Detection of Maize Chlorotic Mottle Virus Serotypes by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Two strains of maize chlorotic mottle virus, Kansas (MCMV-K) and Peru (MCMV-P), were compared in double immunodiffusion (DID) and enzyme-linked immunosorbent assay (ELISA) systems against respective antisera (ie, MCMV-K AS and MCMV-P AS) and antiserum prepared to a mixture of both strains (MCMV-K + P AS). In DID, all antisera showed antibody titers of 1/2,048 against both viruses. Homologous reactants spurred over the precipitin line formed against heterologous virus and MCMV-K + P AS against MCMV strains K and P produced bilateral spurs. In ELISA, antisera MCMV-K and -P, but not MCMV-K + P, differentiated homologous from heterologous virus strains. Differences in absorbance values at 405 nm were two to four times higher for homologous than for heterologous reactants, but MCMV-K + P AS produced similar substrate intensity for each virus. Also, results approximating those of MCMV-K + P AS were achieved with an equal mixture of MCMV-K AS and MCMV-P AS (MCMV-mixed AS). The relative sensitivities of DID and ELISA for detecting MCMV were 100 and 0.1 μg/ml, respectively.

Enzyme-linked immunosorbent assay (ELISA) is a sensitive technique for the detection of plant viruses (3,19). This procedure is capable of detecting low-titered and tissue-limited viruses, which would be difficult to assay by conventional means (2). The assay also may be used to differentiate viral serotypes (1,7,16). To investigate the latter application in more detail, with respect to maize chlorotic mottle virus (MCMV), strains from Kansas and Peru afforded a suitable model system. These virus strains were shown in double immunodiffusion tests (DID) to be closely related serologically but not identical (10). In ELISA, differences between these same cultures were even more distinct (16), and this paper presents further ELISA results with MCMV serotypes.

MATERIALS AND METHODS

Source of virus cultures and antiserum. Maize chlorotic mottle virus-Kansas (MCMV-K) and -Peru (MCMV-P), and MCMV-K antiserum (MCMV-K AS) were supplied by C. L. Niblett (Kansas State University, Manhattan). Viruses were increased in corn (*Zea mays* L. inbred line N28Ht), and infected tissues were harvested after 14 days. Virus purification procedures followed those described by Niblett and Paulsen (11) for processing large volumes of tissue extract. Briefly, following polyethylene glycol precipation, virus was sedimented at 78,000 *g* for 90 min and then resuspended in 0.01 M potassium phosphate buffer, pH 7.6, and 0.01 M NaCl; 2 ml/100 g tissue. Insoluble material was removed by low-speed centrifugation (9,000 for 10 min) and, after adjustment to 35% sucrose, the supernatant was zone-electrophoresed (18) in a sucrose gradient made up in the above resuspension buffer.

Preparation, titer determination, and absorption of antisera. Antiserum to the Peru strain and to a mixture of Kansas and Peru strains was prepared in rabbits with purified preparations as follows. Initial injections of 5 mg MCMV in 2.5 ml of Freund's incomplete adjuvant (2:1, v/v) were administered intramuscularly. After 1 wk, a series of three 1-ml intravenous injections was given at 10-day intervals. The virus preparation was mixed (0.9:0.1, v/v) with the antihistamine, Benadryl (diphenhydramine hydrochloride [10 mg/ml; Parke, Davis, and Co., Detroit, MI]) to prevent anaphylactic shock. Antiserum collection began after an additional 10 days; antiserum taken on day 55 was used in this study.

Antibody titers were determined in double immunodiffusion (DID) plates (0.75% Ionagar, 0.85% NaCl, and 0.02% NaN_3, in 0.01 M tris-HCl, pH 7.2) against both purified virus and 14- to 30-day infected and healthy corn tissues. Although the antisera did not react with healthy plant extracts in these tests, all were absorbed with host protein prior to use in ELISA.

Host antigen was prepared as reported by Shepard (13) and combined with antiserum (2:1, v/v); the mixture was incubated overnight at 4 C. Following centrifugation at 78,000 *g* for 90 min, the supernatant was mixed with an equal volume of a saturated $(NH_4)_2SO_4$ solution and incubated 2–4 hr at room temperature. The precipitate was sedimented at 9,000 *g* for 20 min, resuspended in a small volume of PBS (0.01 M sodium-potassium phosphate buffer, pH 7.0, and 0.85% NaCl), and dialyzed against three changes of the same buffer. The dialysate was passed through a 2–3 cm bed of DEAE cellulose (3), and the immunoglobulin fraction was adjusted to 1 mg/ml ($E_{280\ nm}^{mg/ml} = 1.4$).

