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Ultrastructural Aspects of the Mixed Infections of Watermelon Mosaic Potyvirus and Cucumber Green Mottle Mosaic Tobamovirus Isolated from Watermelon

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Symptoms on 4 varieties of watermelons inoculated with watermelon mosaic potyvirus II isolated from watermelon (WMV-W) were severe mosaic and leaf malformation while those inoculated with cucumber green mottle mosaic tobamovirus from watermelon (CGMMV-W) were mild mosaic and chlorotic spots. Inoculation of the mixture of WMV-W and CGMMV-W produced extremely severe mosaic along with necrotic spots and general necrosis. Doubly infected plants were also stunted. Cells infected with WMV-W or CGMMV-W alone exhibited the intrinsically ultrastructural properties of each virus infection. WMV-W induced potyvirus-characteristic cylindrical inclusions in the cytosol. Virus particles were orderly aligned along the tonoplasts. CGMMV-W induced tobamovirus-characteristic stacked crystalline arrays of virus particles in the cytosol. Cells infected doubly with WMV-W and CGMMV-W contained striking cytopathic effects that were not present in single infection of each virus. The unique ring structure, nonagon, was that a single potyvirus particle was surrounded by 9 CGMMV-W tobamovirus particles.

Keywords: CGMMV, WMV, mixed infection, nonagon

Mixed infections of plant viruses are common and often cause severe damage to the hosts, and, therefore, can be detrimental to agricultural industry when they occur in economically important crops. The outbreaks of cowpea stunt disease in southwestern states of the United States (Anderson et al., 1994) and oriental cabbage necrotic stunt disease in mid eastern alpine area of Korea (Kim et al., 1993; Cho et al., 1995) are good examples. These two diseases are caused by synergistic interactions between two distinct combinations of different plant viruses. Cytopathological study of cowpea stunt disease revealed that the cells co-infected with the two viruses, cucumber mosaic cucumovirus and blackeye cowpea mosaic (=bean common mosaic) potyvirus, and contained mixed-infection characteristic features in addition to those characteristics of each virus.

Severe viral damage on watermelons grown in green house at Jinju and Haman areas, southern part of Korea, was investigated in 1995. Our studies indicated that these watermelons were infected either singly with cucumber green mottle mosaic tobamovirus (CGMMV-W) and watermelon mosaic potyvirus II (WMV-W) or mixedly with these two viruses. Mixed infection of the two viruses caused synergistic symptom on several watermelon cultivars. This paper presents striking ultrastructural features in cells infected mixedly with CGMMV-W and WMV-W, which were not present in single infection of each virus.

Materials and Methods

Host range study. Necrotic locals, produced on *Chenopodium amaranticolor* by mechanical inoculation with leaves of watermelons showing mosaic symptom collected from fields, were isolated by three transfers of single local for biological purification. The biologically purified virus isolates were tested for symptomatology and host range using 13 indicator plants including *Cucumis sativus* by mechanical inoculation.

Electron microscopy. Quick dip preparation was used for the observation of virus particle morphology in samples from fields and purified materials. For ultrastructural studies of virus-infected cells, diseased plants of watermelon and cucumber were made by mechanical inoculation with WMV-W, CGMMV-W and mixture of the two viruses. Systemically infected tissues passed 2 weeks after inoculation were fixed with 2.5% glutaraldehyde in Millonigs phosphate buffer, pH 7.3. Dehydration was done with 50-100% ethyl alcohol over six steps after treatment of 2% osmium tetroxide. The dehydrated tissues were embedded in Epon 812 and hardened at 60°C for overnight and then at 90°C for 2 hours. Ultrathin sectioning of 80-100 nm thickness was done with a diamond knife, and the ribbons were stained with uranyl acetate and lead citrate for 5 minutes and 7 minutes, respectively.

Making of mixedly infected plants. Plants used were 4 cultivars of watermelon and 2 cucumber cultivars for single or mixed infec-

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tion with WMV-W and CGMMV-W. The same volume of leaf infected singly with WMV-W and CGMMV-W was macerated with mortar for the mixed inoculum. Tissues of singly and mixedly infected plants for the specimen preparation of electron microcopy were collected in 2 weeks after inoculation.

