Plant Pathology Bulletin 1: 184-189, 1992

Host Range and Cytology of a Virus Causing Mild Mosaic on Carnation

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Accepted for publication: October 9, 1992

ABSTRACT

Liang, Y. G., and Ko, N. J. 1992. Host range and cytology of a virus causing mild mosaic on carnation. Plant Pathol. Bull. 1:184-189.

An isometric virus about 29 nm in diameter was isolated from carnations grown near Taichung with mosaic symptoms. Based on host range studies and cytological results by light and electron microscopy, it was concluded that this virus was different from six previously described isometric carnation viruses. This virus is tentatively named carnation mild mosaic virus (CMMV) due to the symptoms induced on carnations. In host range studies, CMMV can be mechanically transmitted to 3 species of Chenopodiaceae, inducing local lesions, and 5 species of Caryophyllaceae, inducing mild mosaic symptoms or no symptoms. Various sized crystals and vacuolate inclusions were detected in leaf strips from CMMV-infected plants stained with calcomine orange - Luxol brilliant green or Azure A. In addition, in longitudinal sections of leaf veins, xylem cells filled with many crystalloid inclusions were detected. Electron microscopy of CMMV-infected tissues showed these crystals to be aggregates of virus particles. The antiserum titer prepared against virus particles was determined to be 1:512 in ring tests. In immunospecific electron microscopy, 400-fold diluted antiserum used for trapping or decorating virions proved most effective for detection. In immunolabelling, the combination of 400-fold diluted antiserum and protein A-gold complex ($A_{520nm}=0.2$) gave the best results in either ultrathin section or negative staining of electron microscopy.

Key words: Carnation mild mosaic virus, Crystal inclusion bodies, Immunoelectron microscopy, Protein A-gold complex.

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.), a worldwide cutflower, is susceptible to six spherical and three filamentous viruses. The most important of these are carnation mottle (10), carnation ringspot (21), carnation etched ring (16), and carnation vein mottle viruses (11). These viruses induce economic losses in carnation production. Other viruses, such as carnation cryptic (18), carnation Italian ringspot (12), carnation yellow stripe (8), carnation latent (25), and carnation necrotic fleck viruses (13), are less important pathogens of carnation cultivation.

Carnation plants with severe mosaic symptoms were observed in the Taichung area during field surveys in 1986. Plants with symptoms were collected and isometric virus particles about 29 nm in diameter were observed in negatively stained extracts.

The isometric virus, referred to herein as carnation mild mosaic virus (CMMV), was similar in size to carnation mottle, carnation ringspot, carnation cryptic, and carnation Italian ringspot viruses. In light microscopic studies, CMMV induced crystals in the cytoplasm of infected carnation cells, unlike those induced by the previously described viruses (5,10, 18,21).

In this paper, we described the isolation, identification, cytological characterizations and applications of protein A-gold complex (pAg) in detection and localization of CMMV.

MATERIALS AND METHODS

Virus isolation and host range

Carnation plants (*D. caryophyllus* var. Maiko kgr) with severe mosaic symptoms were collected from the field and planted in an insect-proof cage. Leaves with symptoms were triturated in 10 parts (w/v) of 0.1 M phosphate buffer (pH 7.0) and inoculated mechanically to carborundum (400 mesh) dusted *Chenopodium amaranticolor* Coste and Reyn., *Nicotiana sylvestris* L., *N. tabacum* L., *Gomphrena globosa* L., *Silene pendula* L., and *Saponaria vaccaria* L. Local lesions occurred only on *C. amaranticolor* and the virus was isolated by three times single-lesion isolation. Isolated virus was inoculated to carnation for propagation and maintainance. For host range tests, 25 species of 8 families (Table 1) were inoculated with sap from local lesions. Susceptibilities were determined by symptom expression and light microscope examination using Azure A stain.

Light microscopy

Epidermal leaf strips and longitudinal sections of inoculated carnation leaf veins were stained in calcomine orange - Luxol brilliant green or Azure A and examined under a light microscope for virus induced inclusion bodies as previously described (2).

