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Construction of Antibodies for Detection and Diagnosis of *Cucumber green mottle mosaic virus* from Watermelon Plants

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We immunized BALB/c mice with purified *Cucumber green mottle mosaic virus* isolate HY1 (CGMMV-HY1). Through the selection of positive clones that were grown on the HAT medium, four sensitive monoclonal clones (CG99-01, CG99-02, CG99-03, and CG99-04) were selected from 500 Hypoxanthine-guanine phosphoribosyltransferase positive hybridoma cells. Four sensitive clones of CGMMV-HY1 were determined as IgM type of the subclass of mouse immunoglobulins Ig group. The titer of monoclonal antiserum against CGMMV-HY1 was estimated 1:12,800 by the indirect ELISA. Although monoclonal antibodies (MAbs) from CG99-01 and from CG99-04 cross-reacted with *Zucchini green mottle mosaic virus* and *Kyuri green mottle mosaic virus*, MAb from the cell line CG99-03 was highly specific to CGMMV. No MAbs cross-reacted with *Cucumber mosaic virus*-Fny. Only CG99-04 reacted with *Pepper mild mottle virus* weakly and CG99-02 reacted with both CGMMV and KGMMV. CGMMV was detected from the rind of watermelon fruit by DAS-ELISA of CGMMV-HY1, but not from the flesh of watermelon. Average seed transmission rate of CGMMV in watermelon was 24% from symptomatic watermelon collected from 5 regions of Gyeongnam province. CGMMV was detected by DAS-ELISA with specific MAb of CGMMV-HY1 periodically from root stock, during the sequential process for nursery seedling in Haman. Necrotic spots on cotyledons of root stock seedling progressed to reveal the typical symptomatology on the primary leaves of scion upon grafting. Here, we have established MAb based ELISA system, which could accurately detect CGMMV from watermelon seeds, nursery seedlings, transplants and field samples from greenhouse or open out door field as well.

Keywords : BALB/c mice, CGMMV-HY1, DAS-ELISA, MAb & PAb

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Among several methods for identifying viruses, serological techniques are frequently favored because of their accuracy and efficiency. However, conventional serological techniques can not be used effectively for certain viruses because of limitations such as low virus concentrations, the presence of virus inactivators or inhibitors in tissue extracts. The microplate method of enzyme-linked immunosorbent assay (ELISA) helps largely overcome these limitations (Clark and Adams, 1977; Cochet et al., 2000).

Clark and Adams (1977) found that the double antibody sandwich form of ELISA (DAS-ELISA) was suitable for plant viruses. The most significant characteristic of ELISA is the ability to detect plant pathogens occurring in much lower concentrations than is possible by classical immunoprecipitation methods. It is also more strain-specific and enables quantitive analysis (Voller et al., 1979). Thus, ELISA is frequently used for testing field samples of large scale, for example, survey of virus disease or epidemiological investigations. Im et al. (1991) attempted to monitor pepper viruses by DAS-ELISA. Park and Cha (2002) attempted to use polyclonal antibody based ELISA system to detect *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV) and *Cucumber mosaic virus* (CMV) from Tomato seed and plants.

Recently, Ko et al. (2004) monitored the virus diseases of *Cucumber green mottle mosaic virus* (CGMMV), *Watermelon mosaic virus* (WMV), and *Zucchini yellow mosaic virus* (ZYMV) associated with watermelon cultivation in Jeonnam province by RT-PCR analysis from early growth stage to later fruiting stage. ELISA is often preferred over PCR because PCR test is costly and time-consuming for testing large number of samples.

Lee et al. (1990) first reported occurrence of CGMMV on watermelon in Korea in 1989, which caused epidemic in watermelon field resulting in what is called 'blood flesh' of fruit and considerable economic damage in 1995 (Lee, 1996). According to Choi (2001), CGMMV occurred in Korea in 463 ha in 1998, 17.3 ha in 1999, and 27.2 ha in

2000. Previously, we reported that CGMMV-HY1 causing mottle mosaic, mosaic, growth stunting, necrosis and deformed fruit on the watermelon plants in greenhouse from Gyeongnam province (Shim et al., 2005).

