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Zantedeschia mosaic virus causing leaf mosaic symptom in calla lily is a new potyvirus

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Summary. A novel virus, Zantedeschia mosaic virus (ZaMV-KR), causing mosaic and malformation symptoms was isolated from calla lily (*Zantedeschia* spp.) in Korea and its biological and molecular properties were characterized. The virus was distinct from *Dasheen mosaic virus*, an Araceae-infecting potyvirus, by serological and sequence analyses. Multiple alignments of the CP amino acid sequence between the virus and other potyviruses showed 51.8 to 62.1% identity. Phylogenetic analyses of the CP revealed that the virus could be clustered with *Plum pox virus* and *Turnip mosaic virus*. Sequence comparison of the CP gene between the virus and three other ZaMV isolates from Taiwan showed over 93.9% identity, and most of amino acids changes occurred in the N-terminal region. Sequence comparison of 3′ NTR revealed homology levels of 27.0 to 47.9% between the virus and other potyviruses. Our results support ZaMV as a distinct species of the genus *Potyvirus*.

Introduction

In Korea, several varieties of calla lily (*Zantedeschia* spp.) have been introduced from New Zealand, Australia and the Netherlands and have been cultivated as cut-flowers for its excellent flower type. A new virus disease has occurred in the calla lily culture regions in Kangwon Province in Korea since 2000. The causal agent for the disease was first considered to be a strain of *Dasheen mosaic virus* (DsMV) simply based on the leaf mosaic and malformation symptoms and virus particle morphology. We have surveyed the occurrence of virus disease on the cultivated calla lily plants during the last two years. During the investigation, the

causal potyvirus did not react with DsMV antiserum and no PCR product was amplified with the DsMV-specific diagnostic primers (our unpublished data).

In this study, we have investigated the properties of the new virus infecting calla lily and have determined the nucleotide sequence of the coat protein (CP) gene and 3′ nontranslated region (NTR) of the virus. Comparisons of sequence analysis between the virus designated Zantedeschia mosaic virus (ZaMV) and other potyviruses are discussed in this report.

Materials and methods

Virus sources, virus purification and viral RNA preparation

A Korean strain of ZaMV (ZaMV-KR) was isolated from diseased calla lily var. Black Magic (Fig. 1a). The virus was propagated in virus-free tissue-cultured calla lily. Inocula of *Turnip mosaic virus* (TuMV) and *Lettuce mosaic virus* (LMV) were obtained from the Plant Virus GenBank (http://www.virusbank.org) of Seoul Women's University, Seoul, Korea. Virions were purified from infected leaves as described by Berger and Shiel [2]. Ultraviolet absorption spectra of virions were determined using a UV spectrophotometer (UV2000, Pharmacia). Viral genomic RNA was isolated from the purified virion preparations by SDS/proteinase K-phenol extraction method as described by Ryu et al. [9] and Berger and Shiel [2]. Antiserum against ZaMV was produced in immunized white rabbit. ELISA kit for DsMV was purchased from Agdia, Inc (Indiana).

Host range test

To determine the host range of ZaMV, crude sap (1:100, w/v) from leaf extracts of virus-infected calla lily were mechanically inoculated to 14 plant species representing 5 families. The plants were predusted with Carborundum (320 grit, Fisher Scientific). The plants were maintained in a glasshouse and observed for symptom development. Virus infection was checked by RT-PCR and ELISA [15] with ZaMV-specific primers and antibodies, respectively, 20 to 30 days post inoculation.

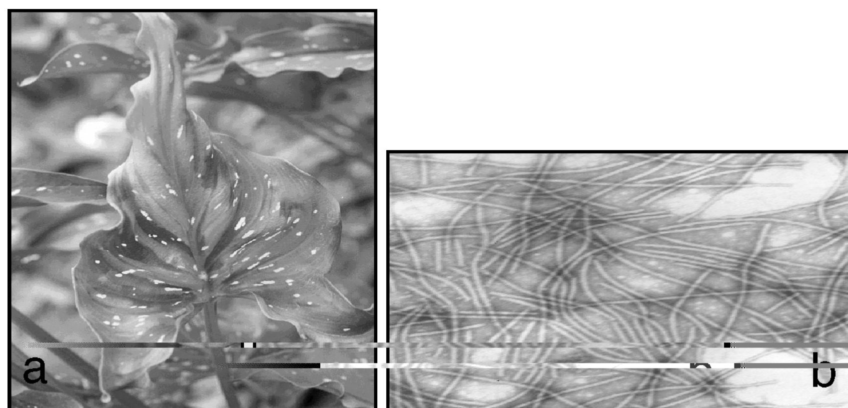

Fig. 1. Calla lily plant cv. Black Magic showing mosaic symptom (**a**), and electron micrograph of purified virus particles of ZaMV (**b**)

Electron microscopy of ZaMV

Purified virus particles of ZaMV were placed onto Formvar-coated copper grids and stained with 2% uranyl acetate. The specimens were examined with a LEO 906 transmission electron microscope at 80 kV.

