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Molecular and serological characterization of a distinct potyvirus causing latent infection in calla lilies

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ABSTRACT. A virus (isolate: Ca-M19) capable of inducing local lesions on *Chenopodium quinoa* Willd. was isolated from calla lilies (*Zantedeschia* spp.). Subculture of Ca-M19 was easily maintained in *C. quinoa*, but a back inoculation from single lesion of *C. quinoa* to calla lilies has so far not been successful. Typical potyvirus-like flexuous particles were consistently detected in Ca-M19 infected plants, and a 1.3-kb DNA fragment was amplified from these plants by reverse-transcription polymerase chain reaction (RT-PCR) using potyvirus degenerate primers. The PCR product was cloned and its sequence analyzed (AF469171). The amplicon was revealed to correspond to the 3′ terminal region of a potyviral genome. After comparing this sequence with known potyvirus sequences in the GenBank, we considered the virus a new species of *Potyvirus* based on the uniqueness in its coat protein gene (CP) and the 3′ non-coding region (NCR). Comparative studies showed that *Soybean mosaic virus* (SMV) and *Watermelon mosaic virus* 2 (WMV 2) were the two most similar potyviruses with Ca-M19, but they shared only 80% of nucleotide identities in CP and NCR with Ca-M19. Attempts to purify a sufficient quantity of Ca-M19 from *C. quinoa* for preparation of antibodies were unsuccessful. Alternatively, Ca-M19 CP was expressed by the vector pET28b and purified from *E. coli* culture, and polyclonal antibodies were prepared in rabbits. The antibody was applied in ELISA, Western blotting, SDS-immunodiffusion and immuno-specific electron microscopy for the detection of Ca-M19 in calla lilies. It did not react with at least five calla lily infecting potyviruses, including *Dasheen mosaic virus*, *Bean yellow mosaic virus*, *Konjak mosaic virus*, *Turnip mosaic virus*, and *Zantedeschia mild mosaic virus*. Indirect ELISA and SDS-immunodiffusion tests showed that Ca-M19 was serologically related, but distinct from *Bean common mosaic virus* (BCMV), *Black cowpea mosaic virus* (BlCMV), *Melon vein banding mosaic virus* (MVbMV), *Passionfruit mottle virus* (PaMV), *Passionfruit crinkle virus* (PCV), *Passionfruit woodness virus* (PWV), *Soybean mosaic virus* (SMV), *Watermelon mosaic virus* 2 (WMV 2), and *Zucchini yellow mosaic virus* (ZYMV). Besides serological techniques, a primer pair (M19u/M19d) and a DNA probe were designed which could also specifically detect and differentiate Ca-M19 from other viruses. By the use of specific antibodies in ELISA, Ca-M19 was frequently detected in calla lily plants collected from several major calla lily production townships in Taiwan. Among 86 field samples positively reacting to the antibody, 77 of them exhibited evident systemic mosaic symptoms, but these symptomatic plants were confirmed to be infected simultaneously by other viruses. Nine plants were found to be infected by Ca-M19 alone. These plants were confirmed to have remained symptomless throughout a 6-month observation period. Therefore, we propose naming this isolate *Calla lily latent virus* (CLLV) for its inability to develop any visible symptoms on calla lily.

Keywords: Calla lily; Coat protein expression; Potyviruses; Sequence analysis; Serology.

INTRODUCTION

Calla lily (*Zantedeschia* spp.), an aroid plant species, is a popular ornamental crop in Taiwan and many parts of the world. Viral diseases and bacterial soft rot are the major factors limiting its production. Among viruses, *Dasheen mosaic virus* (DsMV) is considered the most prevalent and widespread in the family *Araceae*, including *Zantedeschia* spp. (Zettler et al., 1978; Zettler and Hartman, 1995). In addition to DsMV, several *Potyvirus* species—including *Bean yellow mosaic virus* (BYMV) (Pham et al., 2002), *Konjak mosaic virus* (KoMV) (Chang et al., 2001; Hu, 2001; Kwon et al., 2002; Pham et al., 2002), *Turnip mosaic virus* (TuMV) (Chen et al., 2003a), and *Zantedeschia mild mosaic virus* (ZaMMV) (Chen et al., 2003b;

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Huang and Chang, 2002, 2005)—were identified from calla lilies. These potyviruses always induce systemic mottle or mosaic symptoms with variable degree of severity on leaves. Diagnosing the causal potyviruses simply based on visual inspection of symptoms is difficult. Besides potyviruses, spherical viruses such as *Carnation mottle virus* (Chen et al., 2002b), *Calla lily chlorotic ringspot virus* (Lin et al., 2003), *Cucumber mosaic virus* (CMV), and *Tomato spotted wilt tospovirus* have also been reported to occur naturally in calla lilies (Zettler and Hartman, 1995). In general, these spherical viruses induce more severe symptoms, such as chlorotic spotting, necrotic streaks, ringspotting, and distortion of leaves, than those caused by potyviruses (Chen et al., 2002b; Zettler and Hartman, 1995). Based on our observation, some calla lily cultivars develop color breaking on their inflorescences when infected by CMV (unpublished data).

In 2001, we isolated an unknown virus from a calla lily plant dually infected with DsMV and KoMV. A pure culture was established by single lesion isolation on *Chenopodium quinoa* Willd. Although back inoculation using the extract of the lesion to calla lily was not successful, the virus was consistently detected from calla lily plants in the field. The virus was later found to be biologically, serologically and molecularly different from previously described calla lily-infecting potyviruses. In this paper, we provide evidence that this virus is unique. Due to its inability to induce symptoms on calla lily, we have christened this novel virus *Calla lily latent virus* (CLLV) (Chen et al., 2004).