In this study, we have constructed the monoclonal antibodies raised against CGMMV-HY1 and screened for their specificity to tobamoviruses via indirect ELISA. We also validated the efficiency of monoclonal antibody based DAS-ELISA system, for detecting viruses from watermelon seed and fruits, rind and internal discolored and decomposed fruit flesh (IDD), root stock, nursery seedlings, and whole transplants from greenhouse and/or from the field samples.

Materials and Methods

Preparation of antigen. The CGMMV-HY1 obtained from watermelon leaves with severe mottle mosaic and isolated from single local lesion from *Chenopodium amaranticolor* was maintained in cucumber and purified as previously reported (Shim et al., 2005). The purified CGMMV-HY1 particles were used as antigens for raising polyclonal (PAbs) and monoclonal antibodies (MAbs), respectively.

Construction of antibody. In preparation for the first injection to construct a PAb and MAb, a 400 μl of purified CGMMV-HY1, (5-10 μg for each rabbits and mouse) was mixed with an equal volume of Freund's complete adjuvant (Gibco BRL, USA) to make emulsion by 3-way stopcock. The New Zealand white rabbits and BALB/c mice were immunized by injecting three times at 2 weeks intervals, respectively, with a 700 μl and 400 μl of antigen-adjuvant mixtures.

Immune status was checked by a test bleed from ear vein of rabbit for PAb, three days after second boosting injection. By indirect ELISA method, the titer of CGMMV-HY1 antisera was determined to be suitable when OD value of antisera was higher by 0.2 over that of negative control at the 490 nm wavelength. For production of MAb, three days after 2nd boosting injection, the titer of mice antisera in a test bleed from tail of mouse was determined; those that yield higher OD_{490} 0.2 over that of negative control were used for cell fusion. Three days after final boosting, 5-10 μg of antigen of CGMMV-HY1 without adjuvant, the mice splenocytes were fused with V653 myeloma cells (P3X63-Ag8.V653) using poly ethylene glycol (PEG 1500). Hybridoma cell lines were selected by HAT selective medium [100 μl 20% DMEM, 1 μl HAT (hypoxanthine-aminopterin-thymidine, Gibco BRL, USA)]. Hybridoma cell-culture supernatants were screened for the presence of specific antibodies by indirect-ELISA assay.

Indirect-ELISA. Indirect ELISA was performed using purified virus as antigen-coated plates as described by Koenig (1981). The purified CGMMV-HY1 particles were diluted in 0.05M sodium carbonate buffer (62 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.0), and 96 well ELISA plates were coated with 50 μl of diluted CGMMV-HY1 particles and incubated at 4°C overnight or 2 hrs at 37°C. The plates were washed three times with PBST (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.05% Tween 20, pH 7.4), and blocked with 1% skim milk for 30 minutes at room temperature. After washing three times with PBST, the hybidoma supernatants were diluted in 1% bovine serum albumin (BSA) and were dispensed. The 96 well plates were then incubated at 37°C for 2 hrs. The plates were washed three times with PBST, then incubated at 37°C for 1 hr with secondary antibody (Sigma A0412, goat anti-mouse polyvalent IgG, IgA, IgM, peroxidase conjugate) diluted 1:10,000 in 1% BSA in PBS. After five washings, 50 μl substrate solution (*o*-phenylenediamine 4 mg/5 ml PCB 0.1 M phosphate-citrate, 10 μl H_2O_2) was added per well, after 30 min the reaction was stopped by adding 50 μl of 2 N H_2SO_4 in 15 min, followed by OD reading in ELISA reader (BIO-RAD, Model 550) at 490 nm. A threshold value for positive samples was set at three times the value of the uninfected control.

Determination of Ig subclass. The Ig subclass of MAb from selected cell line was determined with a commercial mouse MAb isotyping kit according to the manufacturer's instruction (Sigma, ISO-1, USA).

Specificity and cross reactivity of monoclonal antibodies. Reference strains (CGMMV-W, ZGMMV, KGMMV, CMV-Fny, PMMoV) were kindly supplied by the Plant Virus GenBank (PVGB) of Seoul Women's University, Seoul, Korea. To determine the serological relationships of the four tobamoviruses (CGMMV, ZGMMV, KGMMV, and PMMoV) and a cucumovirus (CMV-Fny), indirect ELISA was carried out with four hybridoma cell lines secreting monoclonal antibody against CGMMV including CG99-01, CG99-02, CG99-03, and CG99-04.