Electron microscopy

For negative staining, leaf extracts from carnation or local lesions of *C. amaranticolor* were stained in 2 % uranyl acetate (UA) and examined with a JEOL 200 CX electron microscope. In ultrastructural studies, small pieces of diseased carnation tissue were prefixed in 5 % glutaraldehyde and postfixed in 2 % osmium tetroxide, after dehydration in an ethanol series. The tissues were then embedded in LR White medium and polymerized at 60 C for 24 hr (14). Ultrathin sections about 70 nm in thickness were collected on copper grids and stained with 2 % UA and 0.5 % lead citrate.

Virus purification

The following procedures were conducted at 4-6 C. Diseased leaves harvested from inoculated carnations were homogenized in 2 parts (w/v) of 0.1 M phosphate buffer (pH 7.0). The slurry was squeezed through 2-layers of cheesecloth and clarified by centrifugation at 10,000 *g* for 15 min. Polyethylene glycol 6,000 and NaCl were then added to the supernatant to a final concentration of 6 % and 0.2 M, respectively, and stirred for 1 hr. After centrifugation at 10,000 *g* for 15 min pellets were resuspended in 1/5 original volume of 0.01 M phosphate buffer (pH 7.0) and clarified by centrifugation at 10,000 *g* for 15 min. The virus was then subjected to one cycle of differential

TABLE 1. Host range of Carnation mild mosaic virus

Test Plant	Symptom[1]	L. M.[2]
Aizoaceae		
Tetragonia expensa Murr.	-	-
Amaranthaceae		
Gomphrena globosa L.	-	-
Caryophyllaceae		
Dianthus caryophyllus L.	MM	+
D. barbatus L.	MM	+
D. chinensis L.	YN	+
Saponaria vaccaria L.	-	+
Silene pendula L.	-	+
Chenopodiaceae		
Chenopodium amaranticolor Coste and Reyn.	LL	+
C. quinoa Willd.	LL	+
C. murale L.	LL	+
Cucurbitaceae		
Cucumis sativus L.	-	-
Citrullus vulgaris Schrad.	-	-
Labiatae		
Salvia splendens Ker-Gawl.	-	-
Leguminosae		
Cassia occidentalis L.	-	-
Vigna unguiculata (L.) Walp. subsp. *unguiculata*	-	-
Phaseolus vulgaris L.	-	-
Pisum sativum L.	-	-
Solanaceae		
Nicotiana tabacum L. Xanthi	-	-
N. edwardsoni L.	-	-
N. glutinosa L.	-	-
N. rapanda Willd and Lehm.	-	-
N. rustica L.	-	-
Lycopersicum esculentum Mill.	-	-
Capsicum annuum L.	-	-
Physalis floridana Rydb.	-	-

1. Symptoms on inoculated plants.
   LL: Local lesion.
   MM: Mild mosaic.
   YN: Yellowing and necrosis.
   -: No symptom.
2. Light microscopic results of Azure A stained leaf strip.
   +: Inclusion bodies observed.
   -: No inclusion body observed.

centrifugation (22). The final suspension was layered on 10-40 % continuous sucrose gradient and centrifuged at 85,000 *g* for 2 hr. Virus bands were collected and virus concentrations were determined spectrophotometrically (1).

Preparation of antiserum

A New Zealand white rabbit was injected intramuscularly with 1 mg purified virus preparation emulsified with Freund's complete adjuvant in equal volume. After 3 injections at weekly intervals, a booster injection of 1 mg virus preparation with 1 ml Freund's imcomplete adjuvant was administered intravenously. Ten days after the booster the rabbit was bled. Antiserum titers against the homologous virus were determined by ring test (24).

Immunoelectron microscopy

Trapping was done as described by Derrick (4). Grids were floated on CMMV antiserum diluted in 1:400 with 0.02 M phosphate buffered saline (PBS) for 10 min, rinsed with 10 drops of PBS, then floated on 20 times diluted crude sap for 10 min, rinsed with PBS and stained with 2 % UA. In decoration tests (20), grids were pretreated with diluted virus suspensions for 10 min and rinsed with PBS. They were then floated on 1:400 diluted CMMV antiserum for 10 min. After PBS rinsing, grids were stained with 2 % UA and examined by electron microscopy. In these tests, preimmune normal serum and protein A were used as controls.

