf extracts prepared from infected hibiscus. Two individual types of local lesions - chlorotic and yellowish -are consistently formed on inoculated leaves of *C. quinoa* 4-5 days after inoculation. The chlorotic local lesions showed chlorosis first and turned necrotic while yellowish local lesions remained yellow but never became necrotic. The two virus cultures were established after three successive single lesion isolations from chlorotic or yellowish lesions and designated Hibiscus virus-chlorotic isolate (HV-Ch) and Hibiscus virus-yellowish isolated (HV-Y), respectively, in this study. Not all of the plants inoculated were infected with HV-Ch or HV-Y. Some plants in the family *Malvaceae* and genera *Chenopodium* and *Nicotiana* are susceptible to the virus (Table 1).

Table 1. Reactions of experimental plants after mechanical inoculation with hibiscus viruses (strains HV-Y and HV-Ch)

Plant	Reactions [1]	
	Inoculated leaves	Upper leaves
Chenopodiaceae		
Chenopodium amaranticolor	LL	/
C. quinoa	LL	/
Malvaceaea		
Abelmoschus esculentus	MM and DRS	MM
Gossypium hirsutum	MM and DRS	MM
Hibiscus cannabinus	MM	MM
H. sabdariffa	MM	MM
Solanaceae		
Nicotiana benthamiana	MM	/
N. rustica	MM	/
N. tabacum	MM	/

[1] LL: local lesion; MM: mild mosaic; DRS: dark red spot; /: no infection.

All malvaceous plants tested were systemically infected by HV-Y and HV-Ch although with various degrees of infection in individual plant species. In general, the systemic symptoms were very mild, and lesions ranging from dark red to purple colors developed on inoculated leaves 10-14 days after inoculation. Interestingly, both HV-Ch and HV-Y infected the inoculated leaves of *Nicotiana benthamiana*, *N. rustica*, and *N. tabacum* but failed to spread systemically. Mild mosaic developed on inoculated leaves of *Nicotiana* spp. but no symptoms were observed on upper leaves. The infections on the inoculated lower leaves, but not on the systemic upper leaves, were confirmed by ELISA and bioassay in *C. quinoa*.

SDS-PAGE and immunoblotting

PAGE of SDS dissociated proteins of purified virus revealed a major polypeptide band of about 18 kDa, larger than CP of TMV (17.5 kDa). Immunoblotting identified the protein bands are the CP of the hibiscus virus (Fig. 1). Test with antisera to HLSV, HLFPV, CGMMV, and TMV showed

Table 2. Accession numbers of nucleotide and deduced amino acid of the movement protein (MP) and the coat protein (CP) ORFs and their molecular weight (MW, kDa) of some tobamoviruses used in this study

Viruses	MP			CP			Acce. No.
	nt	aa	MW	nt	aa	MW	
HV-Ch	846	282	30.85	489	163	18.20	AY546636, AY546635
HV-Y	846	282	30.92	489	163	18.20	AY546634, AY546633
HLSV	846	282	30.89	489	163	18.20	AF395899, AF395898
HLFPV	855	285	31.10	474	158	17.59	AY250831
CFMoMV	741	247	27.77	489	163	17.39	AF321057
CGMMV	795	265	28.88	486	162	17.40	D12505
FrMV	771	257	28.46	525	175	19.01	AF165884
KGMMV	789	263	28.28	486	162	17.19	AJ295948
NTLV	867	289	31.72	474	158	17.24	AY137775
ObPV	825	275	30.62	486	162	17.98	D13438
ORSV	912	304	33.58	477	159	17.78	X82130
PMMoV	774	258	28.51	474	158	17.24	AB084456
RMV	795	265	29.75	474	158	17.67	AF254924
SHMV	852	284	30.97	495	165	18.38	J02413
TMV	807	269	30.00	480	160	17.62	V01408
TMGMV	771	257	28.44	480	160	17.59	M34077
ToMV	795	265	29.29	480	160	17.75	AB083196
TVCV	804	268	30.11	474	158	17.60	U03387
ZGMMV	786	262	28.15	486	162	17.18	AJ295949