DAS-ELISA for CGMMV detection and diagnosis. A DAS-ELISA was performed for detection of the CGMMV as described by Adkins et al. (2003). CGMMV-HY1 specific Monoclonal antibody (CG99-03) was diluted in 0.05 M sodium carbonate buffer by 1:10,000 and was dispensed in 96 well plates with 50 μl and incubated at 4°C over night, or at 37°C for 1hr. Coated wells were washed three times with PBST and were blocked with 1% skim milk in PBS for 30 min at room temperature and negative controls in all ELISA assays included sample buffer only.

From watermelon seed. Total seeds from each fruit were harvested and air dried at room temperature for one week. Single seed was individually homogenized in PBS buffer and centrifuged at 12,000 rpm at 4°C for 5 min. The supernatants of homogenized seed tissues were diluted in 1% BSA and dispensed 100 μl per well and incubated at 37°C for 1 hr.

From the fruit rind, IDD, and flesh tissue adjacent to seed. As shown in Fig. 4, fruit tissue was cut out with 5 mm cork-borer sequentially from rind to core, designated 1-9 for a, b, c and 1-3, for d, e, f, respectively. Mid-portion represent the internal discolored and decomposed tissue (IDD) and flesh tissue adjacent to seedset. Each sample were individually subjected the procedure for ELISA as above.

From root stock, nursery seedling and transplant. For etiological study, three leaf disks, 0.5 cm in diameters, were homogenized, in PBS and centrifuged at 12,000 rpm at 4°C for 5 min. tissues supernatants were diluted in 1% BSA in PBS and dispensed 100 μl per well and incubate at 37°C for 1hr. Over the period of 2003-2004, hundreds of commercial field samples from 8 location of southern horticulture area were also subjected to above-mentioned process to monitor CGMMV infection to determine accuracy and efficiency of ELISA kit.

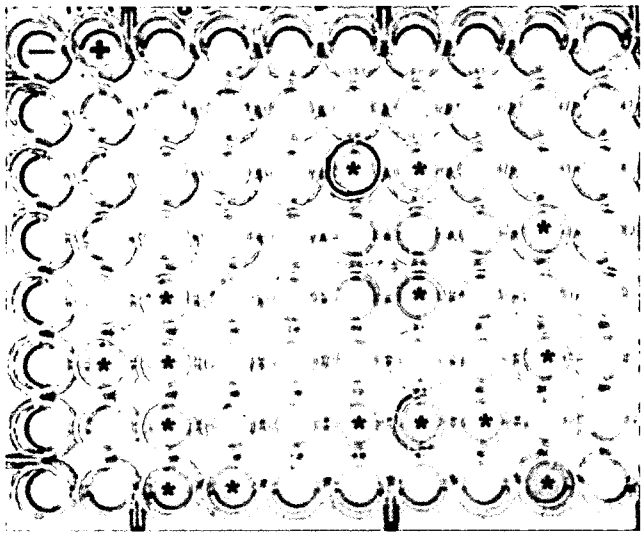

Fig. 1. Screening of specific hybridoma cells by indirect ELISA against CGMMV-HY1. Tentatively selected 480 clones were subjected to three more cycles of cloning for high specificity and hybridoma cell proliferation. Plus (+) and minus (−) signs represent positive and negative control, respectively, while asterisk (*) represents specific positive clones of CGMMV-HY1. Four clones (CG99-1, CG99-2, CG99-3, and CG99-4) were selected for further analysis.

Results

Construction of polyclonal antibody and monoclonal antibody. Four sensitive monoclonal clones (CG99-01, CG99-02, CG99-3, and CG99-04) were selected from 480 HGPRT positive hybridoma cells (Fig. 1). The titer of polyclonal antiserum and monoclonal antisera of CGMMV-HY1 were estimated 1:25,600 and 1:12,800 respectively, by the indirect ELISA against CGMMV-HY1. This result suggested that titers of polyclonal and monoclonal antibodies were higher than those reported by other researchers (Lee et al., 1990) and thus should be sufficient to detect virus.

