**Identification and molecular characterization
of *Zantedeschia* mild mosaic virus, a new calla
lily-infecting potyvirus**

Brief Report

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Summary. Tissue-cultured seedlings of calla lily (*Zantedeschia* spp.) with mild mosaic symptom were observed in Taiwan. A new potyvirus, *Zantedeschia* mild mosaic virus (ZaMMV), was identified in the diseased plants based on the result of ELISA, virion morphology and sequence data. In the host range test, ZaMMV only infected *Philodendron selloum* and *Zantedeschia* spp. causing systemic mosaic and mild mosaic symptom, respectively. The 3'-terminal region of the virus was amplified by RT-PCR from total RNA using a potyvirus-specific degenerate primer and an oligo-dT primer. Sequence analysis revealed that the highest amino acid sequence identity of the capsid protein (CP) gene between ZaMMV and thirty potyviruses was 55%, and the highest nucleotide identity of 3' untranslated region (3'UTR) was 52%. Therefore, in terms of species demarcating criteria, ZaMMV is a new species of the genus *Potyvirus*. From the result of phylogenetic analysis, ZaMMV should be a member of the *Bean common mosaic virus* subgroup. According to the deduced amino acid sequence of ZaMMV, the N terminus of the CP contained 39 glutamine residues before DAG motif. This unique sequence has never been discovered in plant viruses.

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Zantedeschia spp., which originated from Africa and are commonly called calla lily, pig lily and arum lily, belong to the Araceae family and are classified into seven species and two subspecies [10]. In Taiwan, many cultivars of calla lily have been imported from New Zealand, the Netherlands and USA for producing cut flowers, breeding and also for establishing a micropropagation system. Viral diseases are one of the limiting factors for calla lily production in Taiwan as

well as in other countries. Many viruses have been reported to infect calla lily including isolates of *Dasheen mosaic virus* (DsMV) [4, 17], *Cucumber mosaic virus* (CMV) [13], *Konjak mosaic virus* (KoMV), *Bean yellow mosaic virus* (BYMV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Alfalfa mosaic virus* (AMV), *Arabidopsis mosaic virus* (ArMV), *Potato virus X* (PVX), *Tobacco rattle virus* (TRV) [16], *Turnip mosaic virus* (TuMV) [3] and *Zantedeschia mosaic virus* (ZaMV) [2, 9]. Among them, DsMV, a member of the genus *Potyvirus*, is the most important virus of aroid plants and has been studied for a long time [22]. Although ZaMV was discovered only recently, it has become more prevalent in calla lilies than DsMV in Taiwan and Korea [9].

In 2001, tissue-cultured seedlings of *Zantedeschia* sp. cv. 'Black Magic' purchased from the Taiwan Seed Improvement and Propagation Station (TSIPS) were grown in a greenhouse. After one growing season, tubers were harvested, stored and replanted in the same condition. A new mild mosaic symptom was observed in some seedlings of calla lily (Fig. 1). A virus isolate, ZUN, was successfully transferred to *Philodendron selloum* by mechanical inoculation and then maintained on this systemic host plant. To determine the host range of ZUN isolate, inoculum was prepared by grinding the infected *P. selloum* tissues in 10 volumes of 0.01 M sodium phosphate buffer (pH 7.2) and then rubbed onto 36 plant species representing 13 families (Table 1) which were predusted with 600-mesh Carborundum. At least five plants of each species were tested. Inoculated plants were kept at 25 °C, and monitored for symptom expression for one month and also for the contamination with CMV, DsMV, TuMV or ZaMV by indirect ELISA (I-ELISA) according to the protocol of Agdia Inc. (Agdia Inc., IN, USA). Crude sap of ZUN-infected leaf tissue was placed onto the carbon

Fig. 1. Mild mosaic symptoms on the leaf of ZaMMV-infected calla lily (right). Healthy plant is shown on the left