MATERIALS AND METHODS

Virus source and maintenance

In the beginning of our study, an unknown virus designated Ca-M19 was isolated from a calla lily plant doubly infected by DsMV and KoMV, as determined by enzyme-linked immunosorbent assay (ELISA). Leaf extracts from this plant, however, induced local lesions on *C. quinoa* which normally was not susceptible to DsMV or KoMV. After three consecutive single lesion passages, a pure culture of Ca-M19 in the infected leaves of *C. quinoa* was preserved in 50% glycerol and stored in a freezer at -20°C. The bulb of the original Ca-M19 infected calla lily plant was grown in 7-inch pots under greenhouse conditions for subsequent studies.

Host reaction tests

Ca-M19 induced lesions on *C. quinoa* were ground in potassium phosphate buffer (50 mM, pH 7.5) at 1:10 dilution (g/ml) and used to mechanically inoculate tissue-culture derived calla lily plantlets cv. Black magic, *Nicotiana benthamiana* Domin, *Glycine max* (L) Merrill., and *C. quinoa* was used as control. Plants were kept in an insect-proof greenhouse for 2 months for symptom development. Ten plants each were inoculated, and inoculation tests were repeated thrice.

Virus purification

Ca-M19 was mass propagated in *C. quinoa*, and the inoculated leaves were collected 7-10 days after inoculation for virus purification. Virus was purified according to a procedure described previously (Chen and Chang, 1998). Yields of virions were determined by absorbance at 260 nm (HITACHI 220S spectrophotometer), and calculated using an extinction coefficient of 2.4 for potyviruses without light scattering correction (Holling and Brunt, 1981). Purified virus samples were treated with an equal volume of dissociation buffer (250 mM Tris-HCl, containing 2% [w/v] of SDS, 4% [v/v] of 2-mercaptoethanol and 10% [w/v] of sucrose) (Laemmli, 1970) before analysis by 12% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook and Russell, 2001).

Electron microscopy

Virus particles of Ca-M19 from leaf dips of *C. quinoa* or purified samples were negatively stained with 2% PTA (phosphotungstic acid) (Hall et al., 1945) and examined with an electron microscope (HITACHI H-7000). Modal length of Ca-M19 virus particles was calculated by measuring 100 virions. Immuno-sorbent electron microscopy was used to determine the reaction of Ca-M19 with its homologous antiserum. Grids were first coated with Ca-M19 infected tissue extract, incubated with 1:1000 (v/v) diluted immunoglobulin G against Ca-M19 CP, and finally stained with 2% PTA before observation.

Cloning of 3´-terminal region of Ca-M19 and nucleotide sequence analysis

A potyvirus-degenerate primer Hrp-5 (5´-ATgATH-gARKCNTgggg) designed by Pappu et al. (Pappu et al., 1998) and an oligo $d(T)_{14}$ primer were used to amplify the 3´-terminal region of Ca-M19 by the reverse-transcription polymerase chain reaction (RT-PCR). Extraction of viral RNA and reverse transcription of the first strand cDNA were conducted as described previously (Chen et al., 2003a, Chen et al., 2002a). PCR amplification was carried out by 26 repeated cycles (Perkin Elmer GeneAmp system 2400): denaturing at 94°C for 1 min, annealing at 50°C for 45 s, and DNA synthesis at 72°C for 1 min 30 s. An elongation step at 72°C for 6 min was conducted at the last cycle. DNA fragment amplified by RT-PCR was cloned into a pCRII-TOPO vector (Invitrogen, California) according to manufacturer's instructions. Nucleotides were sequenced by an automatic DNA sequencer (ABI PRISM 377, Perkin-Elmer, CA). Three independent clones were selected for alignment to determine the complete nucleotide sequence. Sequence data were analyzed and compared with potyvirus species in GenBank using the Vector NTI Suite (InforMax, Inc., Bethesda, MD). In order to confirm the existence of Ca-M19 in infected calla lilies, a specific primer set was designed for RT-PCR detection. A biotinylated DNA probe specific to Ca-M19 prepared by a BrightStar™ kit (Psoralen-Biotin, Ambion Inc. Texas) was further used to hybridize with

RT-PCR products for confirmation. Hybridization were determined by SEQ-Light Chemiluminscent Sequencing system (Tropix Inc., MA, USA) and exposed to Kodak intensifying screen (Eastman Kodak Co., New York). One field isolate (Ca2) that positively reacted with Ca-M19 specific primers was cloned, sequenced, and compared with that of the type culture.

Expression of viral coat protein in *Escherichia coli*

Based on the sequence data, a primer set was designed for the amplification of Ca-M19 putative complete CP gene. The recognition sites for restriction enzymes, *Nco*I and *Xho*I, were created at the 5′ end of the upstream (M19CPu: 5′-TCACATACCATGGGCTCGGGAG AAAAGACAGGT, *Nco*I site underlined) and downstream (M19CPd: 5′-CCTGCCCTCGAGTTACTGCGGT GGACCCATAC, *Xho*I site underlined) primers, respectively. Viral RNAs from purified virions were used as a template for RT-PCR amplification of Ca-M19 CP gene, which was then constructed into expression plasmid vector pET28b (Novagen, Inc., Madison, WI) by the directional cloning technique (Chen et al., 2002a). Constructed pET plasmids were subsequently transformed into *Escherichia coli* strain DH5*α* and later into *E. coli* strain BL21 (DE3) p*Lys*S (Novagen) for protein expression. Protein expression was induced by the addition of isopropyl *β*-D-thiogalactopyranoside (IPTG) as an inducer, and the results were analyzed by SDS-PAGE on 12% gels (Sambrook and Russell, 2001). The size and expression level of Ca-M19 CP were determined by western blotting analysis using the potyvirus-specific monoclonal antibody (Agdia Inc., Elkhart, IN). Bacteria expressed Ca-M19 CP was eluted from the SDS-PAGE gel as described previously (Chang et al., 1988).