Determination of Ig subclass and specificity of MAbs. Immunoglobulins are grouped into classes or isotypes according to differences in their heavy chain or light chain constant domains. To determine the immunotypes, MAbs diluted in 100X PBS were subjected to the strips, precoated individually with antimouse Ig subclasses, *i.e.*, IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. Four sensitive hybridoma clones of CGMMV-HY1 belonged to IgM types of the subclass of mouse immunoglobulins Ig group by Mouse Monoclonal Isotyping Kit (Sigma, ISO-1, USA) (Fig. 2).

To determine the serological relationships of the four tobamoviruses (CGMMV, ZGMMV, KGMMV, and PMMoV) and a cucumovirus (CMV-Fny), indirect ELISA was carried out with four hybridoma cell lines secreting monoclonal antibody against CGMMV, such as CG99-01, CG99-02, CG99-03, and CG99-04. MAbs from CG99-01 and from CG99-04 cross-reacted with ZGMMV and KGMMV. MAb from the cell line CG99-03 was highly specific to CGMMV discriminating other viruses tested. No MAbs were cross-

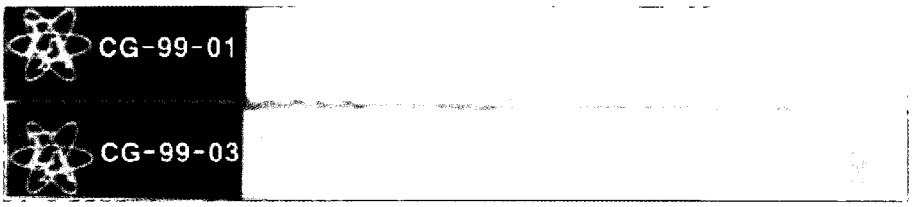

Fig. 2. Determination of subclass of mouse immunoglobulin Ig group for CGMMV-HY1 monoclonal antibody by mouse monoclonal isotyping kit (Sigma, ISO-1, USA). CG-99-01 and CG-99-03 clone were determined IgM type. The red cross represents quality control of Mouse Monoclonal Isotyping Kit.

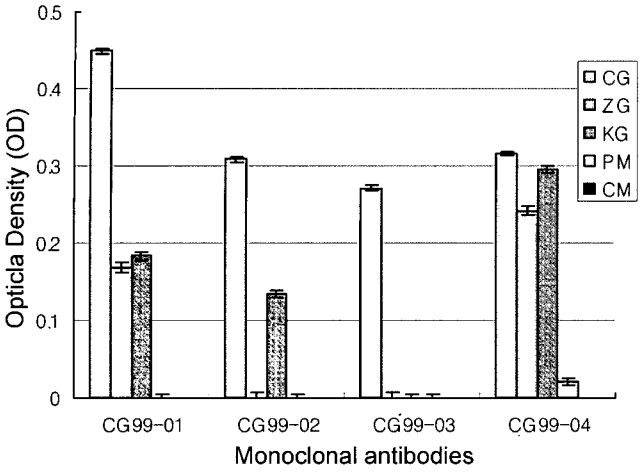

Fig. 3. Cross reactivity of CGMMV specific monoclonal antibodies for reference virus strains such as tobamovirus (ZGMMV, KGMMV, and PMMoV), and cucumovirus (CMV-Fny) by indirect ELISA method which was colonized with *o*-phenylenediamine (4 mg/5 ml) in 0.1 M phosphate-citrate buffer (PCB). Cloned hybridoma cell lines secreting monoclonal antibodies against CGMMV: CG99-01, CG99-02, CG99-03, and CG99-04. CG, CGMMV; ZG, ZGMMV; KG, KGMMV; PM, PMMoV; CM, CMV-Fny.

reacted with CMV-Fny. Only MAb from the cell line CG99-04 reacted with PMMoV weakly and CG99-02 reacted with both CGMMV and KGMMV (Fig. 3).