Results

Disease incidence. The occurrence of virus disease averaged 45.0% on watermelon showing mosaic and yellowing symptoms, but was as high as 95.0% in Jinju area. Two different types of virus particles had been existed with long flexuous rod and short straight rod having a central canal that discovered through leaf-dip electron microscopy. Watermelon collected from fields had virus particles of flexuous rod for 25.9% and rigid rod for 48.2%, and both of these two types for 25.9% in total plants observed.

Viruses occurred on watermelon. The two types of virus particles from watermelon were identified as WMV-W for flexuous rod and CGMMV-W for straight rod having a central canal by biological characteristics and ultrastructural characteristics (Cho, 1998). The two type viruses could infect locally *Chenopodium amaranticolor* and *C. quinoa,* and systemically *Nicotiana benthamiana, Cucumis sativus* and *C. melo.* WMV-W produced chlorotic local lesions on *Gomphrena globosa* and *N. occidentalis*, however, CGMMV-W could not. Six plants including *Physalis floridana* were not infected with both viruses (Table 1).

Symptomatology by mixed infection. Symptom development in watermelons in 4 weeks after inoculation was shown in Table 2. CGMMV-W produced mild symptoms of mosaic and chlorotic spots (Fig. 1A), and WMV-W produced severe mosaic and malformation (Fig. 1B). However, in mixedly infected watermelon, devastating symptoms of severe mosaic along with necrotic spots, stunting and general leaf necrosis were produced (Fig. 1C). In cucumber, CGMMV-W begot chlorotic spots with yellowing and WMV-W caused severe mosaic and malformation. The mixed virions of WMV-W and CGMMV-W produced the mixed symptom of severe mosaic, yellowing and malformation. Average plant height of watermelon inoculated with WMV-W, CGMMV-W and mixture of both viruses was 28.8 cm, 30.0 cm and 15.6 cm, respectively. Watermelons infected mixedly were stunted as 61.2% compared with non-inoculated control plants and, 45.8% and 48.0% for the plants infected singly with WMV-W and CGMMV-W, orderly.

Table 1. Host range and symptomatology of WMV-W[a] and CGMMV-W[b]

Plant	Symptoms[c] by	
	WMV-W	CGMMV-W
Chenopodium amaranticolor	CL/–	CL/–
C. quinoa	CL/–	CL/–
Gomphrena globosa	CL/–	–/–
Physalis floridana	–/–	–/–
Nicotiana benthamiana	CL/M	–/M
N. clevelandii	–/–	–/–
N. glutinosa	–/–	–/–
N. occidentalis	CL/–	–/–
N. tabacum 'BY'	–/–	–/–
N. tabacum 'Ky-57'	–/–	–/–
Tetragonia expansa	–/–	–/–
Cucumis sativus	–/CS, M	–/M
C. melo	–/CS, M	–/M

[a] Watermelon mosaic potyvirus isolated from watermelon.
[b] Cucumber green mottle mosaic tobamovirus isolated from watermelon.
[c] CL; Chlorotic local, CS; Chlorotic spot, M: Mosaic, –; Non reaction, Inoculated leaf/Upper leaf.

Table 2. Symptom development in watermelon varieties inoculated with WMV-W[a], CGMMV-W[b] or their mixed inoculum

Variety	Systemic symptoms[c] by		
	WMV-W (W)	CGMMV-W (C)	W+C
Citrullus vulgaris 'Olympia'	SM, Mal	M, CS	M, NS, St, N
C. vulgaris 'Daiborum'	SM, Mal	M, CS	M, NS, St, N
C. vulgaris 'Hosan'	SM, Mal	M, CS	M, NS, St, N
C. vulgaris 'Kichan'	SM, Mal	M, CS	M, NS, St, N

[a] Watermelon mosaic potyvirus isolated from watermelon.
[b] Cucumber green mottle mosaic tobamovirus isolated from watermelon.
[c] CS; Chrolotic spot, M; Mosaic, Mal; Leaf malformation, N; Leaf necrosis. NS; Necrotic spot, SM; Severe mosaic, St; Stunt.

Ultrastructure for single infection of WMV-W. The potyvirus of WMV-W induced typical inclusions of pinwheel, scroll and laminated aggregates in the cytosol of mesophyll cells (Francki et al., 1985). WMV-W particles arranged lineally at the tonoplasts and presented intimately with the potyvirus inclusions (Fig. 1D).

Ultrastructure for single infection of CGMMV-W. CGMMV-W located typically lineally as band-structural crystalline in the cytosol of all type cells (Fig. 1E). The virus particles of CGMMV-W scattered in vacuoles of mesophyll and phloem parenchyma cells (Fig. 1F). Especially, xylem vessel was full of the virus particles of CGMMV-W. The virus particles of CGMMV-W were also observed in mitochondria.