Antiserum preparation and serological tests

Antiserum against bacterial expressed Ca-M19 CP was prepared by immunizing New Zealand white rabbits. The immunization protocols were the same as those described (Chang, 1992; Chen and Chang, 1998). Serological relationships between Ca-M19 and 20 known potyviruses were analyzed by an SDS-immunodiffusion test and ELISA as described previously (Chen and Chang, 1998; Purcifull and Batchlor, 1977). Antigens of four calla lily-infecting potyviruses were used for comparison. These included: S isolate of DsMV (Chen et al., 2003a), R7 isolate of KoMV (Chen et al., 2003a), YC5 isolates of TuMV (Chen et al., 2003a), and T17Q isolate of ZaMMV (15) and the following potyviruses: *Bean common mosaic virus* (BCMV) (Chang, 1993), Scott isolates of BYMV (BYMV-Scott) (Chang, 1993), *Blackeye cowpea mosaic virus* (BlCMV) (Chang, 1993), TP1 isolate of *Lily mottle virus* (LiMV-TP1) (Chang et al., 1995), *Lycoris mild mottle virus* (LyMMV) (Chang et al., 2002), *Lycoris potyvirus* (LPV) (Chang et al., 2002), *Melon vein banding mosaic virus* (MVbMV) (Liu et al., 1999), type W strain of *Papaya ringspot virus* (PRV-W) (Purcifull and Hiebert, 1979), *Passionfruit crinkle virus* (PCV) (Chang and Lin, 1989), *Passionfruit mottle virus* (PaMV) (Chang, 1992), *Passionfruit woodness virus* (PWV) (Chang, 1992), Ts isolate of *Peanut stripe virus* (PStV-Ts) (Chang et al., 1990), *Pea seed-borne mosaic virus* (PSbMV) (Chang et al., 1989), *Soybean mosaic virus* (SMV) (Chang, 1993), *Tuberose mild mosaic virus* (TMMV) (Chen and Chang, 1998), *Watermelon mosaic virus* 2 (WMV 2) (Purcifull and Hiebert, 1979), and *Zucchini yellow mosaic virus* (ZYMV) (Purcifull et al., 1984).

Surveys for the occurrence of Ca-M19 in calla lily fields

Symptomatic and asymptomatic plants were randomly collected from various calla lily fields and indexed with antisera against calla lily-infecting potyviruses by indirect ELISA as described previously (Chen et al., 2003a).

RESULTS

Host reaction and symptomatology

Among the test plant species inoculated with Ca-M19 in the experiment, only *C. quinoa* responded consistently with local lesion reactions. *Nicotiana benthamiana*, *G. max*, and tissue-culture-derived calla lily plantlets, did not show symptoms throughout the 2-month observation period, nor did they react with antibody against Ca-M19 in ELISA within one month of mechanical inoculation. Again, five different calla lily cultivars were subsequently inoculated with Ca-M19, but none were infected, as confirmed by ELISA.

Virus purification and electron microscopy

Flexuous rod particles, typical of potyviruses, were consistently observed in negatively stained leaf dips of Ca-M19 infected calla lilies and *C. quinoa* (Figure 1A). Inoculated leaves from *C. quinoa* 7-10 days after inoculation were used for virus purification, and approximately 0.6 mg Ca-M19 per 100 g of leaf tissue was obtained. The UV absorption ratio (260/280) was 1.02, indicating that the purity of the sample was not satisfactory (Stace-Smith and Tremaine, 1970). EM examination showed numerous flexuous rod particles similar to those observed in negatively stained leaf dips, measured about 755 nm in length as calculated from 100 purified virus particles. Particles either purified or from leaf dips were readily decorated by the Ca-M19 CP antibodies (Figure 1B).

Nucleotide sequences and molecular characterization

A 1.3-kb DNA product was consistently amplified from the RNA templates of purified Ca-M19 virions or total RNA extracted from Ca-M19 infected tissue by RT-PCR using the potyvirus degenerate primer sets (data not shown). The amplicon was cloned, sequenced, and filed

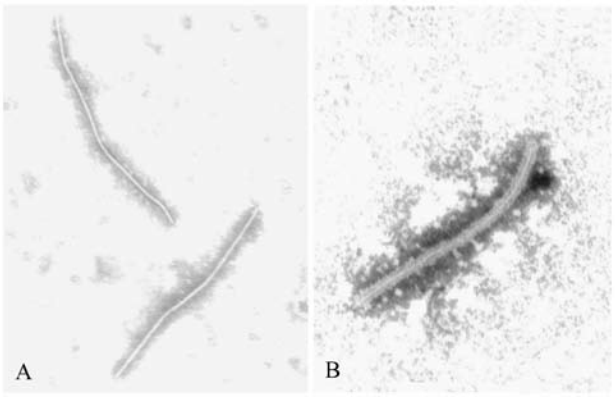

Figure 1. Electron micrographs of virus particles of *Calla lily latent virus* isolate M19 (Ca-M19). (A) Negative staining of filamentous virus particles observed in leaf dip of Ca-M19 infected calla lily; (B) Virus particles decorated with antiserum against bacteria expressed coat protein of CLLV.

to the GenBank with the accession number AF469171. Disregarding the length of the poly A tail, the amplified sequence comprises 1339 nucleotides (nts) corresponding to the 3′-terminal region of a potyvirus. The deduced amino acid sequence contains 362 residues encoding part of the 3′-terminal region of the nuclear inclusion b gene (80 residues) and the complete sequence of the coat protein (CP) gene (282 residues). A 253 nts non-coding region (NCR) was found at the 3′-terminal region of the DNA.