Coat protein and viral RNA analysis

Coat proteins of ZaMV, LMV and TuMV were separated in 12.5% SDS-PAGE gel and visualized by Coomassie blue R-250 staining [1]. SDS-PAGE separated coat proteins were transferred onto nitrocellulose membrane and immunoprobed with ZaMV antibody by Western analysis [15]. Viral RNAs of ZaMV and the two other potyviruses were separated on a 5% polyacrylamide gel containing 8M urea and visualized by ethidium bromide staining [11].

RT-PCR detection

RT-PCR was performed for molecular characterization of ZaMV. Nucleotide sequences of conserved amino acid regions for 8 potyviruses were aligned for primer selection. Degenerate upstream and downstream primers, Poty-P (5′-ATGGT/G,T or C/TGGTG/C or T/AT/A, T or C/GA-3′) and Poty-M (5′-CCATCCAG/G or C/CC/G or A/AA/C or T/A-3′), respectively, were designed to amplify a partial CP gene sequence about 340 bp of virus.

cDNA synthesis and cloning

The cDNA was synthesized from 10 μg of viral genomic RNA using the Plasmid Choice cDNA Synthesis & Cloning kit (Gibco BRL, MD) with oligo dT primer-adapter system [14]. Double stranded cDNA molecules were cloned into *Not*I/*Sal*I site of pSPORT1 vector (Gibco BRL, MD). The recombinant plasmids were transfected into competent *E. coli* strain JM109 [11].

Nucleotide sequencing and sequence analysis

Subclones containing cDNA molecules of genomic RNA of ZaMV were generated by restriction enzyme hydrolysis. Overlapped cDNA clones containing the viral genes were sequenced in both directions by the dideoxynucleotide chain termination method using the Model 377 automatic DNA sequencer (ABI). Nucleotide sequences and deduced amino acid sequences of the viral genomic RNAs were analyzed using the DNASTAR software package (Madison, WI). Sequences of species of the genus *Potyvirus* were obtained for comparison from the GenBank/EMBL databases under the following accession numbers: *Bean common mosaic virus* (BCMV), U19287; *Bean common mosaic necrosis virus* (BCMNV), U37076; *Zucchini yellow mosaic virus* (ZYMV), L31350; *Peanut mottle virus* (PeMoV), AF023848; *Clover yellow vein virus* (ClYVV), AB011819; *Plum pox virus* (PPV), NC_001445; *Tobacco vein mottling virus* (TVMV), NC_001768; *Papaya ringspot virus* (PRSV), NC_001785; *Peanut stripe virus* (PStV), U34972; LMV, X97704; *Pepper mottle virus* (PepMoV), NC_001517; *Potato virus Y* (PVY), NC_001616; *Tobacco etch virus* (TEV), NC_001555; *Soybean mosaic virus* (SMV), S42280; TuMV, D10927; ZaMV-Zan, AF332872; ZaMV-DB, AY026464; ZaMV-BG, AY026463; DsMV-ND, AJ298034; DsMV-M13, AJ298033; and DsMV-DK, AJ298035. Phylogenetic analysis was performed to address the relationship of ZaMV and other potyviruses using the neighbor-joining algorithm in the DNAMAN package (version 5.1, Lynnon Biosoft, Canada) according to Saitou and Nei [10]. The data set was subjected to 1,000 bootstrap replicates.

Results and discussion

Incidence and properties of ZaMV

Virus infection was frequently detected in cultivated calla lilies in Korea during the last two years, at an infection rate from 24.9 to 31.9%. DsMV was not detected in 150 calla lily samples showing mosaic and malformation symptoms while over 95% of these samples were infected by ZaMV based on serology, electron microscopy and RT-PCR. It has been reported that DsMV is the prevalent viral pathogen of many aroid plants such as calla lily in the world [4, 6, 8, 16]. ZaMV was originally isolated from calla lily plants and showed leaf mosaic and malformation symptoms. The virus was characterized by host reactions, serology and sequence analysis. Virus was easily purified from the collected leaf tissues by differential centrifugation. The average Amax/min and A260/280 ratios of purified virion preparations were 1.06 and 1.25, respectively. In an electron microscope, ZaMV particles appeared as long filaments about 850 nm in length and 12 nm in width (Fig. 1b), which is the typical morphology of members in the genus *Potyvirus* [12, 13].