Ultrastructure for mixed infection of WMV-W and CGMMV-W. The leaf cells of 4 cultivars of watermelon and cucumber plants systemically infected with WMV-W and CGMMV-W had the same cytopathic effects of each virus. The nonagon ring formed by two different virus particles of CGMMV-W and WMV-W was observed for the first time. The unique ring structure was nonagon that a

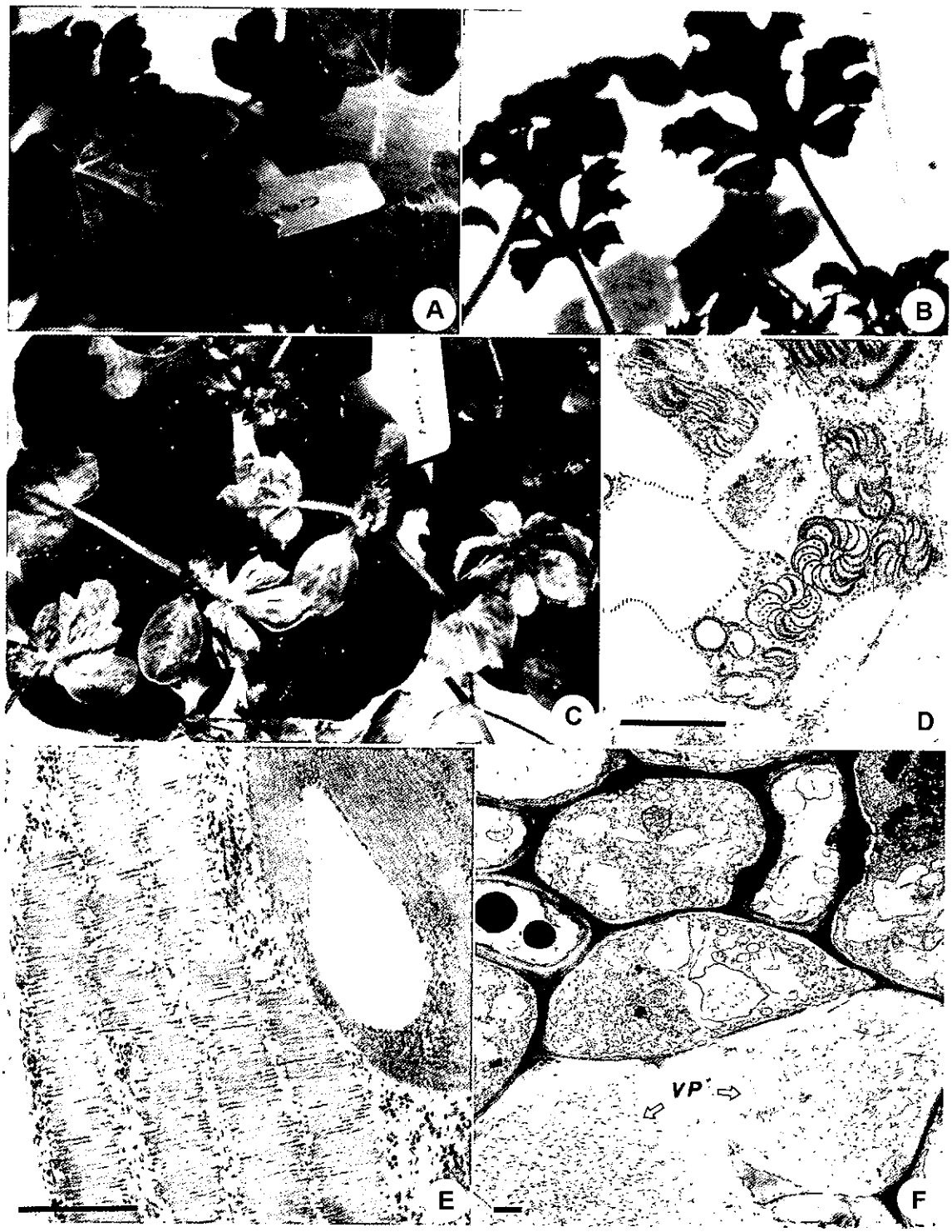

Fig. 1. The singly infected watermelon with CGMMV-W produced mild mosaic and chlorotic spots on the upper leaves in 4 weeks after mechanical inoculation (A). The watermelons infected singly with WMV-W were produced severe mosaic and malformation (B). The watermelons infected doubly with WMV-W and CGMMV-W were shown the synergistic symptoms of stunting and leaf necrosis (C). The typical inclusions of potyvirus were pinwheel, scroll and laminated aggregate, and the virus particles of WMV-W arranged lineally in tonoplast (D). The cells infected singly with CGMMV-W had the stacked-band structure in cytosol (E) and the virus particles (VP) were scattered in xylem vessel (F). Black bars = 500 nm.