Table 1. Symptoms of Zantedeschia mild mosaic virus (ZaMMV) on test plants

Family	Species	Symptoms ^a
Aizoaceae	<i>Tetragonia tetragonioides</i>	—
Amaranthaceae	<i>Amaranthus mangostanus</i>	—
	<i>A. viridis</i>	—
	<i>Celosia argentea</i>	—
	<i>Gomphrena globosa</i>	—
Apocynaceae	<i>Catharanthus roseus</i>	—
Araceae	<i>Philodendron selloum</i>	SM, VC, LD
	<i>Zantedeschia</i> spp.	SMM, VC
Brassicaceae	<i>Arabidopsis thaliana</i>	—
	<i>Brassica chinensis</i>	—
	<i>B. juncea</i>	—
	<i>Raphanus sativas</i>	—
Caricaceae	<i>Carica papaya</i>	—
Chenopodiaceae	<i>Chenopodium amaranticolor</i>	—
	<i>C. quinoa</i>	—
Compositae	<i>Crassocephalum crepidioides</i>	—
Convolvulaceae	<i>Ipomoea aquatica</i>	—
Cucurbitaceae	<i>Cucurbita pepo</i>	—
	<i>Cucumis sativus</i>	—
Fabaceae	<i>Cassia occidentalis</i>	—
	<i>Phaseolus calcaratus</i>	—
	<i>P. limensis</i>	—
	<i>P. radiatus</i>	—
	<i>P. vulgari</i>	—
	<i>Pisum sativum</i>	—
	<i>Vigna unguiculata</i>	—
	<i>Datura stramonium</i>	—
Solanaceae	<i>Lycopersicon esculentum</i>	—
	<i>Nicotiana benthamiana</i>	—
	<i>N. clevelandii</i>	—
	<i>N. occidentalis</i>	—
	<i>N. tabacum</i> var. Samsun	—
	<i>N. tabacum</i> var. White burley	—
	<i>Petunia hybrida</i>	—
	<i>Solanum tuberosum</i>	—
Umbelliferae	<i>Coriandrum sativum</i>	—

^aAbbreviation of symptoms: leaf distortion (LD), systemic mosaic (SM), systemic mild mosaic (SMM), veinal chlorosis (VC) and no infection (—)

film-coated copper grid, and stained with 2% phosphotungstic acid (PTA). The virus particles were observed and photographed by an electron microscope. Plant total RNAs were extracted from 100 mg of tissue following the protocol of Plant Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA). The first-strand cDNA was then synthesized using AMV reverse transcriptase (Promega, Madison, WI, USA) and dT-Bam primer (5'-AGCTGGATCC(T)₁₈-3').

PCR reactions were carried out using dT-Bam and PNIbF1 (5'-GGBAAYA ATAGTGGNCAACC-3') primers [8] and DyNAzymeTM II DNA polymerase (Finnzymes Inc., Espoo, Finland) according to the manufacturer's protocol. RT-PCR products were analyzed in a 1% agarose gel and desired cDNA fragments were purified from the agarose gel by GFXTM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The purified fragments were ligated with pGEM-T[®] Easy Vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5 α . Two independent clones were selected for sequencing. DNA sequencing was performed by ABI PRISM[®] 310 Genetic Analyzer using ABI PRISM[®] BigDyeTM terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were assembled and amino acid sequences were predicted using Wisconsin GCG Package version 10.3 (Accelrys Inc., San Diego, CA, USA). Multiple sequences were aligned by PILEUP program in Wisconsin GCG Package and verified by ClustalX [19]. Phylogenetic analysis was performed with neighbor-joining (NJ) method using programs in PHYLIP version 3.573c [6]. Genetic distances between pairs of amino acid sequences were calculated using PROTDIST (Kimura formula). Phylogenetic trees were constructed by a distance matrix method (NEIGHBOR) using the original data set and 1000 bootstrap data sets generated by the program SEQBOOT from the original set. The bootstrap values and the consensus tree were generated by the program CONSENSE. The program TREEVIEW was used to draw the phylogenetic tree [14].

Among 36 plant species belonging to 13 families tested, ZUN successfully infected two aroid plants, calla lily (*Zantedeschia* spp.) and *P. selloum* (Table 1). Inoculated calla lilies produced veinal chlorosis and later became systemic mild mosaic symptoms similar to the symptoms observed before. Whereas, *P. selloum* appeared systemic mosaic symptoms, veinal chlorosis and sometimes leaf distortion (Table 1). In order to know if these symptomatic plants in host range test were contaminated with calla-infecting viruses commonly found in Taiwan, I-ELISA assays were performed. None of the samples reacted positively for the presence of CMV, DsMV, TuMV or ZaMV; however, the anti-poty group monoclonal antibody (Agdia Inc., Elkhart, IN, USA) gave a positive result (data not shown). In addition, the remaining 34 plant species did not produce symptom nor react to anti-poty group monoclonal antibody one month after inoculation. When crude sap of the ZUN-infected leaves was stained with 2% PTA, flexuous filaments about 680 nm in length were observed by TEM (Fig. 2). From the results of I-ELISA and virion morphology, ZUN was a potyvirus, and did not react with antibodies to DsMV, TuMV or ZaMV, the potyviruses found in Taiwan.

