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First Report on *Carnation vein mottle virus* in *Dianthus barbatus* in Korea

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A potyvirus causing chlorotic mottle and yellow spots on leaves of *Dianthus barbatus* was isolated and identified as an isolate of *Carnation vein mottle virus* (CVMV). Purified preparations of *Chenopodium quinoa* infected with CVMV-K showed filamentous particles between 695 and 785 nm long. Many cytoplasmic inclusions were observed, and these consisted of pinwheels, dense bands, loops, and circles. The coat protein of CVMV-K was about 32 KDa in western blot analysis using a CVMV antibody. The nucleotide sequence of coat protein gene showed 97.6% homology with a Japanese isolate. The genome size of CVMV-K was about 9.0 kb by dsRNA analysis. These results indicate that the virus is an isolate of CVMV. This is the first report on CVMV in Korea.

Keywords : Carnation, *Dianthus barbatus*, *Carnation vein mottle virus*, dsRNA, *Potyvirus*

Carnation (*Dianthus caryophyllus*) has an important position in the world floral industry. The total carnation production area in Korea is approximately 124.2 ha (Ministry of Agriculture and Forestry, 2002). The largest cultivation region of carnation within Korea is Kyeongnam Province with cultivation area of 43.7 ha (Ministry of Agriculture and Forestry, 2002).

Carnations, being multiplied by cuttings, are prone to many viruses. Fourteen fully identified viruses have so far been isolated from carnation (Lovisolo and Lisa, 1978). Among these, the most important are *Carnation mottle virus* (CarMV), *Carnation vein mottle* virus (CVMV), *Carnation ringspot virus* (CRSV), *Carnation latent virus* (CLV), *Carnation etched ring virus* (CarERV), and *Carnation necrotic fleck virus* (CNFV) (Lisa, 1995; Luisoni et al., 1988; Sánchez-Navarro et al., 1999).

CVMV was first reported in *D. caryophyllus* from the USA by Kassanis (1955). The partial nucleotide sequence of CVMV from *D. japonicus* was determined in Japan (Sasaya et al., 2000; GenBank accession no. AB017630) and from *D. caryophyllus* in India (GenBank accession no. CVE549329). This is the most common virus in *D. barbatus* (Lisa, 1995). The disease associated with CVMV shows symptoms such as presence of chlorotic, dark green spots, flecks, and mottling; flower breaking and malformation in *D. caryophyllus*; and mottling in *D. barbatus* (Lovisolo and Lisa, 1978; Weintraub and Ragetli, 1970).

In this study, we report for the first time that we have identified CVMV-K in *D. barbatus* grown as a pot plant in Korea. Our findings are based on biological assay, particle morphology, western blot analysis, electron microscopy, dsRNA analysis, and nucleotide sequence determination of coat protein gene.

Materials and Methods

Source of virus. Collection of *D. barbatus* showing chlorotic spots, yellowing, and mottling (Fig. 1) was done in a commercial farm in Kyeonggi Province. Sap of leaf samples was inoculated on indicator plants of *Chenopodium quinoa*. Single local lesion from the inoculated leaves was used as an inoculumn on *C. quinoa* for three successive times. The filamentous virus isolate was propagated in *C. quinoa* and was designated as CVMV-K in this study.

Fig. 1. *Dianthus barbatus* showing chlorotic spots, yellowing, and mottling by spontaneous infection with CVMV-K.

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Virus purification. The researchers harvested, 14 days after inoculation, the *C. quinoa* plants which they systemically infected with CVMV-K. The virus particles were purified as described previously (Berger and Shiel, 1998) by negatively stained these with 0.5% phosphotungstic acids, pH 7.0. These were then examined by using a LEO 906 transmission electron microscope.

Host range test. Nine indicator plants were inoculated with crude sap of *C. quinoa* infected with CVMV-K in 0.01 M phosphate buffer, pH 7.2. Test was conducted in October in a glass house.