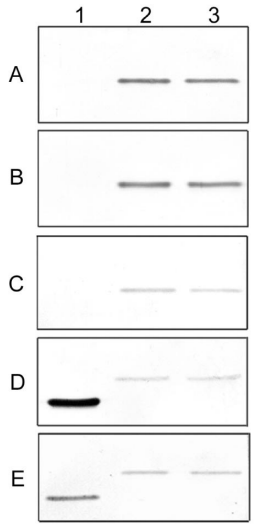

Fig. 1. Immuno-blotting of purified tobamoviruses CGMMV (1), HV-Y (2), and HV-Ch (3). Viral coat proteins were probed with polyclonal antibodies of HV-Y (blot A), HLSV (blot B), HLFPV (blot C), CGMMV (blot D), and TMV (blot E).

that the antisera reacted with various degrees with the dissociated protein of HV-Ch and HV-Y, indicating that both HV-Ch and HV-Y are members of *Tobamovirus*. Both isolates reacted strongly with antiserum of HLSV than with that of HLFPV.

Sequence analysis

The cDNA of the viral CP and MP genes were cloned and sequenced. The CP genes of HV-Ch and HV-Y contain 492 nucleotides and encode a peptide of 164 amino acid residues with a molecular weight of 18.2 kDa; whereas the MP genes of both isolates contain 849 nucleotides and encode a peptide of 283 amino acid residues with a molecular weight of 31 kDa (Table 2). The CP and MP sequences were compared with those of HLSV, HLFPV, TMV, ORSV and CGMMV. Sequence identity at the nucleotide level of CP and MP between HV-Ch, HV-Y and non-malvaceous tobamoviruses is less than 65% (54.99-64.96%) and 60% (53.5-59.6%), respectively (Table 3), whereas those of the deduced amino acid level are less than 46% (41.77-45.91%) and 32% (29.33-32.04%), respectively (Table 4). Sequence identity at the nucleotide and amino acid levels of both CP and MP among malvaceous tobamoviruses is more than 71%. The CP and MP identities among HV-Ch, HV-Y and HLSV are higher than 99% indicating that HV-Ch and HV-Y are

