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DIFFERENTIATION OF TWO SEROTYPES OF CUCUMBER MOSAIC VIRUS IN JAPAN BY $F(ab')_2$ ELISA WITH CROSS-ABSORBED ANTIBODIES*

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INTRODUCTION

Cucumber mosaic virus (CMV) has a very wide host range including both herbaceous and woody plants and causes economically important diseases of many crops throughout the world. In Japan, CMV is one of the most common viruses occurring on vegetable and ornamental crops. CMV isolates found in Japan are serologically classified into two distinct groups, designated Y and P serotypes[4,11]. The determination of CMV serotypes has been done by a gel double-diffusion test. In this test, purified or partially purified virus preparations are usually used as antigens because this test needs relatively higher concentrations of antigens to develop reactions. Recently, the various forms of enzyme-linked immunosorbent assay (ELISA) have been applied to detect many plant viruses as very sensitive procedures. The application of ELISA for identifying virus serotypes previously has been reported for some plant viruses[2,7,13,17] including CMV[10,12,14]. However, differentiation of CMV serotypes by ELISA seems to be unreliable when crude sap from infected plants or virus samples of unknown concentration are used, as ELISA values for each strain may vary depending on antibody specificities and antigen concentrations used in the tests.

This paper describes the differentiation of two Japanese serotypes of CMV by $F(ab')_2$ ELISA with cross-absorbed antibodies with heterologous strains and its application to serotyping of CMV isolates obtained from Japan.

MATERIALS AND METHODS

1. *Viruses*

The yellow strain of CMV (CMV-Y)[16] and the strain from *Zinnia elegans* (CMV-Z)[11] were used as the standard strains for Y and P

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serotypes [4], respectively. Viruses were propagated in *Nicotiana tabacum* cv. White Burley and were purified by the method previously described[11]. CMV isolates obtained from various plants in Japan were maintained in *N. tabacum* cv. White Burley or in *N. clevelandii* after serial local lesion transfers on *Chenopodium quinoa*.

2. *Production of Antisera*

Antisera to CMV-Y and CMV-Z were prepared in rabbits. Each animal received four intramuscular injections of purified virus (total 10 mg) emulsified with an equal volume of Freund's complete adjuvant at 2-week intervals and was bled at 120 days after the initial injection. Both antisera to CMV-Y and CMV-Z had homologous and heterologous titers of 1 : 256 and 1 : 128, respectively, in double-diffusion tests. Spurs were formed between the precipitin lines of two serotypes in reciprocal combinations of antisera and antigens. The antisera were absorbed with insolubilized healthy tobacco proteins before use[3].

3. *Absorption of Antisera with Heterologous Strains and Preparation of Immunoglobulin*

The antiserum to each serotype was diluted ten fold with phosphate-buffered saline (PBS) and purified heterologous virus was added to the antiserum (5mg of virus/1m*l* of undiluted antiserum). After incubation for 1hr at 37℃, the reaction mixtures were centrifuged at 65,000 $\times g$ for 1.5hr to remove virus-antibody complexes. This absorption was repeated four times. IgG was isolated from the native and the cross-absorbed antisera by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography. $F(ab')_2$ fragments of IgG were prepared by digesting IgG with pepsin (1 : 60,000, Sigma)[1].

4. *$F(ab')_2$ ELISA*

$F(ab')_2$ ELISA was performed essentially as described by Barbara and Clark[1]. Wells of polystyrene microtiter plates (Nunc-Immuno plate U II, Inter Med) were incubated with $F(ab')_2$ (2 μg/m*l*, 180 μ*l*/well) in 0.05M sodium carbonate buffer, pH 9.6, for 3hr at 30℃. Plant extracts were prepared by homogenizing tissues with PBS containing 0.02% sodium azide, 0.05% Tween-20, 2% polyvinylpyrrolidone (mol wt 40,000) and 0.2% bovine serum albumin (PBS/Tween/PVP/BSA). The homogenate was filtered through a filter paper (Whatman No. 2). Purified virus or plant extract at 100-fold dilution in PBS/Tween/PVP/BSA was added to the $F(ab')_2$ coated wells and was then incubated for 16hr at 4℃. After washing the wells, IgG from native or cross-absorbed antiserum was added as detecting antibodies and incubated for 3 hr at 30℃.

Protein A (Nakarai Chem. Co., Kyoto) was conjugated simultaneously with horseradish peroxidase (Type I-C, 264u/mg, RZ : 3. 29, Toyobo Co., Osaka) and BSA at a ratio of 0. 5 : 1 : 0. 75 (w/w) by the periodate oxidation procedure[1]. Following the addition of protein A conjugate diluted 1 : 4, 000, the plates were incubated for 3hr at 30℃ and then the enzyme substrate (0. 5g/*l* o-phenylenediamine in 0. 025M sodium acetate buffer, pH 5. 5, containing 0. 06% hydrogen peroxide)was added. The reaction was stopped by the addition of 50 μl of 3M sulphuric acid to each well after 30min at 30℃ and absorbance of reacted mixture was measured at 490nm.