The virus induced systemic mosaic symptoms only on calla lily plant and did not infect 13 species of indicator plants (Table 1). *Tetragonia expansa*, *Colocasia esculenta*, *Cucumis sativus*, *Chenopodium amaranticolor*, *C. quinoa*, *Glycine max*, *Phaseolus vulgaris*, *Vicia faba*, *Vigna unguiculata*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, *N. tabacum* (cv. Samsun, cv. White Burley) showed no symptoms and no virus accumulation occurred in their inoculated and

Table 1. Comparison of experimental host range and symptoms of ZaMV and DsMV

Family	Species	Symptoms[a]	
		ZaMV	DsMV
Tetragoniaceae	*Tetragonia expansa*	–/–	LL/–
Araceae	*Colocasia esculenta*	–/–	–/M
	Zantedeschia aethiopica	–/M	–/M
Chenopodiaceae	*Chenopodium amaranticolor*	–/–	–/–
Leguminosae	*Glycine max*	–/–	–/–
	Phaseolus vulgaris	–/–	–/–
	Vicia faba	–/–	–/–
	Vigna unguiculata	–/–	–/–
Solanaceae	*Nicotiana benthamiana*	–/–	–/–
	N. clevelandii	–/–	–/–
	N. glutinosa	–/–	–/–
	N. rustica	–/–	–/–
	N. tabacum cv. Samsun	–/–	–/–
	N. tabacum cv. White Burley	–/–	–/–

[a]Symptoms on inoculated leaves and upper uninoculated leaves are to the left and right of the slash, respectively. *M*, mosaic; *LL*, local lesion; –, no infection

upper leaves. DsMV produced local lesions in inoculated leaves of *T. expansa*, and systemic mosaic symptoms on *Z. aethiopica* and *C. esculenta* (Table 1). DsMV did not infect other tested plants. The two viruses overlap natural host ranges of aroid plants. Our results indicate that the host ranges of ZaMV and DsMV are very narrow and that *T. expansa* plant can be used for differentiating the two viruses.

Viral CP and RNA analysis and serological relationship

Purified ZaMV CP gave one major protein band of about 32 kDa in SDS-PAGE (Fig. 2A). The 32 kDa protein reacted strongly with ZaMV CP antibody in Western blot analysis (Fig. 2B). In RNA analysis, a single-stranded genomic RNA of about 10 kb was found in virion preparations of ZaMV (data not shown). Electrophoretic pattern of the viral RNA was similar to those of known potyviruses such as TuMV and LMV.

Gel double diffusion tests revealed that ZaMV was not serologically related to DsMV. In double antibody sandwich ELISA tests ZaMV reacted strongly with its homologous antibody but did not react with DsMV antibody (Fig. 3A). Similarly, DsMV reacted strongly with its homologous antibody but did not react with ZaMV antibody (Fig. 3B). This clearly indicated that ZaMV and DsMV are not serologically related and may be different species in the same genus.

The 360 bp DNA product obtained by RT-PCR with the viral RNAs and degenerate potyvirus-specific oligonucleotide primers was cloned and its nucleotide sequence was determined. In the BLAST analysis, the viral sequence was closely related to known potyviruses (data not shown), and further sequences were determined by using the viral genomic clones from the cDNA library.

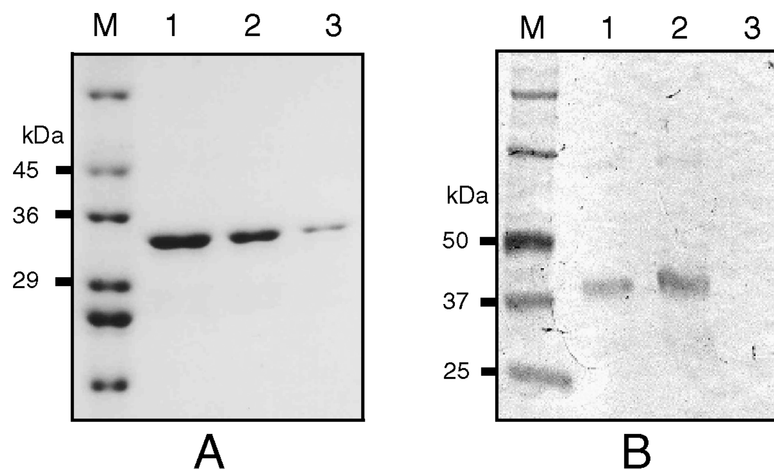

Fig. 2. SDS-PAGE of coat proteins of ZaMV and other potyviruses (**A**) and Western blot analysis (**B**). Coomassie stained gel (**A**) and immuno-probed NC membrane with ZaMV CP antibody (**B**). **A**: *1*, LMV; *2*, TuMV; *3*, ZaMV; *M*, prestained protein standards markers. **B**: *M*, prestained protein markers, *1*, ZaMV-infected leaf sample; *2*, purified ZaMV; *3*, healthy calla lily leaf sample