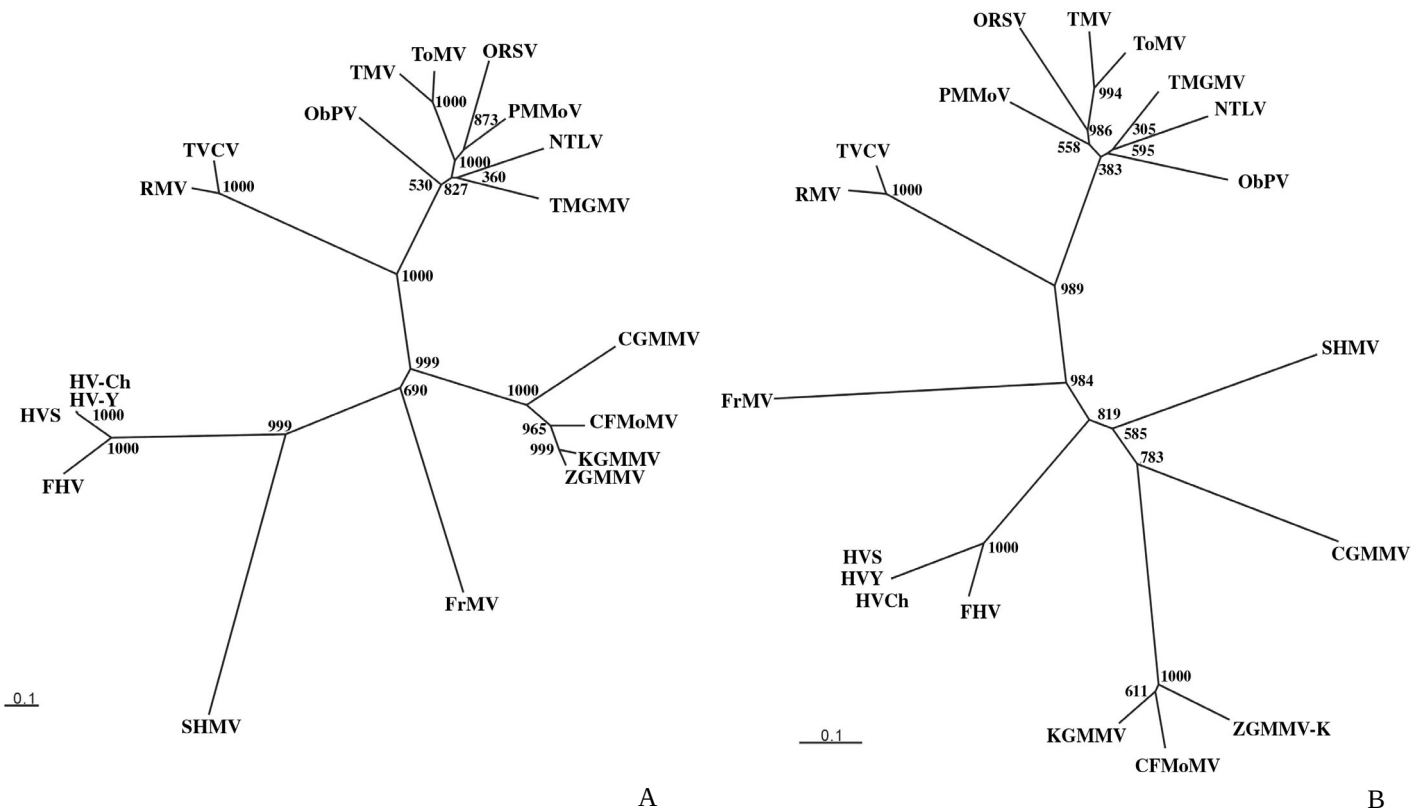

Fig. 2. Phylogenetic tree based on deduced amino acid sequence comparisons of movement protein (A) and coat protein (B) showing the relationship of HV-Y and HV-Ch to other nineteen tobamoviruses in Table 2. Values at the forks indicate the numbers of times out of 1000 tree that this grouping occurred after bootstrapping the data. The scale bar shows distances at substitutions per base.

Table 3. Comparisons of homology (%) of nucleotide sequences of coat protein (CP) and movement protein (MP) genes of HV-Ch and HV-Y with some tobamoviruses

Virus	HV-Ch		HV-Y	
	CP	MP	CP	MP
HV-Ch	-	-	100	99.76
HV-Y	100	99.76	-	-
HVS	99.39	98.94	99.39	99.18
FHV	71.82	71.26	71.82	71.69
CGMMV	54.99	53.52	54.99	53.52
TMV	61.09	57.69	61.09	57.69
ORSV	64.96	59.60	64.96	59.60

Table 4. Comparisons of homology (%) of deduced amino acid sequences of coat protein (CP) and movement protein (MP) of HV-Ch and HV-Y with some tobamoviruses

Virus	HV-Ch		HV-Y	
	CP	MP	CP	MP
HV-Ch	-	-	100	99.29
HV-Y	100	99.29	-	-
HVS	100	98.94	100	99.65
FHV	76.92	82.97	76.92	82.97
CGMMV	45.91	31.94	45.91	31.94
TMV	41.77	31.55	41.77	32.04
ORSV	41.83	29.33	41.83	29.33

strains of HLSV. Based on the deduced amino acid sequences of MP and CP from 17 tobamoviruses, phylogenetic trees were generated (Fig. 2-A and 2-B).

Both CP and MP sequences of HV-Y and HV-Ch are deposited in the Genbank database with accession numbers of AY546633, AY546634, AY546635, and AY546636, respectively.

DISCUSSION

Hibiscus plants showed symptoms of vein yellowing and mosaic had been observed in Taiwan for many years and the causal agent was identified as a tobamovirus-like agent [6]. Serological tests with antisera to many known tobamoviruses indicated that it appeared to be a new species [6]. In current studies, the tobamovirus causing vein yellowing and mosaic symptoms on hibiscus was biologically, serologically, and molecularly characterized. Results show that the previously reported hibiscus-infecting tobamoviruses in Taiwan [6] are strains of *Hibiscus latent Singapore virus* (HLSV, previously Hibiscus virus S) [21, 23].

Based on the genomic locations and the origin of virion assembly (OAS), tobamoviruses have been classified into two subgroups [10]. In subgroup 1 (Solanaceous) tobamoviruses, the OAS is located within the MP ORF, whereas in subgroup 2 (Cucurbitaceous) tobamoviruses, the OAS is within CP ORF. A third subgroup (Cruciferous) of tobamoviruses includes those with different nucleotide sequence and different organization of MP and CP ORFs [3, 14]. Hibiscus-related tobamoviruses (HLSV and HLFPV) are newly emerging viruses in Asia and America, and have distinct characteristics from the rest of the tobamoviruses [1, 2, 6, 21, 23]. The (GXX)n-rich sequences were found within the CP ORF of HLSV [21] and HV-Ch (data not shown) indicating that these Asian hibiscus-infecting tobamoviruses are members of subgroup 2. The CP genes and deduced proteins of hibiscus-related tobamoviruses are only about 50% similar, at nucleotide and amino acid levels, to all other tobamoviruses. Host range studies showed that they are restricted to malvaceous plants and *Chenopodium* spp.. Because of the aforementioned difference, HLSV and HLFPV were re-classified as members of the newly proposed malvaceous subgroup of the genus *Tobamovirus* [1].