Immunolabelling for negative staining

Crude leaf extracts applied drop-wise on grids were incubated for 10 min, followed by an incubation of 1 % BSA (w/v) in 0.1 M sodium acetate (pH 5.0) for 10 min and 0.01 mg/ml purified gamma globulin diluted in 0.1 M sodium acetate for 10 min. After washing with 40 drops of 0.1 M sodium acetate, the grid was incubated for 10 min in 15 nm pAg diluted to $A_{520nm}=0.2$ in 0.1 M sodium acetate. Washed as above and rinsed with 10 drops of deionized water, stained with 2 % UA and examined by electron microscopy (23).

Immunolabelling for ultrathin section

Diseased carnation tissues were embedded as described except that osmium tetroxide was omitted. Ultrathin sections were collected on copper grids (100 mesh) coated with carbon-backed Formvar films. For immunolabelling, grids were attached to double-sided cellophane tape on slides (3). Sections on grids were treated with 1 % BSA diluted in 0.02 M PBS for 10 min to prevent nonspecific binding of antibodies. After 30 min, incubation with antiserum diluted in 1:400 with 0.02M PBS, grids were washed first with PBS and then with deionized water. The grids were incubated with pAg diluted to $A_{520nm}=0.2$ in 0.02 M PBS for 30 min in the dark and washed with PBS and deionized water(15). Grids were poststained with UA and lead citrate and examined by electron microscope. Healthy tissue treated as described above and preimmuned normal serum were used as controls.

RESULTS

Symptomatology and host range

Symptoms of field-grown carnation plants infected with CMMV were severe mosaic, but showed mild mosaic after they were transferred to screenhouses. In inoculated indicator plants, CMMV infected members of the Caryophyllaceae systemically and caused local lesions on Chenopodiaceae. Infections were diagnosed by symptoms and light microscopy. CMMV could not infect inoculated species of Amaranthaceae, Cucurbitaceae, Leguminosae, Solanaceae, Aizoaceae, and Labiatae (Table 1). In Caryophyllaceae, CMMV induced mild mosaic symptoms on *D. caryophyllus* and *D. barbatus* L., yellowing and necrosis on *D. chinensis* L., and failed to induce symptoms on *S. vaccaria* and *S. pendula*. The virus induced yellow spotted local lesions 1-2 mm in diameter on *C. amaranticolor*, *C. quinoa* Willd., and *C. murale* L. Those lesions on *C. amaranticolor* and *C. quinoa* developed a necrotic center about 5 days after inoculation.

Light microscopy

Many crystals were seen in the cytoplasm of CMMV-infected tissues. They were olive green after staining with calcomine orange - Luxol brilliant green and red-violet (Fig. 1A) after staining in Azure A. In *S. pendula*, vacuolate, red-violet inclusion bodies were observed. In longitudinal sections, crystalloids could be observed in vessels (Fig. 1B). These crystals were distributed in epidermal, mesophyll, phloem cells and vessels.

Electron microscopy

Isometric virus particles, *av* 29 nm in diameter, were detected in negatively stained leaf extracts of systemically infected carnations and in local lesions of *C. amaranticolor*. In ultrathin sections, virus particles were occasionally dispersed in the cytoplasm, although in most cases, they aggregated to form crystals (Fig.2A). Virus aggregates varied in size from small to very large. Aggregated virus particles distributed in vascular tissues were also found in sectioned materials (Fig. 2B).