Electron microscopy. CVMV-K – infected leaves of *D. barbatus* were prefixed in 1% Karnovsky's fixative solution for 4 hr, and washed three times for 20 min in each step in 0.05 M Cacodylate buffer, pH 7.2. The specimens were postfixed for 2 hr in 1% Osmium tetroxide in 0.1 M Cacodylate buffer, pH 7.2. Fixed specimens were dehydrated in an ethanol series of 50, 75, 90, 95 and 100% for 30 min in each step. The tissue was then embedded in Spurr resin (Electron Microscopy Science, Washington, PA). Ultrathin sections were stained with 2% uranyl acetate and 0.08 M lead citrate buffer, pH 12.0, and examined with a Carl Zeiss LEO 906 transmission electron microscope.

Western blot analysis. Purified virus and crude sap of CVMV-K – infected *C. quinoa* was separated on 12.5% polyacrylamide gels containing 0.4% SDS. After electrophoresis, gel was transferred into immuno-blot PVDF membrane (0.2 um, BIO-RAD, CA) by using an electro-blot apparatus (BIO-RAD) according to the manufacturer's instructions. The membrane was blocked with 0.5% Bovine serum albumin and immunoprobed with CVMV antiserum, provided by Plant Virus GenBank (PVGB stock obtained from DSMZ Plant Virus Collection (Braunschweig, Germany), and alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega, USA), a secondary antibody.

For color development, Western Blue stabilized substrate was used (Promega, USA). The primary was used at a concentration of 0.01 mg/ml for 2 hr, and the secondary antibody was used at a dilution of 1:7,500 following the supplier's instruction (Promega, USA).

dsRNA analysis. Viral dsRNA was extracted from CVMV-K – infected *C. quinoa* by using CF-11 cellulose column chromatography (Morris and Dodds, 1979). Two dsRNA preparations of *Zucchini yellow mosaic virus* (ZYMV) and *Cucumber mosaic virus* (CMV) were used as molecular weight size markers. DsRNAs were separated in 6% polyacrylamide gel in 44.5 mM Tris-borated buffer containing 1.0 mM EDTA, pH 8.0. The dsRNA bands were made visible by silver staining these (Promega, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Specific primer was synthesized for amplification of coat protein gene of CVMV-K, based on the nucleotide sequence reported previously (GenBank accession no. AB017630). The sequence of the forward primer was 5'-GTTGATGCACTACATGCAGCAT-3', and that of the reverse primer was 5'-GTGATCACATCCGA-ACGCCG-3'.

RT-PCR was conducted by using GeneAmp RNA PCR kit (PerKin Elmer Cetus Inc., Norwalk, CT) according to manufac-