The re-inoculation of the hibiscus plants with the viruses isolated in current studies was not possible due to lack of virus-free hibiscus plants. The field observation showed that symptoms on hibiscus in Taiwan are severe and conspicuous comparing to the symptomless infections in hibiscus plants caused by HLSV or HLFPV alone. The environmental factors, the age of the plants when infected, and/or the latent period after infection can all affect the symptom expression. Multiple infections with different isolates or with other viruses are other possibilities that cannot be excluded since *Hibiscus chlorotic ringspot virus* (HCRSV) is another hibiscus-infecting virus found in Taiwan [16].

Different types of local lesions on *C. quinoa* appeared after inoculation with crude sap from diseased hibiscus indicating that there might have multiple infection of different strains of *Tobamovirus* in the field. It now appears that the 126/183 kDa gene is somehow involved in the prevention of normal chloroplast development and thus the formation of yellows and mosaic symptoms in infected plants and that the induction of necrosis (hypersensitive reactions, HR) was localized in the CP ORF [7, 11, 18, 19]. Sequence comparisons between HV-Ch and HV-Y show that the CP ORFs of the two viral strains are identical suggesting that sequences of 126/183 kDa genes is a potential factor responsible for the formation of local lesions with different appearances. The full length sequences of genomic RNA of HV-Y and HV-Ch are now being analyzed.

Serological relationships between the viruses in this investigation and other reported tobamoviruses were analyzed by immunoblotting. The antisera to TMV (subgroups 1 tobamovirus) and CGMMV (subgroup 2 tobamovirus) reacted weakly with hibiscus-infecting viruses. In reciprocal tests, the antisera of hibiscus viruses did not react with CGMMV indicating that serological relatedness among tobamoviruses is dependent on how the tests are being conducted [22].

Since hibiscus is propagated vegetatively and tobamoviruses are transmitted mechanically, there is a high

risk of wide spread of virus infection in hibiscus. An appropriate and effective means of disinfecting cutting tools during propagation is therefore essential to prevent virus transmission. Dipping cutting tools and pruning equipment in 10% sodium hypochlorite and 20% non-fat dry milk has shown completely preventing transmission infection of hibiscus to HLFPV [12].

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摘 要

劉芳麟[1]、陳宗祺[1]、葉錫東[1]、徐惠迪[2]、陳慶忠[3]、包慧俊[4]、陳煜焜[1,5]. 2004. 台灣地區感染朱槿的似菸草嵌紋病毒特性之分析. 植病會刊 13：283-290. ([1.] 台中市 國立中興大學植物病理系；[2.] 美國馬里蘭州 美國農部；[3.] 彰化縣大村鄉 台中區農業改良場；[4.] 雲林縣斗六市 環球技術學院；[5.] 聯絡作者，電子郵箱：ykchen@nchu.edu.tw；傳眞：886-4-22877585)

感染朱槿 (*Hibiscus rosa-sinensis* L.) 的菸嵌病毒屬病毒 (Genus *Tobamovirus*) 近來在國外陸續被報導，分析其寄主範圍、血清反應及鞘蛋白 (coat protein, CP) 和移動蛋白 (movement protein, MP) 基因的核苷酸序列等特性，顯示感染朱槿的桿狀病毒爲 *Tobamovirus* 屬的新種病毒。台灣地區也曾有朱槿受疑似菸嵌病毒感染之記錄。本研究針對感染台灣地區朱槿的菸嵌病毒之寄主範圍、血清親緣關係及鞘蛋白和移動蛋白等基因的核苷酸序列等特性進行進一步之分析。試驗結果顯示感染台灣地區朱槿的桿狀病毒與Tobamoviruses 有血清類緣關係。在CP 和 MP 的核苷酸序列及胺基酸序列上與 *Hibiscus latent Singapore virus* (HLSV) 有高達 98 % 的相似度；而與 *Hibiscus latent Fort Pierce virus* (HLFPV) 則僅有 71 % 的相似度。本研究之結果顯示台灣地區所分離之朱槿菸嵌病毒爲HLSV 的一個分離株。

關鍵詞：朱槿、菸嵌病毒