Preparation for ELISA. Alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO 63178) was conjugated to purified anti-MCMV immunoglobulin with glutaraldehyde (3). Unlabeled and enzyme-labeled immunoglobulin fractions were diluted to 10 μg/ml, respectively, with sodium carbonate buffer, pH 9.6, and PBS buffer, pH 7.0, containing 0.05% Tween®-20, 0.2% ovalbumin, and 2% PVP (polyvinylpyrrolidone, mol wt 44,000); 250 μl/well was placed in microELISA™ (Dynatech Laboratories, Inc.,

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Alexandria, VA 22314) plates (3). The enzyme substrate, *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8, was used at 0.6 mg/ml with 300 µl added to each well and allowed to incubate for 30 min. The reaction was stopped with 50 µl per well of 3 N NaOH.

Test samples included purified virus preparations and crude extracts from infected and healthy corn tissue in PBS buffer, pH 7.0, 0.05% Tween-20, and 2% PVP. All virus concentrations and sap dilutions were replicated twice and serologic tests were repeated two or more times.

RESULTS

During zone electrophoresis, two opalescent bands were found migrating toward the anode. Both bands were strongly reactive in DID-MCMV-K AS serologic tests and were pooled. Average yield of virus was 0.3–0.4 mg/g infected tissue. An $E^{0.1\%}_{260\,nm} = 5.85$ for southern bean mosaic virus (14) was used for MCMV based on similarity in certain physical properties (coat protein and RNA molecular weights) (5) and biological behavior (beetle vectors) (9) of the two serologically unrelated viruses (Uyemoto, *unpublished*).

In homologous and heterologous virus-DID systems, all crude antisera were reactive to 1/2,048 and nonresponsive to normal host antigens. Host antigen-absorbed and ammonium sulfate-fractionated antiserum was reactive to 1/512. Precipitin lines against each virus were produced at purified antigen concentrations as low as 100 µg/ml or at dilutions of infected plant sap of 1/50–100 and the relative intensity of the common precipitin line was similar in appearance. When viral preparations and antiserum (MCMV-K AS or -P AS) were compared in a common well pattern, homologous reactants always produced spurs over heterologous reactants. Use of MCMV-K + P AS resulted in bilateral spurs.

The sensitivity of ELISA was greater than DID. Specific reactions were obtained at antigen concentrations or plant sap dilutions of 1.0–0.1 µg/ml and 1/1,000–2,000, respectively. Furthermore, MCMV antisera clearly differentiated virus isolates and suggested up to a 10-fold increase in relative sensitivity between homologous and heterologous systems (see MCMV-K AS or -P AS results, Tables 1 and 2). Differences in sensitivity were greatest when viral antigens were used at low concentrations (virus preparations at 100 µg/ml or less, or leaf extract dilutions at 1/50 or more). In contrast, use of MCMV-K + P AS gave similar absorbance readings against both virus strains tested at equivalent concentrations. Similarily, an equal mixture of the globulins of MCMV-K AS and -P AS (MCMV-mixed AS, tested at 10 µg/ml containing 5 µg/ml of each globulin AS) yielded a nondifferentiating antiserum resembling MCMV-K + P AS (Table 1).

The reactivity of unlabeled and enzyme-labeled immunoglobulin fractions used in ELISA was tested in microprecipitin (under mineral oil) and DID. The immunoglobulin fractions were diluted in twofold fashion in PBS and viral preparations were adjusted to 0.1 or 0.5 mg/ml. In microprecipitin tests, all unlabeled immunoglobulin fractions were reactive from 1/16 to 1/32 against homologous and heterologous reactants. However, none of the enzyme-labeled MCMV-specific immunoglobulin fractions or unlabeled and enzyme-labeled wheat streak mosaic virus-specific immunoglobulin fractions (J. K. Uyemoto, *unpublished*) reacted with MCMV preparations. In DID, all unlabeled immunoglobulin fractions showed a reactive end-point of 1/64 to viral preparations at 0.5 mg/ml. In contrast, enzyme-labeled MCMV-K AS and -K + P AS, but not MCMV-P AS, reacted 1/8 to 1/16 to both viruses.

DISCUSSION

The increased sensitivity of ELISA compared with DID for the detection of MCMV confirms the findings of other (4,7,15). Specific reactions were realized at MCMV concentrations of 100 (in DID) and 0.1 µg/ml (ELISA), and both testing procedures readily distinguished virus serotypes. Based on the same reactive end-point of each antiserum and immunoglobulin fraction and similarity in intensity of the common precipitin line produced in DID against both viral antigens, the large differences in ELISA values between MCMV-K and -P (at virus concentrations less than 1 mg/ml) suggests that each strain possesses antigenic determinants not present in the other and that these specific determinants contributed heavily in differentiating homologous from heterologous reactants. The remaining determinants are common, highly reactive, and characteristic for the MCMV group.