Localization in fruit tissue and etiology of CGMMV. CGMMV were easily detected from the rind of watermelon fruit by DAS-ELISA of CGMMV-HY1, but not from the flesh of watermelon (Fig. 4). Average seed transmission rate for watermelon was 24% (Table 1). CGMMV was detected by DAS-ELISA with specific monoclonal antibody of CGMMV-HY1 periodically from root stock, during the sequential process for nursery seedling in Haman. Root stock seedling with necrotic spot on cotyledon progressively revealed typical symptomatology on grafted nursery seedlings (Fig. 5). Over the years from, 2003 to 2004, about 360 samples were collected from 8 major watermelon cultivation areas in Gyeongnam province and subjected to DAS ELISA kits, with dependable results for monitoring virus incidences. Our DAS-ELISA kit was useful to detect and identify the CGMMV from commercial field samples efficiently within a short period of time, compared to other techniques (Fig. 6).

Table 1. Seed transmission rates of CGMMV from the diseased watermelon fruits in greenhouse

Sample fruits	Location	No. of seeds/fruit	No. of seeds carrying the virus (%)
WM1	Hadong	232	46 (20)
WM2	Hadong	320	73 (23)
WM3	Changwon	250	70 (28)
WM4	Chngwon	235	58 (25)
WM5	Haman	230	48 (21)
WM6	Haman	245	58 (24)
WM7	Hamyang	223	60 (27)
WM8	Chinju	330	82 (25)
Average		258	62 (24)

Discussion

To establish rapid and accurate detection and identification system, we constructed monoclonal antibody based DAS-ELISA system for CGMMV, by which the epidemiology of

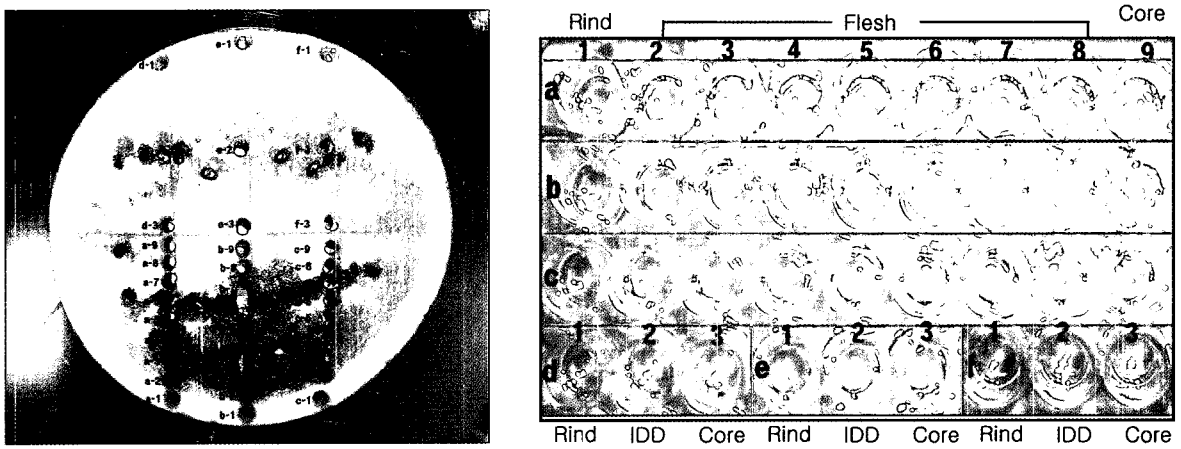

Fig. 4. Detection of CGMMV by DAS-ELISA kit with CGMMV specific monoclonal antibody in fruits (2004). a, b & c represent the cut-out tissue with 5 mm corker-borer sequentially from the rind to core of fruit, designated 1 to 9, respectively; d, e & f represent sampling from rind (1), flesh tissue with internal discolored and decomposed fruit flesh (IDD) (2), and core tissue (3). DAS-ELISA kit was visualized with *o*-phenylenediamine (4 mg/5 ml) in 0.1 M phosphate-citrate buffer (PCB).