When the sequence was compared with known potyviral sequences available in GenBank, the results showed that Ca-M19 is unique in the coat protein gene (CP) and 3′ non-coding region (NCR). Comparisons also showed that *Soybean mosaic virus* (SMV) and *Watermelon mosaic virus* 2 (WMV 2) are most similar to Ca-M19 but sharing only about 80% of nucleotide identities in CP and NCR (Table 1). All other potyviruses share less than 72% sequence identities with Ca-M19. Our analyses of a second isolate of Ca-M19 (Ca2) from calla lily showed that it was serologically and biologically indistinguishable from Ca2 isolate, with a more than 98% sequence homology (Table 1).

Based on the sequence data, we designed a primer set (M19u: 5′-ACAGGTGAGGATTTAGAT / M19d: 5′-AAATAAGTGCGACACAAT) that specifically amplified a 850 bp product from the CP region of Ca-M19 genomic RNA. Using this primer set, Ca-M19 associated calla lilies were identified from the field (Figure 2A). This specific primer did not amplify four different calla lily potyviruses including DsMV, KoMV, TuMV and ZaMMV in RT-PCR (Figure 2A). Reciprocally, the Ca-M19 specific DNA probe did not hybridize with RT-PCR products of these potyviruses amplified by potyvirus-degenerate primers (Figure 2C and 2D). The results confirmed that Ca-M19 has a unique CP gene sequence that is different from other known potyviruses infecting calla lily.

Table 1. Comparisons of nucleotide and amino acid identities of the coat protein (CP) genes and the 3′-noncoding regions (NCRs) of *Calla lily latent virus* isolate M19 (Ca-M19) with those of known potyviruses species.

	Percent identity[a]		
Potyvirus (Acc. No.)[b]	CP nt	CP aa	3′-NCR
Ca-M19 (AF469171)	100	100	100
Ca-Ca2	99	99	98
AzMV (U60100)	70	71	67
BCMV (AJ293576)	69	72	68
BlCMV (AF395678)	69	72	68
BYMV (S77515)	60	56	40
CABMV (AF24123)	69	67	58
DeMV (U23564)	68	68	65
DsMV (AF511485)	59	61	47
KoMV (AF470620)	60	53	25
LiMV-TP1	56	52	45
LMV (Z78227)	61	60	45
LPV (AF511486)	52	40	31
LyMMV (AF399672)	59	47	47
MVbMV	71	71	65
PaMV-tw	68	68	71
PCV-tw	67	63	52
PSbMV (D10453)	57	52	39
PStV (NC001732)	70	72	65
PWV (AF208662)	69	69	67
SMV (NC002634)	77	81	81
SMV (S42280)	78	81	79
SMV (X96665)	77	81	83
TMMV (AF062926)	57	53	29
TuMV (AF530055)	59	54	45
WMV 2 (L22907)	80	82	81
WMV 2 (D13913)	80	82	79
WMV 2 (AB001994)	79	81	81
ZYMV (NC003224)	66	66	49

[a] Percent identities of the respective nucleotide and amino acid sequences were analyzed by the Vector NTI Suite program (InforMax, Inc., Wisconsin).

[b] The potyviruses listed are: Ca-Ca2 (Ca 2 isolate of *Calla lily latent virus*), AzMV (*Azukibean mosaic virus*), BCMV (*Bean common mosaic virus*), BYMV (*Bean yellow mosaic virus*), BlCMV (*Blackeye cowpea mosaic virus*), CABMV (*Cowpea aphid-borne mosaic virus*), DsMV (*Dasheen mosaic virus*), DeMV (*Dendrobium mosaic virus*), KoMV (*Konjak mosaic virus*), LMV (*Lettuce mosaic virus*), LiMV (*Lily mottle virus*), LPV (*Lycoris potyvirus*), LyMMV (*Lycoris mild mottle virus*), MVbMV (*Melon vein banding mosaic virus*), PaMV (*Passionfruit mottle virus*), PCV (*Passionfruit crinkle virus*), PSbMV (*Pea seedborne mosaic virus*), PWV (*Passionfruit woodness virus*), PStV (*Peanut stripe virus*), SMV (*Soybean mosaic virus*), TMMV (*Tuberose mild mosaic virus*), TuMV (*Turnip mosaic virus*), WMV 2 (*Watermelon mosaic virus* 2), and ZYMV (*Zucchini yellow mosaic virus*).

