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Simultaneous Detection of Three Tobamoviruses in Cucurbits by Rapid Immunofilter Paper Assay

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A multi-rapid immunofilter paper assay (multi-RIPA) system was prepared for simultaneous diagnosis of three Tobamoviruses, *Cucumber green mottle mosaic virus* (CGMMV), *Kyuri green mottle mosaic virus* (KGMMV), and *Zucchini green mottle mosaic virus* (ZGMMV) in cucurbitaceous crops. Each of these viruses was specifically detected by the multi-RIPA from cucumber, watermelon, zucchini, and bottle gourd inoculated with the three Tobamoviruses singly or in combination. The three viruses could infect cucumber, watermelon, and bottle gourd; however, CGMMV could not infect zucchini as the latex-coated CGMMV antibody showed a negative reaction in the multi-RIPA of the virus-infected plant extract. When the minimum detection level of multi-RIPA was compared with that of double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) using CGMMV, the latter was 10 times more sensitive than the former. The detection limit of the multi-RIPA for the purified CGMMV was 50 ng/ml. In a survey of the three viruses in cucurbits growing in commercial fields in 1999 and 2000, CGMMV was detected in watermelon and cucumber, and ZGMMV was detected only in zucchini growing in plastic houses at the suburbs of Chonju, Korea. However, KGMMV was not found in the commercially growing cucurbit crops in our study. The results suggest that the multi-RIPA can be a simple, rapid, specific and convenient tool to detect simultaneously the Tobamoviruses.

Keywords **:** multi-RIPA, cucurbit crops, CGMMV, ZGMMV, KGMMV, Tobamovirus.

A number of serological techniques are commonly used for the detection and identification of plant viruses (Clark and Adams, 1977; Flegg and Clark, 1979; Francki et al., 1986; Matthews, 1993). Serological diagnosis of viral diseases is commonly conducted by enzyme-linked immunosorbent assay (ELISA), Ouchterlony immunodiffusion test and immunoelectron microscopy. These methods are not suitable for diagnosis in crop-growing fields because they require special equipments and relatively long time for field diagnosis. Tsuda et al. (1992) developed a rapid immunofilter paper assay (RIPA) that could easily be used for the detection of *Cucumber mosaic virus* (CMV) or *Tobacco mosaic virus* (TMV) with considerably high sensitivity and without any specialized equipments. Tanaka et al. (1997) conducted the RIPA for studies of the occurrence and distribution of *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) in cultivated orchids.

In this work, we conducted a multi-RIPA for detection of Tobamoviruses, *Cucumber green mottle mosaic virus* (CGMMV), *Kyuri green mottle mosaic virus* (KGMMV), and *Zucchini green mottle mosaic virus* (ZGMMV), from cucurbit crops in Korea. The technique revealed a convenient tool for diagnosis of these viruses in the crops growing in the fields.

Materials and Methods

Virus sources and maintenance. CGMMV and ZGMMV (Ryu et al., 2000) were isolated from watermelon and zucchini plants, respectively, showing mosaic symptoms. KGMMV (Francki et al., 1986) was provided from Dr. Ryu, K. H. (Plant Virus GenBank, Seoul Women's University, Korea). CGMMV, KGMMV, and ZGMMV were maintained in bottle gourd, cucumber, and zucchini by mechanical inoculation, respectively.

Preparation of antibodies. Purifications of CGMMV, KGMMV, and ZGMMV were performed according to the conventional method (Gooding and Hebert, 1967). Each of polyclonal antisera to the three viruses was prepared by intramuscular injection of the purified viruses to rabbit four times. The immunoglobulins were purified using immobilized protein A/G affinity column (Pierce Co.).

Preparation of multi-RIPA. A multi-RIPA was prepared for simultaneous detection of Tobamoviruses in cucurbit crops. Three kinds of white latex solutions coated with each of the antibodies to ZGMMV, KGMMV, and CGMMV were applied approximately 1.7 cm, 1.5 cm, and 1.2 cm above the bottom end of 8 × 0.5 cm glass filter paper strips (Whatman GA/A) overlaid with plastic sheet, respectively. Then, the filter paper strips were placed into a plastic box after air drying. Three kinds of dyed latex solutions coated with each virus antibody were prepared according to the

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procedures described by Tsuda et al. (1992). The filter paper strips and the dyed latex solutions coated with antibody were stored at 4°C until use.