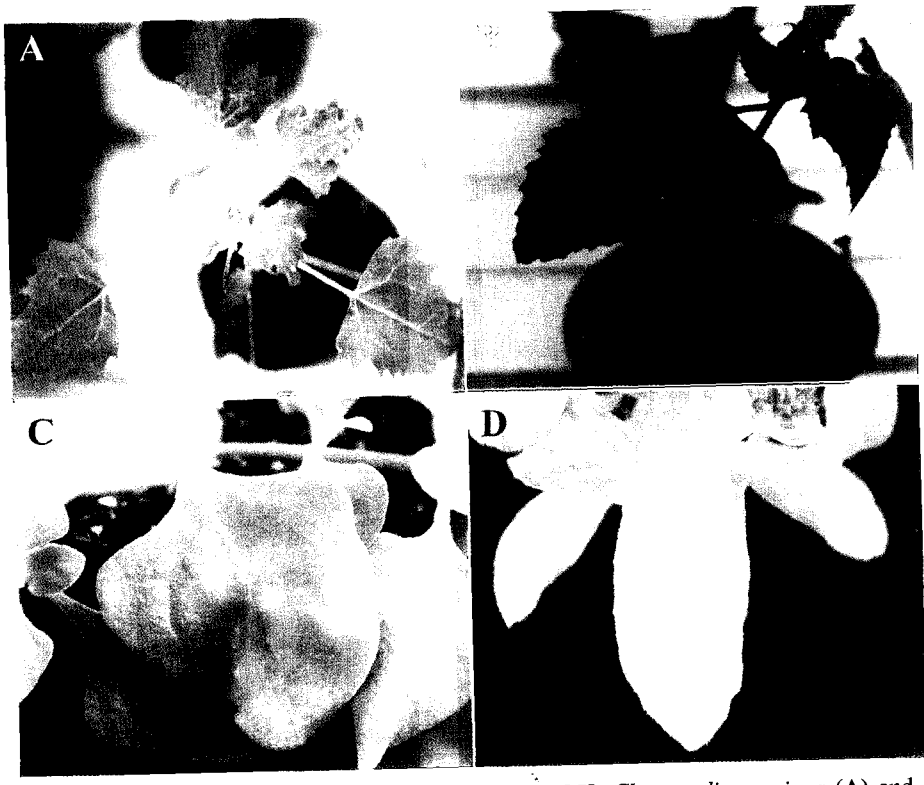

Fig. 2. Symptoms in indicator plants by mechanical inoculation of CVMV-K. *Chenopodium quinoa* (**A**) and *C. amaranticolor* (**B**) showing systemic chlorotic spots. *Tetragonia expansa* showing chlorotic lesion on inoculated leaves (**C**), and *Gomphrena globosa* showing vein clearing on inoculated leaves (**D**).

ture's instruction. PCR was conducted with the following cycles: Denaturation at 94°C for 30 sec, primer annealing at 62°C for 1 min, and extension at 72°C for 1 min for 40 cycles, with a final extension at 72°C for 7 min. Amplified RT-PCR products were analyzed on 1.5% agarose gel in 0.5 × TBE buffer (Sambrook et al., 1989).

Sequence analysis of CP gene. PCR products of 901 bp DNA were gel- purified with the GENECLEAN III KIT (Q-BIO gene, USA), and ligated into the pGEM-T easy vector (Promega, USA) according to the manufacture's instruction. The ligation mixture was used to transform competent cells of *Escherichia coli* JM 109. Recombinants were screened as described previously (Chung et al., 2001). The nucleotide sequence was determined from five independent clones, and was aligned with a CVMV isolate derived from GenBank by using CLUSTAL Method of DNASTAR software version 5.1 (DNASTAR, Madison, WI. USA).

Results

Host range test. CVMV-K induced yellows spots systemically on *Chenopodium quinoa* and *C. amaranticolor* (Fig. 2A, B) CVMV-K infected inoculated leaves of *Tetragonia expansa* with chlorotic lesion and that of *Gomphrena globosa,* with vein clearing symptom (Fig. 2C, D). CVMV-K did not infect *Nicotiana tabacum* cv. Samsun, *N. rustica, N. clevelandii, Cucumis sativus,* and *Vigna unguiculata* (data not shown).

Virus morphology. Purified virions of CVMV-K were filamentous particles which were between 695 and 785 nm long (Fig. 3).

Electron microscopy. Many cytoplasmic inclusions were observed, and these consisted of pinwheels, circles, or dense bands (Fig. 4).

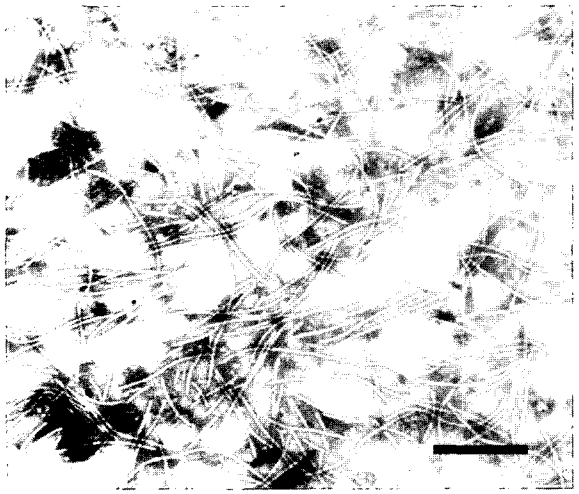

Fig. 3. Electron micrograph of purified CVMV-K. Viral preparations were stained with 0.5% phosphotungstic acids, pH 7.0. Scale bar represents 500 nm.