To identify the unknown potyvirus, ZUN, a potyvirus-specific primer pair was used to RT-PCR amplify the 3' terminal region of the genome. Total RNA of the diseased plant was extracted, a 1.8-kb fragment was amplified, and cloned into pGEM-T[®] Easy vector. Two independent clones, pZUN7 and pZUN8, were sequenced and analyzed. The sequences of both clones had 1845 nt excluding poly(A) tail which included 633 nt of the NIb gene, the complete CP gene of 972 nt and 3'UTR of 240 nt. This nucleotide sequence was first checked against

Fig. 2. Electron micrograph of ZaMMV particles. Crude sap of ZaMMV-infected *Zantedeschia* spp. was stained with 2% PTA. The bar represents 100 nm

the international sequence databases using the BLAST program (National Center for Biotechnology Information). Subsequently, 30 potyviruses revealing high homology with ZUN were selected for sequence comparison. The results of multiple sequence alignment indicated that the percentage identities of the CP amino acid sequences and 3'UTR nucleotide sequences between ZUN and other potyviruses were 42–55% and 9–52%, respectively (Table 2). BCMV-R had both the highest identical amino acids of the CP and nucleotide sequences of 3'UTR among potyviruses compared with ZUN (Table 2). Hence, according to the species demarcating criteria in the 7th ICTV report [20], ZUN is proved to be a new species of the genus *Potyvirus*. It was designated as *Zantedeschia* mild mosaic virus (ZaMMV) according to the mild mosaic symptom it induced on calla lily. The nucleotide sequence of ZaMMV was deposited in GenBank with the accession number AY626825. Phylogenetic analysis of the complete amino acid sequence of the CP revealed that ZaMMV is clustered with BCMV, BCMNV, DsMV, SMV and ZYMV (Fig. 3). Accordingly, it should be a new member of BCMV subgroup that was first proposed by Berger et al. [1].

The conserved NIa-Pro cleavage site, VXXQ/A, was identified between the NIb and CP coding regions of ZaMMV as for DsMV (Fig. 4). The DAG motif relating to aphid-transmission [12] is located at amino acid residues of 51 to 53 of the CP gene. Surprisingly, the N terminus of the CP of ZaMMV contained 39 glutamine residues before the DAG motif (Fig. 4). To confirm the existence of the polyglutamine sequence in the CP of ZaMMV, we did cDNA cloning from another two plants, and all sequenced clones contained this unusual sequence (data not shown). To our knowledge, this is the first time that this kind of polyglutamine sequence was discovered in plant viruses.

Table 2. Percentage of sequence identities between the CP and 3'UTR of ZaMMV and those of 30 distinct potyviruses

Virus ^a	CPaa ^b	CPnt ^b	3'UTR ^c	Accession number
BCMNv-NL3	53	53	42	U19287
BCMV-R	55	56	52	AJ312437
BYMV-MB4	44	48	16	D83749
CIYVV-30	43	46	17	AB011819
DsMV-M13	52	58	29	AJ298033
JGMV	48	52	9	Z26920
JYMV-1	47	49	19	AB016500
LMoV-Sb	45	47	20	AJ564636
LMV-O	48	50	23	X97704
LYSV-Y	46	49	10	AJ307057
MDMV-B	46	48	21	AJ001691
OYDV-Y	44	46	22	AJ510223
PeMoV-M	46	49	20	AF023848
PepMoV-CA	46	48	27	M96425
PPV-M	44	52	18	AJ243957
PRSV-HA	44	53	16	S46722
PSbMV-DPD1	44	50	20	D10930
PVA	45	47	18	Z21670
PVV-DV42	43	48	11	AJ243766
PVY-N	49	47	22	X12456
SCMV-A	46	53	22	AJ278405
SMV-G2	53	53	46	S42280
SPFMV-S	43	53	20	D86371
SrMV-H	48	53	23	U57358
TEV	45	47	17	M15239
TuMV	44	48	18	D10927
TVMV	42	46	25	X04083
YMV-IC	46	49	18	U42596
ZaMV-ZAN	44	48	15	AF332872
ZYMV-RI	52	53	31	L29569