As suggested by Koenig (7), antibody reactivity may be reduced after enzyme conjugation and this in turn may influence the extent of cross-reactions in heterologous ELISA systems. Because of lowered antibody affinity, similar but nonidentical antigenic determinants such as ones occurring on different serotypes of a virus form relatively weak complexes. Such complexes may be dissociated by dilution (rinsing) in the ELISA procedure resulting in an apparently high strain specificity even in a highly cross-reactive antiserum.

The use of ELISA for identifying virus serotypes previously has been reported for other animal (20) and plant virus systems (7,15). In view of recent applications of ELISA in plant virus epidemiology (6,8), in field diagnosis (12), and as reported here, a potential limitation of using a single source of antiserum with high specificity for routine ELISA-virus diagnosis is indicated. ELISA

TABLE 1. Enzyme-linked immunosorbent assay comparison of the Kansas and Peru strains of maize chlorotic mottle virus antisera (MCMV-K AS and MCMV-P AS, respectively) for differentiating virus isolates in purified preparations[a]

Antiserum[b]	Virus isolate	Virus concentration (µg/ml) 10^3	10^2	10^1	10^0	10^{-1}
MCMV-K	MCMV-K	1.05[c]	0.65	0.51	0.13	0.03
	-P	1.02	0.20	0.12	0.09	0.02
MCMV-P	MCMV-P	1.11	1.07	1.06	0.85	0.16
	-K	1.07	0.58	0.49	0.19	0.04
MCMV-K + P[d]	MCMV-K	1.10	1.07	0.97	0.62	0.30
	-P	1.10	1.03	0.95	0.71	0.32
MCMV-mixed[e]	MCMV-K	1.03	0.84	0.65	0.15	0.05
	-P	1.02	0.83	0.76	0.57	0.13

[a] Virus purified by zone electrophoresis.
[b] Antibody titer at 1/512; anti-MCMV immunoglobulin tested at 10 µg/ml.
[c] Absorbance of reacted substrate at 405 nm; healthy corn extracts gave values of 0.02–0.07 at 1/10 dilution.
[d] Antiserum from a single rabbit immunized with a mixture of MCMV-K and MCMV-P antigens.
[e] MCMV-K AS and MCMV-P AS were mixed (1:1, v/v) and tested at 10 µg/ml.

TABLE 2. Enzyme-linked immunosorbent assay comparison of the Kansas and Peru strains of maize chlorotic mottle virus antisera (MCMV-K AS and MCMV-P AS, respectively) for differentiating virus isolates in plant extracts[a]

Antiserum[b]	Virus isolates	Plant sap dilutions 1/10	1/50	1/100	1/1000	1/2000
MCMV-K	MCMV-K	1.24[c]	0.55	0.20	0.01	0.02
	-P	0.44	0.18	0.06	0.03	···
MCMV-P	MCMV-P	1.26	0.92	0.31	0.08	0.06
	-K	1.07	0.24	0.08	0.03	···
MCMV-K + P[d]	MCMV-K	1.26	1.14	0.58	0.22	0.21
	-P	1.26	0.84	0.45	0.23	···
ALL AS	healthy corn	0.07	0.01	···	···	···

[a] Corn tissue infected for 14 days.
[b] Antibody titer at 1/512; anti-MCMV immunoglobulin tested at 10 µg/ml.
[c] Absorbance of reacted substrate at 405 nm.
[d] Antiserum from a single rabbit immunized with a mixture of MCMV-K and MCMV-P antigens.

would be particularly vulnerable for those virus groups known to contain different serotypes, when highly specific low-titered antiserum is used, or when plant tissue with a heterologous virus is sampled, especially in early stages of infection. Under these conditions, it is likely that only homologous (or antigenically similar) virus isolates would be detected. With the recent detection of a second Kansas MCMV serotype, we are currently using MCMV-K + P AS in our virus surveys (17).

The relative sensitivity of homologous ELISA systems is 5- to 10-fold higher than that of heterologous systems. Thus, if MCMV-K AS is used, MCMV-K at 1–10 μg/ml and MCMV-P greater than 100 μg/ml is required for reliable diagnosis. Conversely, with MCMV-P AS, virus concentrations of 0.1–1.0 (MCMV-P) and 1.0–10.0 μg/ml (MCMV-K) are required. In order to ensure that both serologically close and distantly related virus strains are reliably detected, antiserum prepared against a mixture of serotypes or alternatively, a composite of high-titered antisera is recommended. In this study, both kinds of antisera reacted identically with each virus serotype.

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