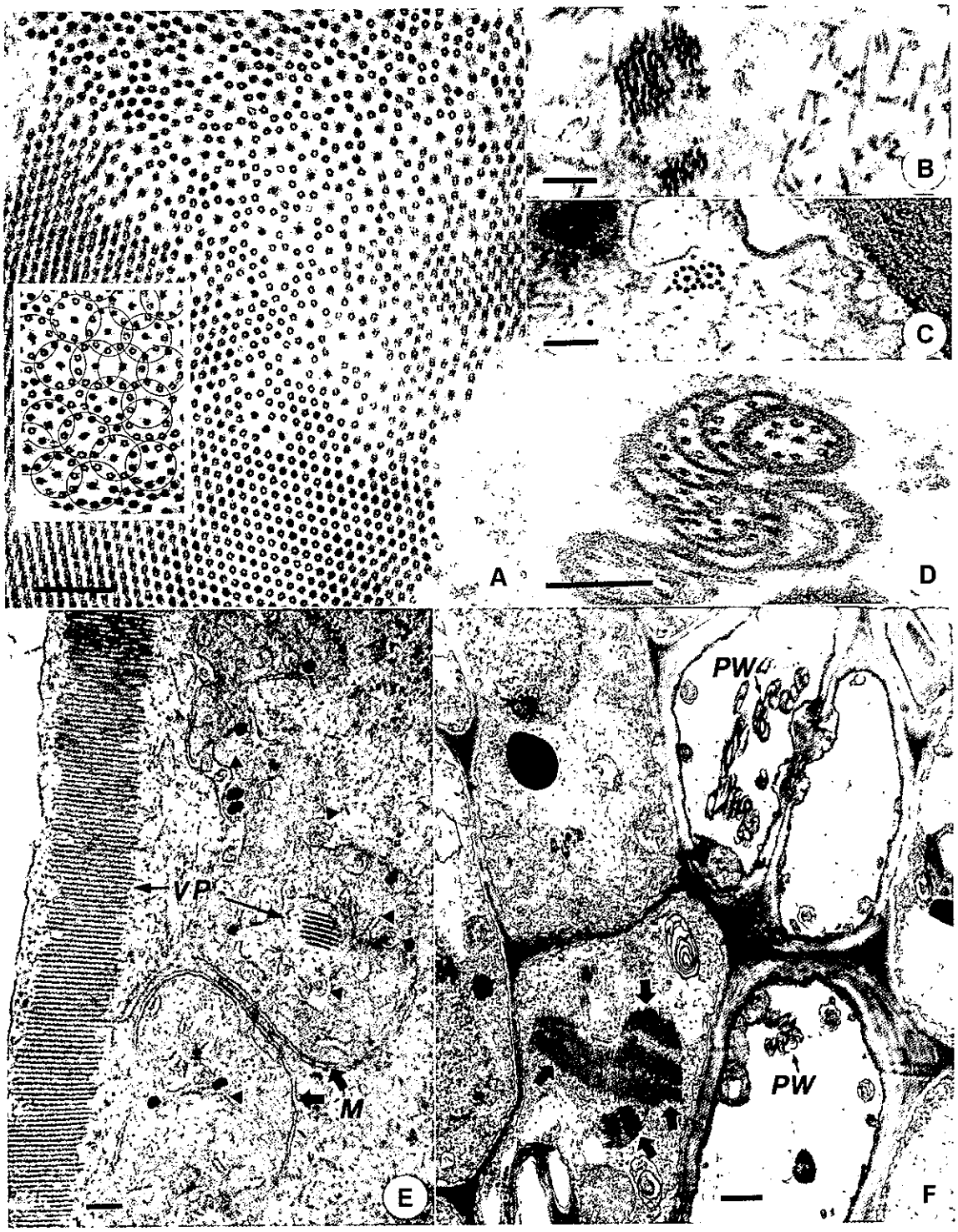

Fig. 2. Two different viruses of CGMMV-W and WMV-W made a unique ring structure of nonagon that a WMV-W particle was surrounded by the evenly spaced 9 CGMMV-W particles. The nonagons formed in crystal of CGMMV (A), and they shared two tobamovirus particles showing canal with adjacent nonagons (Rectangle in A). The nonagons sectioned obliquely formed in vacuole (B). The two nonagons sectioned crossly well in cytosol shared two tobamovirus particles each other (C). The scroll inclusion induced only by potyvirus had also nonagon inner part of the inclusion (D). Membranes of mitochondria (M) were vesiculated severely (Arrowhead) and had virus particles (VP) of CGMMV-W (E). Phloem parenchyma cells had the nonagons sectioned longitudinally (Arrow), and the pinwheels (PW) of potyvirus existed abundantly in sieve tubes (F). Black bars represent 100 nm in A to E and 500 nm in F.

virus particle of WMV-W was surrounded by the evenly spaced 9 CGMMV-W particles. The nonagon located in crystal of CGMMV-W particles (Fig. 2A), vacuole (Fig. 2B), cytosol (Fig. 2C) and inner part of cylindrical potyvirus inclusions (Fig. 2D). The nonagons shared two CGMMV-W particles between adjacent rings that figured in rectangle photo in Fig. 2A. In mixedly infected cells, two different viruses were present together in all kinds of the cells.

The cross-sectioned virus particles of CGMMV-W located much more in the mitochondria of double infection than in those of single infection. Vesiculation of mitochondrial membrane was also occurred severely (Fig. 2E). The potyvirus inclusions were not common generally in sieve tubes of single infection, however, in mixed infection, the inclusions of pinwheel and scroll easily could be seen (Fig. 2F). The high concentration of potyvirus inclusions in sieve tubes revealed that virions were heavily increased by mixed infection.

Discussion

Some of the devastating virus-caused diseases resulted from mixed infections due to synergistic interactions between viruses involved. Many studies have been conducted to measure some changes in virus synthesis, localization, composition and metabolism by mixed infection, however, relatively little is known about the mechanism of synergism.