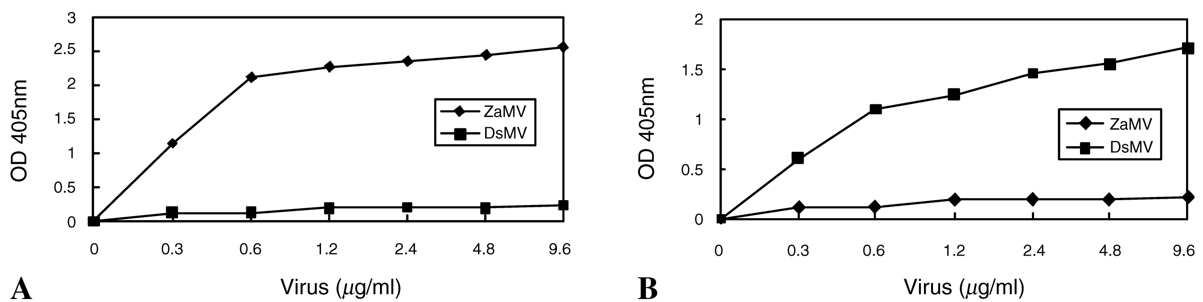

Fig. 3. Serological relationship between ZaMV and DsMV evaluated by DAS-ELISA. ZaMV (**A**) and DsMV (**B**) antibodies were used with the homologous and heterologous virus

Sequence analysis of 3′-terminal region of ZaMV

The cDNA clones containing sequences of viral genomic RNA were selected and the nucleotide sequences of the inserts were determined in both strands. The 1,597 nucleotides from 3′ terminus excluding poly A tract of ZaMV RNA were determined and analyzed. The sequences of the virus revealed over 55% identity with other known potyviruses and over 93% with three isolates of ZaMV at the nucleotide level by BLAST analysis. Although we previously named the virus calla mosaic virus (Kwon, Yoon, Ryu, unpublished results), the sequence similarity between the virus and three virus isolates from Taiwan led us to consider the virus as ZaMV. Thus, these four isolates belong to the same species in the *Potyvirus* genus which appears to be a new species based on sequence data and biological and serological properties.

The nucleotide sequence of ZaMV-KR contained the partial nuclear inclusion body (NIb) gene, entire CP gene (846 bp) and 3′ noncoding region (124 bp). The putative protease cleavage site for the NIb/CP of the ZaMV was found to be Q/S. The CP of ZaMV is composed of 281 (32,378.3 MW) amino acid residues. The CP and 3′-NTR sequences of the virus were compared with those of other species in the genus *Potyvirus*.

Multiple alignments of the CP amino acid sequence between the virus and other potyviruses showed 51.8 to 62.1% identity (Table 2). Sequence alignments and phylogenetic analyses of the CP revealed that the virus clustered with PPV and TuMV (Fig. 4). The two aroid-infecting potyviruses, ZaMV and DsMV, are much more distantly related in the analysis. Sequence comparison of the CP gene between the virus and three other ZaMV isolates from Taiwan showed over 93.9% identity; most of the amino acids changes occurred in the N-terminal region. The alignment of the CP of four isolates of ZaMV showed that ZaMV-KR was shorter (281 aa) due to a deletion of 3 nucleotides while the three Taiwan isolates were identical in size (282 aa). Chen et al. [4] and Pappu et al. [8] reported heterogeneity of CP lengths for DsMV isolates resulting in frequent deletions and insertions responsible for the CP size diversity among DsMV isolates. Gough and Shukla [5] reported that the CP sequence of the K strain of *Passionfruit woodiness virus* differed significantly, particularly in the N terminus, from the sequences of other

Table 2. Percentage sequence identities between the CP gene and encoded amino acid sequence and 3′ NTR of ZaMV and other *Potyviruses*