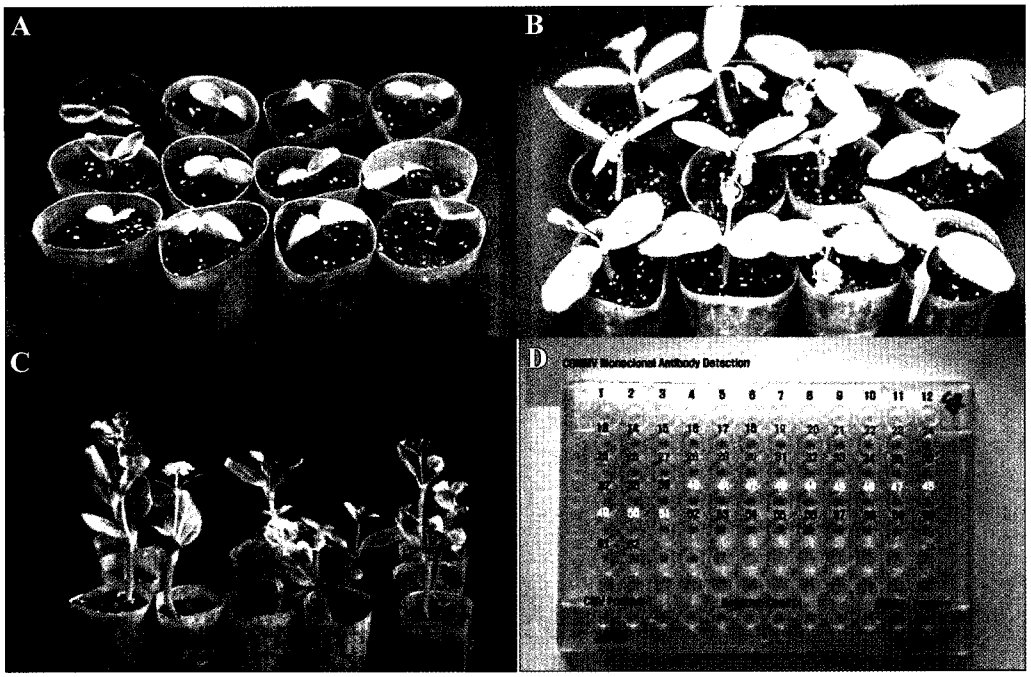

Fig. 5. CGMMV was detected by DAS-ELISA with specific monoclonal antibody of CGMMV-HY1 from root stock of the FR line of Bottleguard nursery seedling in Haman (2003); A, Root stock f the FR line of Bottleguard with necrotic spots on the cotyledon 7 days old before grafting; B & C, 7 days or 10 days old grafted watermelon seedlings with necrotic mottle mosaic on primary leaves; D, 15 days old seedlings with leaf mottling and distortion with stunted growth; D, visualized with *o*-phenylenediamine (4 mg/5 ml) in 0.1 M phosphate-citrate buffer (PCB), where; 1-18, root stock (A); 19-39, 7 days old grafted seedling (B); 40-51, 10 days old grafted seedling (C); 52-62, 15 days old grafted seedling prior to transplanting (D).