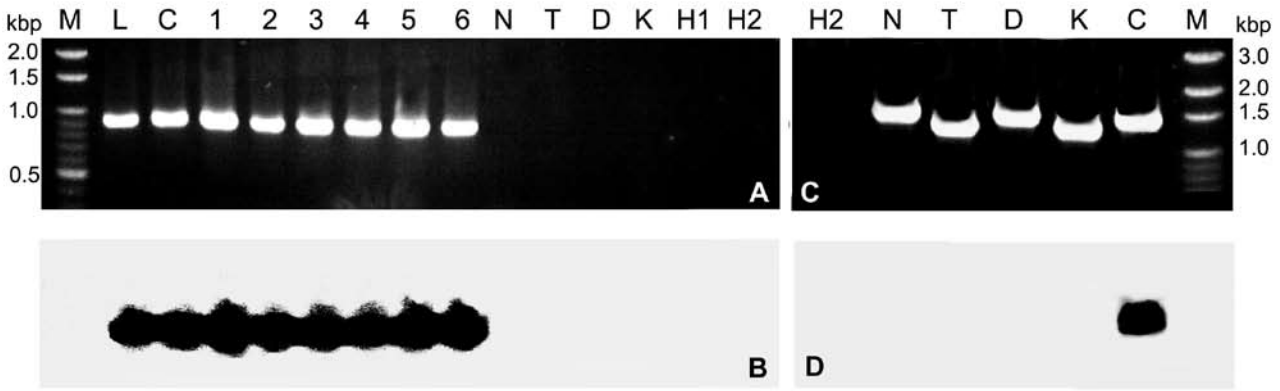

Figure 2. Molecular identification of *Calla lily latent virus* (CLLV) by reverse-transcription polymerase chain reaction (RT-PCR) and DNA probe hybridization. (A) Amplification of CLLV and four different calla lily infecting potyviruses in RT-PCR using CLLV specific primers; (B) Southern blot hybridization of the gel from figure (A) using biotin-labeled DNA probe specific to CLLV; (C) Amplification of four different calla lily infecting potyviruses by RT-PCR using potyvirus-degenerate primer designed by Pappu et al. (1998). (D) Southern blot hybridization of the gel from figure (C) by biotin-labeled DNA probe specific to CLLV. Lane M, kb DNA ladder markers; lanes L and C are CLLV-infected tissues of *Chenopodium quinoa* and calla lily, respectively; lanes 1-6 are field collected calla lily samples infected by CLLV; lanes H1 and H2 are uninfected control of *C. quinoa* and calla lily, respectively; lanes N, T, D, K are four potyvirues infecting calla lily including *Zantedeschia mild mottle virus*, *Turnip mosaic virus*, *Dasheen mosaic virus* and *Konjak mosaic virus*, respectively.

Expression of Ca-M19 CP in *E. coli* and preparation of its antiserum

A 850-bp DNA product equivalent to the entire CP gene of Ca-M19 was amplified by RT-PCR using the aforementioned specific primer set. This product was constructed directionally into expression vector pET28b and transformed into *E. coli* strain BL21(DE3)p*Lys*S for protein expression. A protein about 35 kDa in size, similar to that of purified Ca-M19 CP, was detected by SDS-PAGE in the lysate of bacteria transformed with BL21 clone, but not in the lysate of control bacteria (Figure 3A). Using antiserum against a potyvirus-specific monoclonal antibody in western blotting test, the potyviral CP nature of the 35 kDa protein was confirmed (Figure 3B). The protein was subsequently purified from the lysate of a large volume culture of the transformed bacteria (Figure 3A). The estimated yield of expressed CP in 1000 ml of bacteria culture was about 2 mg. A polyclonal antiserum (#097) against the bacteria-expressed Ca-M19 CP was thus prepared. It reacts strongly with both the CP from purified virions and the bacteria expressed 35 kDa protein (Figure 3C).

Serological tests

The Ca-M19 antiserum (#097) reacted strongly in ELISA, SDS-immunodiffusion, and western blotting tests against homologous antigens of Ca-M19 infected plants but not with uninfected controls (Table 2, Figure 4). In indirect ELISA, the antiserum cross reacted with some potyviruses tested including BCMV, MVbMV, PaMV, PCV, PStV, WMV 2, SMV, and ZYMV (Table 2). Among them, SMV and WMV 2 showed ELISA readings as high as those in homologous reactions. SDS-immunodiffusion tests confirmed that these viruses were serologically

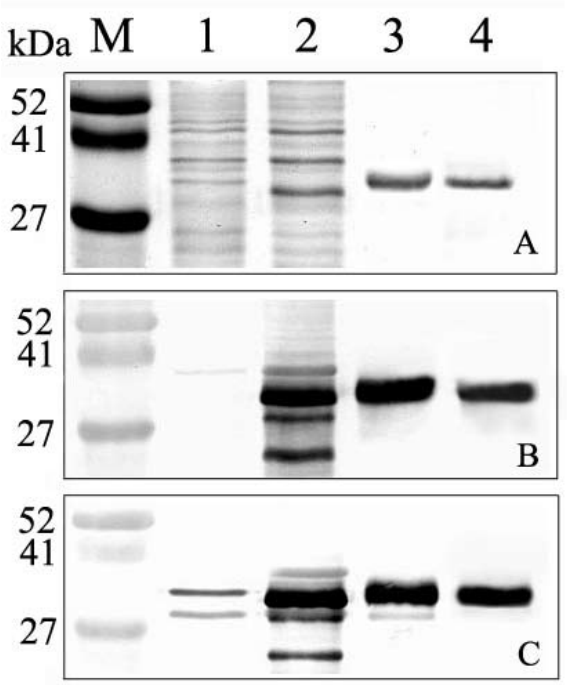

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analyses of the bacteria expressed coat protein (CP) of M19 isolate of *Calla lily latent virus* (Ca-M19). Proteins separated in SDS-PAGE (A) were blotted onto nylon membrane and reacted with the potyvirus-specific monoclonal antibody (Agdia Inc.) (B) and antiserum against Ca-M19 (C); and subsequently detected by alkaline phosphatase conjugated coat anti-rabbit antibody. Lane M, protein markers; lane 1, IPTG-induced bacteria (*E. coli* BL21(DE3)p*Lys*S) lysate containing non-inserted expression vector pET28b; lane 2, IPTG-induced bacteria lysates of pET28b inserted with Ca-M19 CP gene; lane 3, purified bacteria expressed Ca-M19 CP; lane 4, purified Ca-M19 virions.