Virus purification and serology

The diameter of purified viruses examined by electron microscopy were *av* 29 nm. Yields of 17 mg

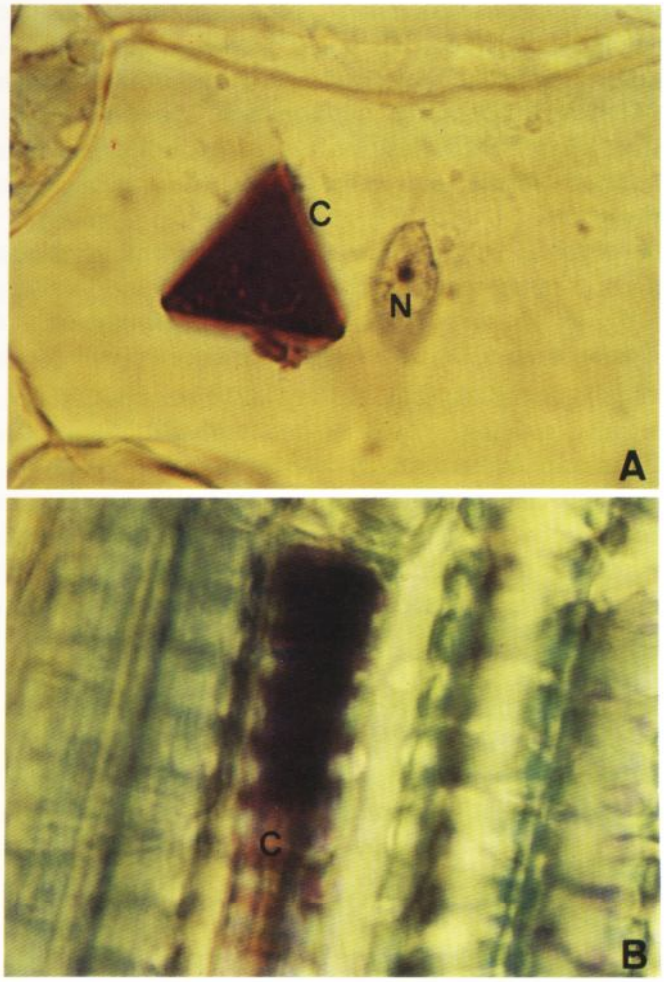

Fig. 1. Carnation epidermal cells (A) and longitudinal section of vessels (B) infected with CMMV stained with Azure A. (C: crystal, N: nuclear).

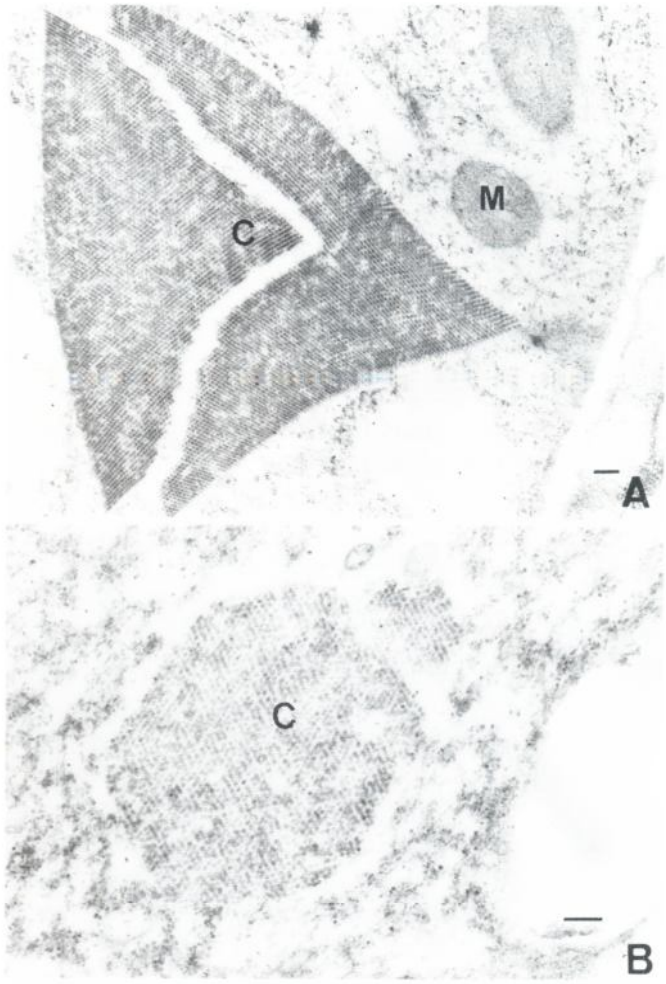

Fig. 2. Electron micrographs of CMMV in carnation cells. A: Large crystal in mesophyll cell. B: Crystal in vessel cell. (C: crystal, M: mitochondria, bar = 200 nm).

virus per 100 g host tissue were obtained in this investigation. An antiserum titer of 512 was determined by the ring test using antiserum collected after the fourth injection.