Virus[a]	CP		3′NTR
	Nucleotide	Amino acid	Nucleotide
BCMV	56.9	53.1	44.1
BCMNV	58.8	56.7	44.1
PStV	58.5	52.7	45.3
SMV-G2	57.7	54.8	47.9
DsMV-ND	56.3	51.8	41.4
DsMV-M13	56.0	52.1	39.7
DsMV-DK	56.9	52.3	44.8
ClYVV	57.5	56.7	30.8
PPV	61.2	62.1	28.9
LMV	57.5	58.5	27.0
PVY	55.9	53.7	31.1
TEV	58.5	57.5	29.8
TVMV	58.5	58.6	30.9
PepMoV	56.0	54.1	28.1
PeMoV	56.2	54.6	29.5
PRSV	60.9	59.1	36.3
ZYMV	56.8	54.1	36.7
TuMV	62.4	59.9	36.7
ZaMV-Zan	93.5	95.0	98.4
ZaMV-DB	93.0	93.9	98.4
ZaMV-BG	93.8	95.4	98.4

[a]Data are from the GenBank/EMBL/DDBJ databases under the following accession numbers: *Bean common mosaic virus* (BCMV), U19287; *Bean common mosaic necrosis virus* (BCMNV), U37076; *Zucchini yellow mosaic virus* (ZYMV), L31350; *Peanut mottle virus* (PeMoV), AF023848; *Clover yellow vein virus* (ClYVV), AB011819; *Plum pox virus* (PPV), NC_001445; *Tobacco vein mottling virus* (TVMV), NC_001768; *Papaya ringspot virus* (PRSV), NC_001785; *Peanut stripe virus* (PStV), U34972; LMV, X97704; *Pepper mottle virus* (PepMoV), NC_001517; *Potato virus Y* (PVY), NC_001616; *Tobacco etch virus* (TEV), NC_001555; *Soybean mosaic virus* (SMV-G2), S42280; TuMV, D10927; ZaMV-Zan, AF332872; ZaMV-DB, AY026464; ZaMV-BG, AY026463; DsMV-ND, AJ298034; DsMV-M13, AJ298033; and DsMV-DK, AJ298035

three strains of the virus resulting in serological difference. Three DsMV isolates were distantly related to ZaMV isolates (Fig. 4). This is consistent with the lack of serological relationship between the viruses.

Sequence comparison of 3′ NTR revealed homology levels of 27.0 to 47.9% between the virus and other potyviruses.

Our results indicate that ZaMV is a previously unknown distinct species of the genus *Potyvirus* causing a significant problem in calla lily production. We have established regeneration of calla lily plants and transformation via particle bombardment techniques. Heat treatment of *Zantedeschia* protocorms was so far

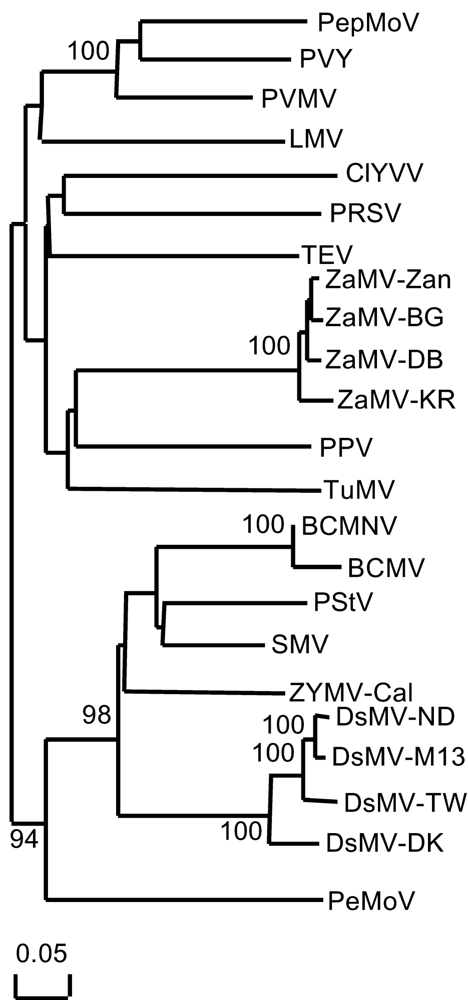

Fig. 4. Phylogenetic relationship of ZaMV and members of the genus *Potyvirus* based on the CP at the amino acid level. Multiple sequence alignment was generated using the DNAMAN package and the tree was constructed by the neighbor-joining algorithm based on calculations from pairwise amino acid sequence distances. The horizontal branch lengths are proportional to the genetic distance, and numbers shown at branch point indicate bootstrap values. The data set was subjected to 1,000 bootstrap replicates

unsuccessful for elimination of the virus (unpublished data). This study was done as a first step in attempts to obtain virus-resistant transgenic plants by CP-mediated molecular breeding program.

EMBL, GenBank and DDBJ accession number

The nucleotide sequence of ZaMV reported in this paper will appears in the EMBL, GenBank and DDBJ sequence databases under the accession number AB081519.

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