Table 2. Reactions of antisera against bacteria expressed coat protein of M19 isolate of *Calla lily latent virus* (Ca-M19) with different potyviruses in enzyme linked immunosorbent assay [a] (ELISA) and SDS-immunodiffusion tests[b].

Antigens[c]	Cross-reactivities			
	ELISA[d]			SDS-immunodiffusion[e]
	25×	50×	100×	1×
Ca-M19 CP	3.08	2.47	1.11	I [e]
Ca-M19	1.13	1.03	1.09	I
Ca-Ca2	1.27	1.14	1.12	I
BCMV	1.58	1.61	1.52	S
BlCMV	0.08	0.09	0.09	-
BYMV	0.18	0.19	0.15	-
DsMV	0.09	0.09	0.08	-
KoMV-R7	0.08	0.09	0.11	-
LPV	0.15	0.15	0.16	-
LyMMV	0.00	0.00	0.02	-
LiMV-TP1	0.14	0.16	0.15	-
MVbMV	0.74	0.37	0.33	S
PaMV	2.10	2.18	2.37	S
PSbMV	0.12	0.13	0.13	-
PCV	0.42	0.33	0.23	-
PRSV-W	0.15	0.14	0.16	-
PWV	2.57	2.62	2.58	S
PStV	1.62	1.46	1.29	-
SMV	2.74	2.51	2.23	S
TMMV	0.13	0.08	0.02	-
TuMV-YC5	0.20	0.19	0.19	-
WMV 2	2.46	2.43	2.25	S
ZYMV	0.86	0.75	0.62	S

[a] Indirect enzyme-linked immunosorbent assay (ELISA), described previously (Chang et al., 1990), was conducted to determine the serological relatedness between Ca-M19 and other potyviruses. Purified immunoglobulins of antiserum #097 were diluted to 1:1000 to react with antigens diluted to 1/25, 1/50, and 1/100.

[b] SDS-immunodiffusion test, described previously (Chen et al., 2003a), was performed by the use of undiluted antiserum (#097) against bacteria expressed Ca-M19 coat protein to react with undiluted SDS-treated antigens of different potyviruses as listed in the table.

[c] The antigens listed are: BCMV (*Bean common mosaic virus*), BlCMV (*Blackeye cowpea mosaic virus*), BYMV (*Bean yellow mosaic virus*), DsMV (*Dasheen mosaic virus*), KoMV (*Konjak mosaic virus*), LPV (*Lycoris potyvirus*), LyMMV (*Lycoris mild mottle virus*), LiMV (*Lily mottle virus*), MVbMV (*Melon vein banding mosaic virus*), PaMV (*Passionfruit mottle virus*), PSbMV (*Pea seedborne mosaic virus*), PCV (*Passionfruit crinkle virus*), PRSV-W (*Papaya ringspot virus* type W), PWV (*Passionfruit woodiness virus*), PStV (*Peanut stripe virus*), SMV (*Soybean mosaic virus*), TMMV (*Tuberose mild mosaic virus*), TuMV-YC5 (YC5 isolate of *Turnip mosaic virus*), WMV 2 (*Watermelon mosaic virus* 2), ZYMV (*Zucchini yellow mosaic virus*), Ca-M19 CP (bacteria expressed coat protein of M19 isolate of *Calla lily latent virus*), Ca-M19 (infected tissue of M19 isolate of *Calla lily latent virus*), and Ca-Ca2 (infected tissue of Ca2 isolate of *Calla lily latent virus*).

[d] Reactivities in ELISA are shown as the absorbance readings (A_{405nm}) taken about 40 min after addition of enzyme substrate solution. The absorbance values are an average of two replicate wells. Readings lower than 0.2 were considered negative reactions and are underlined.

[e] Reactivities in SDS-immunodiffusion tests are shown as the reactions between homologous and heterologous antigens: I = precipitation lines of homologous and heterologous reations fused without spur formation; S = homologous reactions spurred over heterologous reactions; - = no reaction.

related but distinct from Ca-M19 because the precipitin lines formed spurs with those formed by homologous reactions (Table 2). Furthermore, our data showed that those calla lily-infecting potyviruses, including DsMV, KoMV, TuMV and ZaMMV, were serologically unrelated to Ca-M19 in ELISA and the SDS-immunodiffusion test (Table 2, Figure 4), indicating the feasibility of using the antiserum to specifically detect the presence of Ca-M19 in calla lilies.

Surveys for the occurrence of Ca-M19 in calla lily fields

A total of 245 calla lily samples were collected from various fields in Taiwan. They were indexed by ELISA using antisera against five different calla lily infecting potyviruses including Ca-M19, DsMV, KoMV, TuMV and ZaMMV. Ca-M19 was found in 86 of 245 sampled calla lilies. Among these 86 Ca-M19-infected plants, 77 of them exhibited viral symptoms while the other nine samples did not. Interestingly, all these symptomless healthy looking plants were found to be infected only by Ca-M19 while the other 77 symptomatic plants were all found co-infected with other potyviruses. These asymptomatic plants were transplanted from the field and grown in a greenhouse for continuous observation. They remained symptomless throughout a 6-month observation period. During the 6-month observation, we routinely checked these symptomless plants by ELISA and RT-PCR (data not shown). They were confirmed consistently infected by Ca-M19 only.