Immunoelectron microscopy

The best results of trapping were obtained using antiserum dilutions of 1/400. In decoration tests, the surface of virus particles were coated with antibodies. In controls, virus particles were not decorated.

Immunolabelling for negative staining

Gold particles adhered to the surfaces of virus particles and thus were readily identified in leaf extracts (Fig. 3). In control tests, gold particles did not, however, adhere to virus surfaces.

Immunolabelling for ultrathin sections

After immunolabelled with protein A-gold complex, gold particles were specifically labelled on virus particles (Fig. 4). On sections treated with CMMV antiserum, only CMMV virus particles were labelled. Gold particles did not adhere to cell organelles, nor were there any specifically labelled gold particles on those sections treated with preimmune normal serum. No specific gold particles were observed on healthy tissues treated with CMMV antiserum.

DISCUSSION

According to the results, this isometric virus isolated from carnation was different from all other carnation viruses based on morphology, host range, and cytological characters (8,10,11,12,13,16,18,21,25). Because this virus induced mild mosaic symptoms on carnation, we tentatively named it carnation mild moasic virus (CMMV). In symptomatology, diseased carnation plants in field and screenhouse showed severe and mild mosaic, respectively. Those inoculated carnations showed mild mosaic, too. These differences in symptom expression may be owing to different culture conditions.

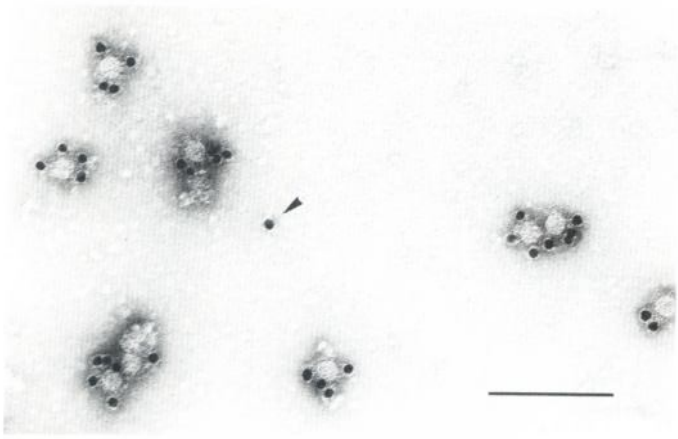

Fig. 3. CMMV particles from leaf extract immunolabelled with protein A-gold. Arrowhead indicated non-specific reaction. (bar = 200 nm).

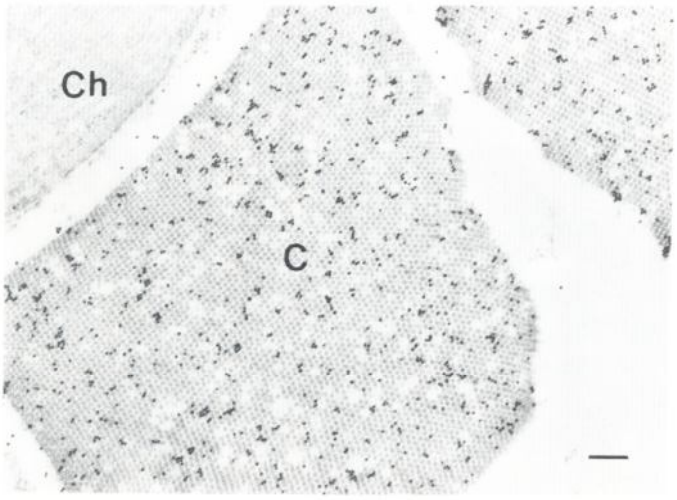

Fig. 4. Section of CMMV infected carnation tissue immunolabelled with protein A-gold. (Ch: chloroplast, C: crystal, bar = 200 nm).