Cytopathological changes were observed in this study when the unrelated two viruses were coinfected in their hosts, especially the nonagon of ring structure is unique. No such virus-induced inclusions have been demonstrated to occur in any other plant virus infections. The rings of nonagon occurred at various locations in a cell, including in large crystalline arrays of CGMMV-W particles where the rings become the members of crystalline arrangement, randomly isolated in the cytoplasmic matrixes in the central vacuole and near or in the round plates of WMV-W inclusion. It is apparent that the ring type was formed by the interactions between the particles of CGMMV-W and WMV-W. This suggests that the rings are formed by the two different virus particles are attracted favorably each other. The two viruses of rod shaped particles apparently attracted by the long axis since rings arranged parallel each virus particles. However, there is a definite pattern in the attraction, in that a central single WMV-W particle attracts 9 CGMMV-W particles *vice versa* in a circular manner maintaining uniform distance. What causes the attraction of these two types of rod particles and what means the regular 1+9 arrangement are not known.

Mixed infection of cowpea mosaic comovirus (CPMV) and bean yellow mosaic potyvirus (BYMV) caused a specifically arranged particle aggregate as a result of an interaction between the particles of these two viruses. In that, six isometric comovirus particles surrounded a single particle of BYMV flexuous rod resulted in the formation of hexagonal structures (Carr and Kim, 1983). Mixed infection of cucumber mosaic cucumovirus (CMV) and blackeye cowpea mosaic potyvirus (BlCMV) caused a devastatingly severe disease in cowpea, the cowpea stunt, due to a synergistic interaction between the two viruses induced also an orderly arranged virion aggregates (Pio-Ribeiro et al., 1978; Anderson et al., 1994). In this case, a single CMV icosahedron was surrounded by eight BCMV flexuous rods, which appeared therefore as octagons.

These three specific ultrastructures of mixed virus particle aggregate indicated that the potyvirus was involved. Because these mixed infections results in severe synergistic effects, there may be a significant correlation between such an orderly arrangement of virus particles and synergistic interactions. The occurrence of these virus aggregates also arouses a question that the unique virus mixture could be an ultrastructural manifestation of phenotypic mixing, genomic masking, or both of which have been shown to be common phenomena when two distinct viruses are present in a single host.

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