DISCUSSION

Based on the size and morphology of virus particles, serological reactivities to potyvirus-specific monoclonal antibodies, and the cross reactivities of the antiserum with other potyviruses, Ca-M19 isolated from calla lilies was identified as a member of the genus *Potyvirus*. Furthermore, the uniqueness in the nucleotide and the amino acid sequences of coat protein gene and 3′-NCR confirm that the virus from calla lilies is a new species of *Potyvirus* (Shukla et al., 1991; Ward et al., 1992). Thus, we conclude that Ca-M19 isolate is a distinct potyvirus and designated it *Calla lily latent virus* (CLLV). Like most potyviruses, Ca-M19 has a very narrow host range. Besides calla lilies, it only infects *C. quinoa* and induces local lesions on the inoculated leaves in our experiment. Recovery inoculation using extracts of an infected single lesion from *C. quinoa* to calla lilies was not successful, making it difficult to conclude what specific symptoms on calla lilies are induced by Ca-M19. Further studies showed that when infected by Ca-M19 alone, calla lilies did not exhibit any symptoms within a 6-month observation period. Our surveys showed that symptomatic calla lilies observed in the fields, when tested positive with Ca-M19 antiserum, were always found co-infected by other viruses. The results confirm our observation that Ca-M19 causes symptomless infection in calla lilies. Therefore, we propose *Calla lily latent virus* (CLLV) as the name of this novel virus (Chen et al., 2004). To our knowledge, CLLV is the first virus reported to cause latent infection in calla lilies. CLLV-associated symptomless calla lilies are indistinguishable from uninfected plants in both growth vigor and flowering potential, including yield and quality. However, possible synergistic effects resulting from simultaneous infection of CLLV with other viruses need to be clarified.

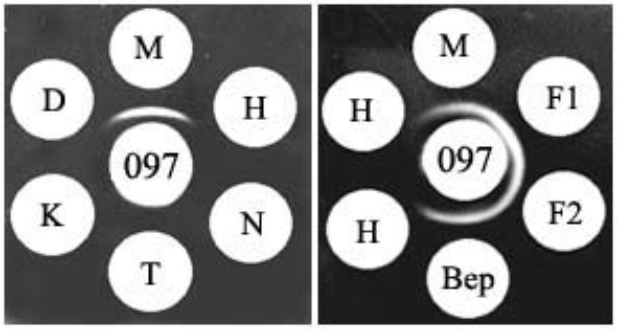

Figure 4. Serological comparison of M19 isolate of *Calla lily latent virus* (Ca-M19) with some potyviruses infecting calla lily in sodium dodecyl sulfate (SDS) immunodiffusion tests. The center wells were charged with antiserum (#097) against bacteria-expressed coat protein of Ca-M19, and the peripheral wells were filled with SDS-treated antigens of *Dasheen mosaic virus* (D), *Konjak mosaic virus* (K), Ca-M19 (M), *Turnip mosaic virus* (T), *Zantedeschia mild mottle virus* (N), healthy calla lily (H), bacteria-expressed coat protein of Ca-M19 (Bep), and two CLLV-infected calla lily antigens collected from fields (F1 and F2).

In this study, we developed DNA probes, specific RT-PCR primers, and antiserum against bacteria-expressed CP for the detection of CLLV. As in our previous studies (Chen et al., 2002a), antiserum prepared to bacteria-expressed CP was useful in ELISA, SDS-immunodiffusion, immuno-specific electron microscopy, and western blotting tests. Using these indexing tools, CLLV was frequently detected in calla lily plants collected from several major calla lily production townships in Taiwan. Although we did not carry out an insect transmission test, we considered these infected plants to be vectored by aphids. This is based on the fact that, as in most potyviruses, a DAG triplet, the genetic code for aphid transmissibility, is found in the N-terminus of CLLV CP.

CLLV is shown serologically related to at least eight potyviruses, including BCMV, MVbMV, PaMV, PCV, PStV, WMV 2, SMV, and ZYMV (Table 2). These potyviruses were shown serologically to be closely related to each other in our previous studies (Chang, 1992; Chang and Lin, 1989; Chang et al., 1990). When the amino acid sequence of CLLV CP gene is examined and aligned with those of WMV 2 and SMV, the two most closely related viruses based on ELISA readings, an extra 17-residues were present at the N-terminus of CLLV CP (Figure 5). In the same region, an extra sequence is present in WMV 2, but it is different from that of CLLV (Figure 5).