Of the six isometric carnation viruses, four could infect *C. amaranticolor* mechanically. Carnation cryptic virus could not be transmitted to any tested plant (18) and carnation etched ring virus infected only members of the Caryophyllaceae (16). Carnation yellow stripe virus infected on *Vigna unguiculata* (L.) Walp. systemically and localized on carnation (8). Carnation mottle virus infected more than 30 species of dicotyledons by mechanical inoculation (10). Carnation ringspot and carnation Italian ringspot viruses could be transmitted to more than 133 and 62 species, respectively (12,21). The host range of CMMV is restricted in Chenopodiaceae and Caryophyllaceae. This indicated CMMV is a new virus occurring on carnations.

In light microscope studies, vacuolated inclusions and crystals were detected in leaf strips. Crystalloid inclusions filling xylem cells in longitudinal sections suggested infection by a comovirus (7). In the electron microscopy, the vacuolate inclusions contained dispersed virus particles while crystals consisted of aggregated virions. These inclusion bodies and distribution resembled the inclusions induced by comoviruses (2,6,7). According to the restricted host range, in comparison, CMMV is possibly a member of comoviruses. Molecular properties and serological relationships between CMMV and other comoviruses remain to be investigated.

Protein A-gold complex was a useful marker in electron microscopy. It is very useful for localizing antigens *in vivo* (9,14,17) or immunolabelling of negative stained virus antigens (19,23). Protein A-gold complex attached to the surface of virions specifically in negative staining as well as in ultrathin sections. This technique enhanced the resolution of serological reactions and enhanced virus identification in electron microscopy. The efficiency and non-specific background of immunolabelling was influenced by combinations between different concentrations of antibodies and pAg.

ACKNOWLEDGEMENTS

This research was supported by the National Science Council, Republic of China. We wish to thank Drs. J. R. Edwardson and F. W. Zettler for reviewing this manuscript critically.

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摘　　要

梁耀光[1]、柯南靖[2]. 1992. 康乃馨輕微嵌紋病毒之寄主範圍及細胞學研究. 植病會刊 1:184-189. (1. 台南市　台灣糖業研究所植物保護系. 2. 美國　佛羅里達大學農藝系)

從台中的康乃馨栽培區中，具有嵌紋病徵的康乃馨植株上分離得到一種球形病毒，直徑約 29 nm，經三次單斑分離，機械接種測定其寄主範圍，以光學及電子顯微鏡研究其細胞學之變化，所得結果與六種已知感染康乃馨的球形病毒皆不相同，根據病徵表現的特性，將此病毒稱爲康乃馨輕微嵌紋病毒 (Carnation mild mosaic virus, CMMV)；此病毒之寄主範圍狹窄，在接種的八科 25 種植物中，可感染三種藜科植物，造成局部病斑；在五種石竹科植物上造成輕微嵌紋或無病徵型的系統性感染；病株之表皮撕片以 Calcomine orange-Luxol brilliant green 或 Azure A 染色後，在感染的細胞中有許多成份爲蛋白質的結晶及空泡狀內含體。維管束經切片、染色後，在導管中亦有許多結晶內含體；超薄切片以電子顯微鏡觀察，顯示這些結晶都是病毒顆粒密集排列而成；純化的病毒經四次免疫注射在紐西蘭白兔上，可以得到力價爲 512 的抗血清。在檢定病毒時，將此抗血清稀釋 400 倍後，作免疫電子顯微鏡之研究，在捕捉法及修飾試驗均有良好效果。在免疫標示試驗中，稀釋 400 倍的抗血清與稀釋 20 倍的蛋白質 A-膠金 (A_{520nm}=0.2) 配合，可以得到最好的結果。超薄切片及陰染法之免疫標示，均可發現金顆粒非常專一地標示在病毒顆粒上。

關鍵字：康乃馨輕微嵌紋病毒、結晶內含體、免疫電子顯微鏡、蛋白質 A 膠金複合物。