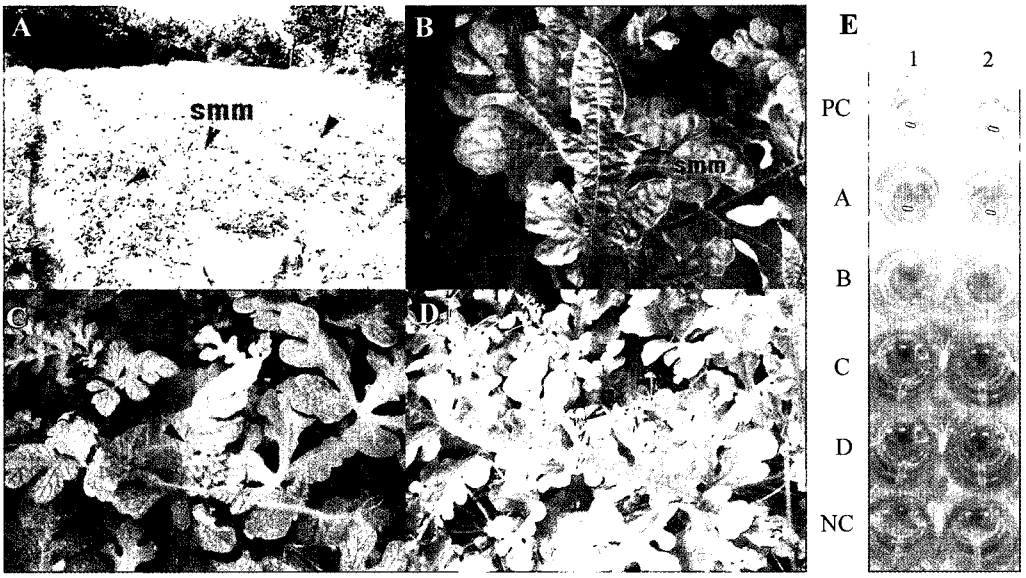

Fig. 6. Detection of CGMMV by DAS-ELISA kit with CGMMV specific monoclonal antibody in watermelon open field in Hamyang. Overview of severe epidemic open field (A), close up view (B, C, and D), and colorization with *o*-phenylenediamine (4 mg/5 ml) in 0.1 M phosphate-citrate buffer (E). PC, positive control; NC, negative control; m, mottle mosaic; smm, severe mottle mosaic; n, necrosis.

CGMMV could be elucidated, as well as preventing influx of exotic plant virus in this country.

Through this study, we have shown that titers of PAb and MAb were estimated to be within the acceptable ranges, 1:25,600 and 1:12,800, respectively as compared to other works (Lee et al., 1990).

This is of interest to note the specificity and cross-reactivity of the MAbs from four clones, such as CG99-01, CG99-02, CG99-03, and CG99-04: Among the tested four MAbs, MAb from the cell line CG99-03 was very specific to CGMMV, while the other three MAbs showed some cross-reactivity to the other viruses tested. Nonetheless, such a diversity of other clones for their specificity and cross-reactivity to tobamoviruses should be of valuable tool to identify the multiple infections.

Four sensitive hybridoma clones of CGMMV-HY1 were immunotyped to be IgM types of the subclass of mouse immunoglobulins Ig group. This should not be of any disadvantage, according to Hsu et al. (2000) who noticed that monoclonal antibodies, whose immunoglobulin class and subclasses were IgM were as good as those of IgG in terms of titer and specificity.

A CGMMV particle was detected to be localized in the rind tissue of watermelon fruit, but not in the fruit flesh of either healthy or internal discolored and decomposed portions (Fig. 4). This result is agreeable to the observation of Lee et al. (1990) who visualized the virus particles being aggregated in the cytoplasm of foliage, and fruit rind tissue by transmission electron microscopy. RT-PCR detection of CGMMV from cucumber fruit revealed that yields of PCR products were higher for fruit wall tissues at basal part toward the stalk and testa end of cucumber fruit then in the middle (Choi et al., 1998).

The rate of seed transmission in this study is was fairly higher compared to that of Hollings et al. (1975) who reported seed transmission rate of about 5%. They also noticed that seed transmission rate for cucumber were usually 8% up to one month after seed harvesting; this rate further decrease to 0.1% in 5 months. Such a difference in seed transmission rate could be attributed to the high sensitivity of our DAS-ELISA system, or to the differences in tolerance of two host plant; watermelon and cucumber. Choi (2001) reported that seed detection rate of CGMMV in bottle gourd seeds tested was 84%, while transmission rate from the virus-contaminated seeds to seedling was 2.0%. Our data would mean the seed borne rate of virus from plants to seed during the fruit development rather than the strict seed transmission rate from seed to plant. This requires further works.

CGMMV was detected by DAS-ELISA with specific monoclonal antibody of CGMMV-HY1 periodically from root stock, during the sequential process for nursery seedling cultivation for transplant production and also from some hundreds of field samples throughout southern horticulture area. This suggests the value of our ELISA kit as a tool to study etiology and epidemiology of CGMMV.

As of 2002, average of greenhouse watermelon in Gyeongnam province represented 2/5 of total acreage of in Korea. The watermelon cultivation is major sources for farm income. Therefore our ELISA kit would be essential to detect the CGMMV and related tobamoviruses (Ryu et al., 2000) at early growth stage of watermelon to prevent the epidemics.

Acknowledgments

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