Detection of the viruses by multi-RIPA. CGMMV, KGMMV, and ZGMMV were mechanically inoculated singly or in combination into the seedlings of watermelon, cucumber, bottle gourd, and zucchini. Detection of the viruses was investigated using multi-RIPA and electron microscopy 15 days after inoculation. Two leaf disks (9 mm in diameter) of cucurbit crops collected in an 1.5 ml microcentrifuge tube were homogenized in 500 µl of extraction buffer (0.1 M phosphate buffer, pH 7.0, with 0.1% mercaptoethanol, 0.01 M EDTA, 0.15% PVP, and 0.1% BSA) using a toothpick. The prepared filter paper strip was wetted with leaf extract by dipping its lower end for 2 min. After cutting off the dipped end of the strip with a pair of scissors, the strip was again dipped into 300 µl of a mixture of dyed latex solutions coated with each virus antibody for about 8 min. In case a blue band was shown on the zone pre-treated with white latex solutions on the strip, the reactions were considered positive.

Comparison of RIPA and ELISA. ELISA was conducted according to the procedure described by Clark and Adams (1977) using purified CGMMV, sap of virus-infected bottle gourd, and its antibody. Absorbance (OD_{405nm}) of microplate wells was measured with a microplate reader (Tecan Spectra) to detect the antigen-antibody reaction.

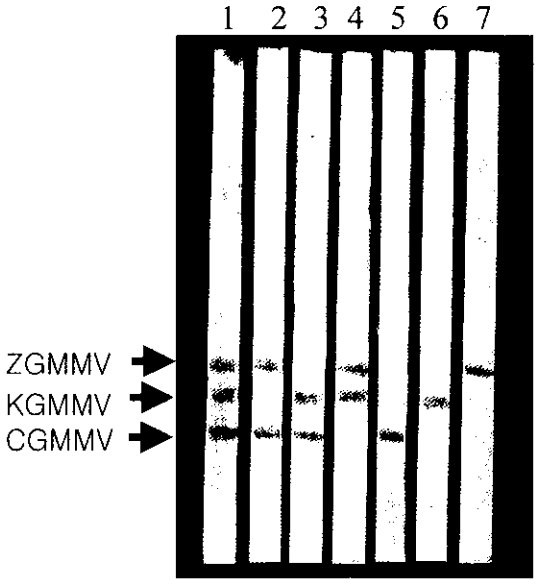

Fig. 1. Detection of CGMMV, KGMMV and ZGMMV from cucurbit crops mechanically inoculated with single or mixed viruses by multi-RIPA. Color bands on the strips show positive reactions. Lane 1, cucumber inoculated with CGMMV, KGMMV and ZGMMV; lane 2, watermelon inoculated with CGMMV and ZGMMV; lane 3, bottle gourd inoculated with CGMMV and KGMMV; lane 4, zucchini inoculated with CGMMV, KGMMV and ZGMMV; lane 5, cucumber inoculated with CGMMV; lane 6, zucchini inoculated with KGMMV; lane 7, zucchini inoculated with ZGMMV.

Results and Discussion

Detection of viruses in mechanically inoculated cucurbit crops. Color bands were formed on the multi-immunofilter paper strips as a result of antigen-antibody reactions, each of which was specific to the respective virus regardless of the plants inoculated (Fig. 1). In cucumber, watermelon, and bottle gourd mix-inoculated with the three Tobamoviruses, three bands corresponding to their antisera appeared simultaneously on the multi-immunofilter paper strips. However, in zucchini KGMMV and ZGMMV were detected from the mix-inoculated plant sap, but CGMMV was not detected either single CGMMV inoculation or mixed inoculation with other viruses (Fig. 1. Lane 4, Table 1). Also CGMMV particles were not detected by electron

Table 2. Comparison of multi-RIPA with DAS-ELISA for diagnosis of Tobamoviruses infecting cucurbit crops[a]

Characteristics	DAS-ELISA	multi-RIPA
Sensitivity		
Purified virus	5 ng/ml	50 ng/ml
Sap dilution	2^{-12}	2^{-11}
Condition for diagnosis		
Time required	6-18 h	5-10 min
Process	Simple	Simpler
Machinery	Reader etc.	None
Place	Lab.	Lab. & field