^aFull names of the viruses are NL3 strain of *Bean common mosaic necrosis virus* (BCMNv-NL3), R isolate of *Bean common mosaic virus* (BCMV-R), MB4 strain of *Bean yellow mosaic virus* (BYMV-MB4), No. 30 strain of *Clover yellow vein virus* (CIYVV-30), M13 isolate of *Dasheen mosaic virus* (DsMV-M13), *Johnsongrass mosaic virus* (JGMV), No. 1 strain of Japanese yam mosaic virus (JYMV-1), Sb isolate of *Lily mottle virus* (LMoV-Sb), O strain of *Lettuce mosaic virus* (LMV-O), Yuhang GYH isolate of *Leek yellow stripe virus* (LYSV-Y), Bulgaria strain of *Maize dwarf mosaic virus* (MDMV-B), Yuhang isolate of *Onion yellow dwarf virus* (OYDV-Y), M strain of *Peanut mottle virus* (PeMoV-M), California strain of *Pepper mottle virus* (PepMoV-CA), M strain of *Plum pox virus* (PPV-M), Hawaii isolate of *Papaya ringspot virus* (PRSV-HA), DPD1 strain of *Pea seed-borne mosaic virus* (PSbMV), *Potato virus A* (PVA), DV42 isolate of *Potato virus V* (PVV-DV42), N strain of *Potato virus Y* (PVY-N), A strain of *Sugarcane mosaic virus* (SCMV-A), G2 strain of *Soybean mosaic virus* (SMV-G2), S strain of *Sweet potato feathery mottle virus* (SPFMV-S), H strain of *Sorghum mosaic virus* (SrMV-H), *Tobacco etch virus* (TEV), *Turnip mosaic virus* (TuMV), *Tobacco vein mottling virus* (TVMV), Ivory Coast strain of *Yam mosaic virus* (YMV-IC), ZAN strain of *Zantedeschia mosaic virus* (ZaMV-ZAN), and Reunion Island of *Zucchini yellow mosaic virus* (ZYMV-RI)

^bFigure represents percentage of amino acid and nucleotide sequence identity

^cFigure represents percentage of nucleotide sequence identity

Fig. 3. Phylogenetic relationship of ZaMMV and members of the genus *Potyvirus* based on the CP at the amino acid level. Multiple sequence alignment was generated using the PILEUP program and verified by Clustal X. The tree was constructed by the neighbor-joining algorithm based on calculations from pairwise amino acid sequence distances. The horizontal branch lengths are proportional to the genetic distance. The number shown at branch point indicates bootstrap value that was subjected to 1000 bootstrap replicates. Only those values above 650 are shown

According to I-ELISA test results, virus particle morphology, and the sequence of the 3' terminal region, a virus identified in the uninoculated tissue-cultured calla lilies was shown to be a new potyvirus referred to as *Zantedeschia mild mosaic virus* (ZaMMV). In addition to DsMV, TuMV [3] and ZaMV [2], ZaMMV is another potyvirus found to infect calla lilies in Taiwan. The 1845-nt sequence of ZaMMV was determined, starting from the 3' terminus of the N1b gene to 3'UTR.

Fig. 4. Alignment of the partial amino acid sequences of ZaMMV and the most closely related potyviruses obtained by PILEUP program in Wisconsin GCG Package and verified by ClustalX. The predicted cleavage site between Nlb and CP is indicated by the arrow. The polyglutamine sequence and the DAG motif in the CP of ZaMMV are underlined

According to the result of multiple sequence alignment of the CP amino acid sequence, BCMV-R, BCMNV-NL3, SMV-G2, DsMV-M13 and ZYMV-RI had 52–55% identities when compared with ZaMMV (Table 2). Because all these potyviruses are classified as members of the BCMV subgroup in the family *Potyviridae* [1], it is reasonable to conclude that ZaMMV belongs to the same subgroup. This speculated relationship was confirmed by phylogenetic analysis (Fig. 3).

Some potyviruses have special amino acid sequences at the N terminus of the CP after the DAG motif. For example, there are repetitive sequences, (GNNTNTNTNT)₃ and six proline residues, after the DAG motif of the CP of DsMV-LA isolate [15]. Similar features, such as (KEKEKQ)₂(KE)₂ occurs in the CP of PRSV-HA [21], and KEKEKE in the CP of ZaMV-ZAN (unpublished data), were also found downstream the DAG motif. In contrast, an unusual, repetitive glutamine sequence at the N terminal region of ZaMMV CP is upstream the DAG motif. This is the first example of such unique feature found in potyviruses or other plant viruses. The mechanism of producing such a polyglutamine repeat is still unknown, but this may be similar to the repetitive sequence of other potyviruses that is thought to be caused by polymerase slippage [7].

The dominant epitopes of potyvirus virions are the variable terminal parts of the capsid protein, especially the N-terminal part. Some reports revealed that about 30 amino acids of the N terminus of CP were exposed on the surface of virus particle [18] and were related to potyvirus movement [5, 11]. The repetitive sequence of ZaMMV CP was composed of 39 glutamine residues which were likely exposed on the surface of virion, if not degraded or processed. Future studies might focus on the function(s) of this polyglutamine tract.

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