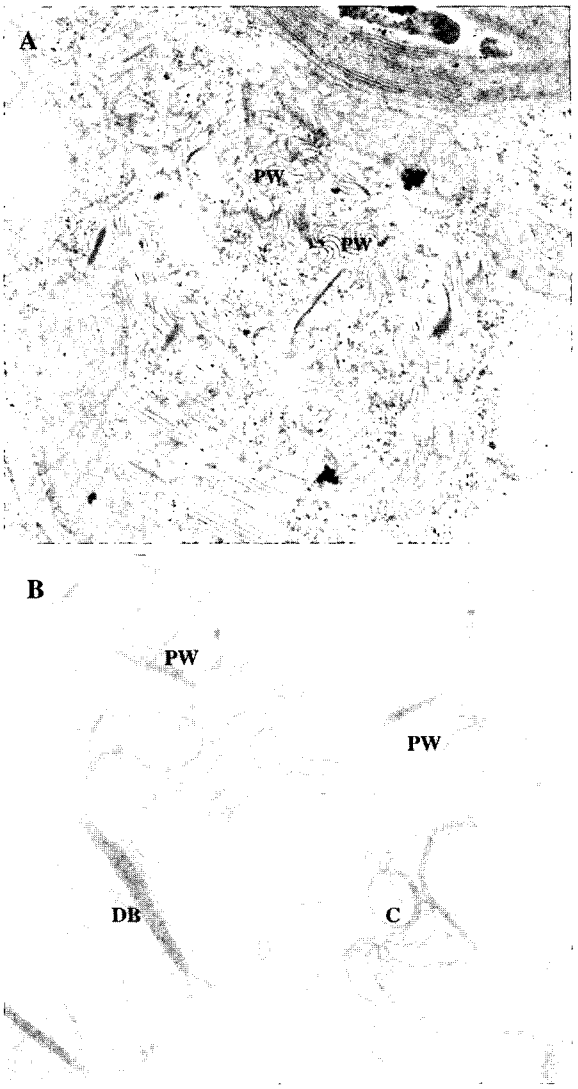

Fig. 4. Ultrathin sections of leaf tissue of *Dianthus barbatus* infected with CVMV-K. A: Inclusions of pinwheels (PW) are shown in the cytoplasm. B: Inclusions of pinwheels (PW), circles (C), and dense band (DB) structures are shown in the cytoplasm.

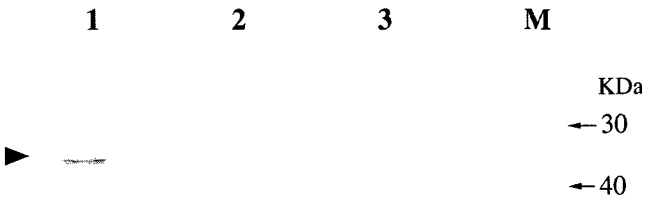

Fig. 5. Western blot analysis of viral coat protein of CVMV-K. PVDF membrane was probed with CVMV antiserum. M, Molecular weight size marker (Invitrogen, USA); lane 1, sap of *Chenopodium quinoa* infected with CVMV-K; lane 2, healthy sap of *C. quinoa*; lane 3, purified virus of CVMV-K. Position of CP is shown by an arrowhead.

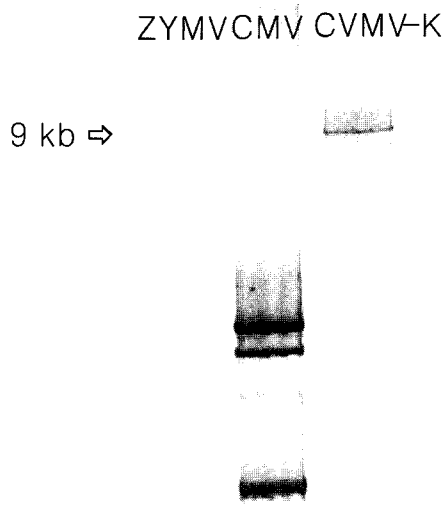

Fig. 6. Double strand RNA (dsRNA) profiles of CVMV-K. DsRNA was extracted from *Chenopodium quinoa* infected with CVMV-K. Electrophoresis was carried out in 6% acrylamide gel, and the gel was made visible by silver staining. Position of dsRNA is indicated to the right of the gel by an arrowhead. ZYMV and CMV were used for molecular weight size marking.