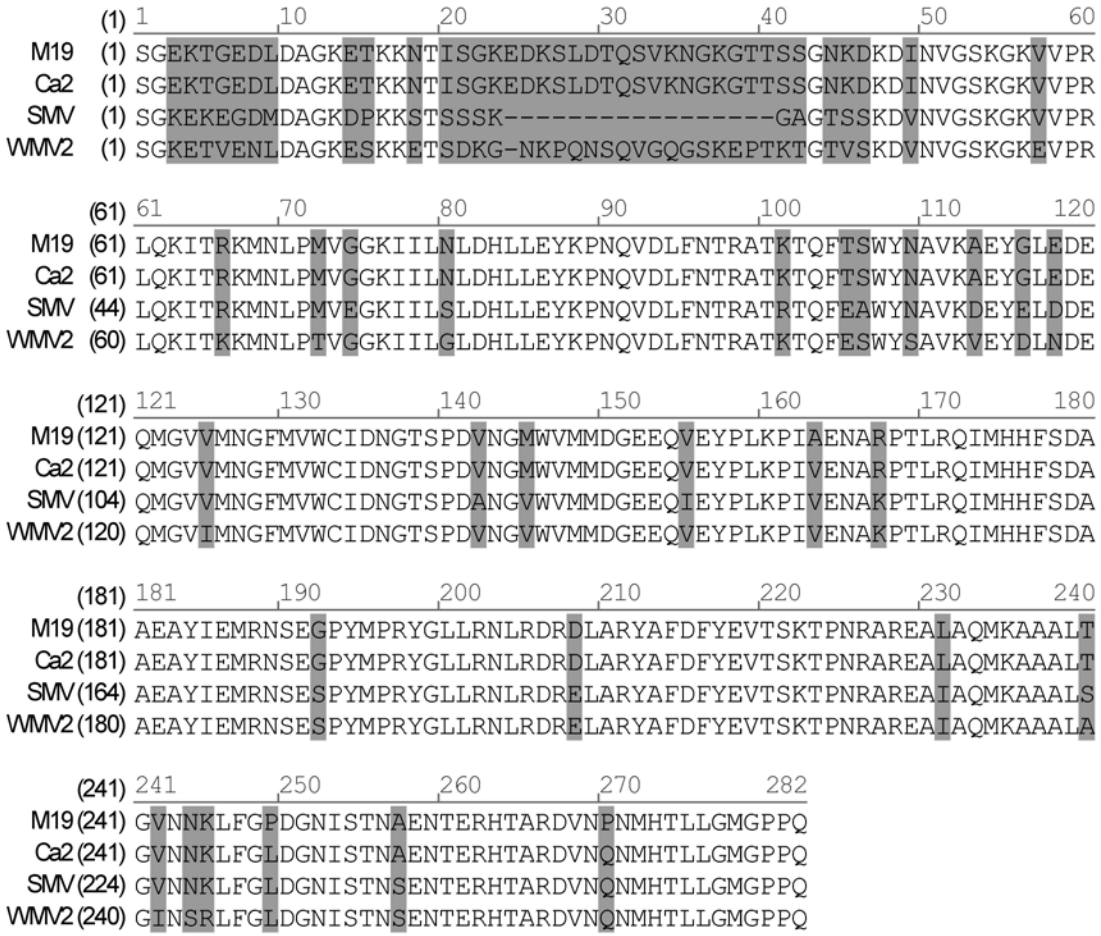

Figure 5. Alignment of the deduced amino acid sequences of coat protein (CP) gene of *Calla lily latent virus* (CLLV), *Soybean mosaic virus* (SMV), and *Watermelon mosaic virus* 2 (WMV 2). Coat protein gene sequences of two CLLV isolates (M19 and Ca2) are aligned with those of SMV (Acc. No. S42280) and WMV 2 (Acc. No. L22907). Those amino acid residues of CLLV different from SMV or WMV 2 are marked. An extra sequence, EDKSLDTQSVKNGKGTT, which is not found on SMV, is observed in the N-terminal region of CLLV. A similar sized but different sequence is located in the same region of WMV 2.

Other than this variable N-terminal portion, the core and C-terminal regions of CLLV CP are very similar to those of SMV and WMV 2. This explains the close serological relatedness among these three potyviruses.

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感染彩色海芋之新種 Potyvirus 病毒之分子及血清學特性

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本研究自彩色海芋 (*Zantedeschia* spp.) 分離到一個具有獨特分子及血清學特性之新種 *Potyvirus* 屬病毒，分離株代號為 Ca-M19。Ca-M19 具典型長絲狀病毒顆粒，可經由機械接種於奎藜（*Chenopodium quinoa* Willd.）葉片造成局部病斑。利用簡併式 *Potyvirus* 屬廣效性引子對可以穩定由感染 Ca-M19 之罹病組織中以反轉錄聚合酶鏈鎖反應增幅出大小約 1.3 kb 之核酸片段。經解序分析後證實此片段符合 Potyvirus 屬病毒 3′ 端基因體特性含有鞘蛋白基因 (coat protein, CP) 及 3′ 端非轉錄區 (3′-NCR)，此序列已正式登錄於 GenBank 中 (AF469171)。 與已知 *Potyvirus* 屬病毒之 CP 基因及其 3′-NCR 序列比對後顯示，與 Ca-M19 親緣最相近之病毒為 *Soybean mosaic virus* (SMV) 及 *Watermelon mosaic virus* 2 (WMV 2)，然而其核酸序列與 Ca-M19 間之相同度僅達 80%。由於利用傳統純化方法自接種 Ca-M19 之奎藜葉片所獲之純化病毒顆粒量不足以製備抗血清，因此本研究將選殖之Ca-M19鞘蛋白基因構築於蛋白表現載體 pET-28b(+) 上，再轉型於 *Escherichia coli* 寄主內以誘導其大量表現 Ca-M19 之 CP，利用純化之細菌表達已成功製備出對應 Ca-M19 之專一性抗體。此抗體可應用於 Indirect ELISA 及 SDS-免疫擴散法中以偵測 Ca-M19 之感染，且發現 Ca-M19 與 *Bean common mosaic virus, Blackeye cowpea mosaic virus, Melon vein banding mosaic virus, Passionfruit mottle virus, Passionfruit crinkle virus, Passionfruit woodness virus, Soybean mosaic virus, Watermelon mosaic virus* 2 及 *Zucchini yellow mosaic virus*等病毒具有血清類緣關係。綜合病毒型態、寄主反應、鞘蛋白序列比對與血清關係，Ca-M19 顯然為 *Potyvirus* 屬中的一種新種病毒，特命名為 *Calla lily latent virus* (簡稱 CLLV)。另外，由田間採樣標本檢測結果顯示，田間呈現明顯病徵之海芋若偵測到 Ca-M19 之感染時，該等植株均被證實同時感染到其他海芋病毒。然而只偵測到 Ca-M19 感染之植株則被證實於整個生長季節中均不會呈現任何可辨識病徵。

關鍵詞：海芋；*Potyvirus* 病毒；核酸序列分析；鞘蛋白表現；血清分析。