[a] Tested using CGMMV, ZGMMV and KGMMV

Table 1. Detection of CGMMV, KGMMV and ZGMMV from cucurbits inoculated mechanically with single or mixed viruses by multi-RIPA

Inoculum	Cucumber			Watermelon			Bottle gourd			Zucchini		
	C	K	Z	C	K	Z	C	K	Z	C	K	Z
CGMMV(C)	+[a]	−	−	+	−	−	+	−	−	−	−	−
KGMMV(K)	−	+	−	−	+	−	−	+	−	−	+	−
ZGMMV(Z)	−	−	+	−	−	+	−	−	+	−	−	+
C+K+Z	+	+	+	+	+	+	+	+	+	−	+	+

[a] + : positive, − : negative.

Table 3. Detection of CGMMV, ZGMMV, and KGMMV from cultivated cucurbit crops by multi-RIPA from 1999 to 2000

Cucurbits	No. of samples[a]	No. of plants infected with the viruses			
		CGMMV	ZGMMV	KGMMV	Mixed infection
Cucumber	186	48	0	0	0
Watermelon	265	90	0	0	0
Zucchini	94	0	87	0	0

[a]Plants showing virus-like symptoms were examined using multi-RIPA.

microscopy in the sap of upper leaves of zucchini mechanically inoculated with CGMMV. The results were corresponding to Horvath's report (1995) that CGMMV was not infecting zucchini (*Cucurbita pepo*). In this respect, the multi-RIPA may be an efficient way for specific diagnosis of three Tobamoviruses infecting cucurbitaceous crops.

Comparison of RIPA with DAS-ELISA. The reliability and efficiency of RIPA were compared with DAS-ELISA (Table 2). In DAS-ELISA, positive reactions were detected at 5 ng/ml for the purified CGMMV and at 2^{-12} dilution for the sap of bottle guard infected with the virus, while in RIPA at 50 ng/ml and at 2^{-11} dilution, respectively. DAS-ELISA was about ten times more sensitive than RIPA in detecting the virus. Tsuda et al. (1992) reported that the sensitivity of RIPA was almost the same as that of ELISA in the detection of CMV. ELISA has been used widely in the diagnosis of viruses, and known to be very convenient and practical especially for routine tests of a large number of samples. However, ELISA requires laboratory equipments including an ELISA reader and is time-consuming, requiring 6 to 18 h for the assay. On the other hand, RIPA needs only minimum tools and very simple moreover it takes no more than 10 min to observe the assay result with the naked eye. These results were similar to those of previous reports for the detection of TMV, CMV, CymMV and ORSV (Tanaka et al., 1997; Tsuda, et al., 1992).

Occurrence of the viruses in cucurbit crops growing in fields. Occurrence of CGMMV, ZGMMV and KGMMV was surveyed in cucumber, watermelon and zucchini plants cultivated for commercial purpose in 1999 and 2000 by multi-RIPA in field or laboratory conditions. The results are shown in Table 3. CGMMV was detected in 138 out of the 545 cucurbit plants showing virus-like symptoms. However, CGMMV was not detected from zucchini. ZGMMV was observed only in zucchini fields at the suburbs of Chonju. KGMMV was not found in all of the samples collected from the fields investigated. Lee et al. (2000) reported the occurrence of KGMMV in zucchini grown in the same location by nucleotide sequence analysis. Comparison of coat protein sequences between KGMMV-Y (Tan et al., 2000) and KGMMV-Z (Lee et al., 2000) revealed 78.4% and 77.6% identities at the nucleotide and amino acid sequences, respectively. However, the coat protein gene of the KGMMV-Z reported by Lee et al. (2000) shares over 98% homology with the ZGMMV, suggesting the two viruses are the same species in the genus *Tobamovirus*. Therefore, we suggest that KGMMV-Z (Lee et al., 2000) may be revised as ZGMMV for the gene sequences homology (Ryu et al., 2000) and serological specificity among the viruses using the multi-RIPA in this experiments.

In conclusion, the multi-RIPA is a simple and rapid diagnostic method for the three Tobamoviruses, CGMMV, ZGMMV and KGMMV, occurring in cucurbit crops, which can be utilized for screening of virus-free plants in seed production as well as virus survey in fields for commercial production.

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