Western blot analysis. The coat protein of the virus was estimated at 32 KDa in western blot analysis using the CVMV antibody provided by DSMZ Plant Virus Collection (Braunschweig, Germany) (Fig. 5).

DsRNA analysis. The genome size of CVMV-K was about 9.0 kb by dsRNA analysis of the virus in 6% polyacrylamide gel (Fig. 6).

Sequence analysis of CP gene. The nucleotide sequences of CP gene of CVMV-K, amplified from diseased *D. barbatus*, were determined and deposited in the GenBank database under the accession no. AY512554. The region encoding the CP gene was 843 nucleotides long, and the deduced protein consisted of 280 amino acids. Nucleotide sequence of the coat protein gene of CVMV-K showed 97.6% homology with that of a Japanese isolate.

Discussion

CVMV thrived in almost all places where carnations were grown and infected *D. caryophyllus* and *D. barbatus* (Hollings and Stone, 1971; Lovisolo and Lisa, 1978). The virus was isolated from *D. barbatus* and was identified by using biological assay, particle morphology, western blot analysis, electron microscopy, dsRNA analysis, and the nucleotide sequences of CP gene. The virus isolate, CVMV-K in this study, is an isolate of CVMV by the following evidences: i) its filamentous particles, ii) symptom development on test plants, iii) weight of coat protein, iv) genome length, v) electron microscopy of ultrathin sections of infected leaf cells, vi) serological relationship, and vii) sequence analysis of CP gene.

CVMV has flexuous particles between 675 and 850 nm (Weintraub and Ragetli, 1970); 750 nm (Mokrá and Götzová, 1994); 790 nm (Hollings and Stone, 1971). CVMV-K was also between 695 and 785 nm long.

The reactions on some test plants including *C. quinoa, C. amaranticolor, Gomphrena globosa, Nicotiana* sp. and *Vigna unguiculata* were the same as those described in the previous reports, infecting only *C. quinoa, C. amaranticolor*, and *G. globosa* (Hollings and Stone, 1971; Jiang et al., 1992) with some differences. CVMV infects only inoculated leaves of *C. amaranticolor* (Hollings and Stone, 1971; Jiang et al., 1992). On the other hand CVMV-K systemically infected *C. amaranticolor* with yellows spots. Up to now *Tetragonia expansa* has not been reported as a host plant of CVMV (Hollings and Stone, 1971; Jiang et al., 1992). However CVMV-K infected *T. expansa* with chlorotic lesion.

In a survey of commercially grown carnations conducted in Korea, CVMV was not detected in 18 cultivars and 454 samples tested (data not shown). Presumably CVMV appears not to be pervasive in commercial carnation cultivars and only widespread in *D. barbatus*. Accordingly, it was assumed that CVMV-K was imported from the Netherlands through cuttings of *D. barbatus*.

Its weight of CP and genome length ranged similar to those of the *Potyvirus* group. *Potyvirus* is known as a single-stranded RNA molecule of 8500-9800 nucleotides, and encapsidated by single species of CP of 30-36 KDa (Berger and Shiel, 1998). *Potyvirus* specific inclusions were found in the cytoplasm, including pinwheels, dense bands, loops and circles, in electron microscopy of thin section of CVMV-K -infected *D. barbatus* leaf cell as reported previously (Weintraub and Ragetli, 1970).

Purified virions of CVMV-K positively reacted with CVMV antiserum provided by DSMZ Plant Virus Collection (Braunschweig, Germany) in agar gel double diffusion test (data not shown). Also, crude sap of *C. quinoa* infected with CVMV-K showed positive reaction in enzyme-linked immunosorbent assay (ELISA) using *Potato virus* Y (PVY) kit (Agdia, Indiana, USA) (data not shown). This result agreed with that of Hollings and Stone (1971), which stated that CVMV belongs to the PVY group. All these results support the conclusion of CVMV-K as an isolate of CVMV, a unique *Potyvirus* infecting *Dianthus barbatus* and carnation.

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