RESULTS AND DISCUSSION

1. *Reactions of Two Serotypes against Homologous and Heterologous Antibodies in F(ab′)$_2$ ELISA*

Two serotype of CMV were compared in F(ab′)$_2$ ELISA against homologous and heterologous antibodies. Purified CMV-Y and CMV-Z at concentrations of ranging from 0. 01 to 10 μg/m*l* were added to the wells

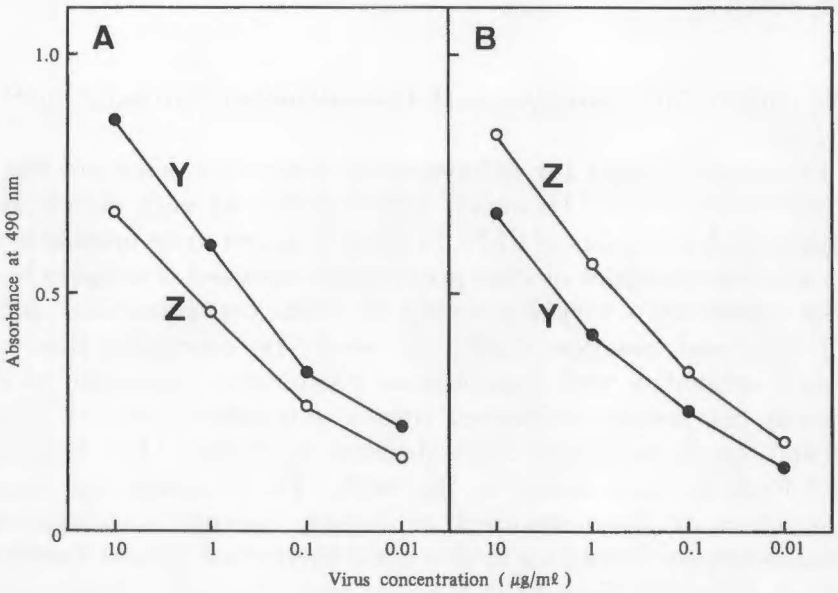

FIG. 1. Comparison of F(ab′)$_2$ ELISA values for two serotypes of CMV in homologous and heterologous systems. Purified CMV-Y(serotype Y) and CMV-Z (serotype P) were added to the wells of microplates precoated with F(ab′)$_2$ (2 μg/m*l*) prepared from antiserum to CMV-Y (A) or CMV-Z (B) and then homologous IgG (2 μg/m*l*) to coated antibody was reacted. The antibodies bound on trapped viruses were detected by protein A-peroxidase conjugate at a dilution of 1 : 8000.

precoated with $F(ab')_2$ fragments of respective antibodies. After incubation, homologous IgG ($2\mu g/ml$) to coated $F(ab')_2$ was added to react with the trapped viruses. The bound antibodies on trapped viruses were detected with protein A-peroxidase conjugate. ELISA values for each serotype were higher in homologous than those in heterologous combinations of antibody and virus within the range of virus concentrations tested (Fig. 1). In this system, the antibodies to CMV-Y and CMV-Z could differentiate homologous from heterologous serotype. However, the difference in ELISA values between the two serotypes was less compared with those of direct DAS ELISA[10]. Indirect ELISA has been observed to exhibit lower specificity in differentiating among serologically related viruses or strains of virus than direct ELISA[8,18]. This may be attributed to the differences in specificities of detecting antibodies used in the two forms of ELISA. In direct ELISA, antibody reactivity may be reduced after enzyme conjugtion and this may reduce the extent of cross-reactions in heterologous systems as suggested by Koenig[7]. On the other hand, indirect ELISA has a wider specificity than direct ELISA because of using native antibodies[8,18].

2. *Reaction of Two Serotypes with Cross-absorbed Antibodies in $F(ab')_2$ ELISA*

The use of ELISA for differentiating plant viral serotypes has been reported[2,7,13,14,17,18]. However, differentiation of very closely related serotypes such as strains of CMV by ELISA seems to be unreliable when unknown concentrations of virus preparations are used as antigens because ELISA values may vary depending on virus concentrations, antibody specificities and reaction conditions used. To overcome this, cross-absorbed antibodies with heterologous strains were examined to detect selectively two serotypes. Purified viruses were reacted in $F(ab')_2$ coated wells and then homologous cross-absorbed antibodies (IgG fraction) to coated $F(ab')_2$ were added to the wells. To determine the optimum concentration of cross-absorbed antibodies, various concentrations of antibodies ranging from $2\mu g$ to $20\mu g/ml$ were tested against homologous serotypes. Relatively high ELISA values were obtained at higher concentrations of IgG up to $10\mu g/ml$. However, only weak color developments were observed at lower concentrations of IgG. Low activities of the cross-absorbed IgG apparently due to elimination of common antibodies to both serotypes after cross-absorption. As $10\mu g/ml$ of IgG concentration seemed to be reasonable for enough color development, this concentration of IgG was adopted for all further experiments. The ability of this system for selective detection of two serotypes of CMV was examined using

purified viruses. As shown in Fig. 2, positive reactions were observed with homologous combinations and gave only weak reactions with heterologous systems. To determine the suitability of this procedure for differentiation of two serotypes using crude sap, preparations from the tobacco plants infected with several strains belong to Y or P type were tested at a sap dilution of 1 : 100. The homologous ELISA values for all the strains tested were higher than those of heterologous reactions (Table 1) and we obtained similar results to those from the experiments employing purified preparation of CMV. This method has some advantages over gel diffusion test. This may be useful for rapidly differentiation of CMV serotypes using crude sap from field-collected samples.

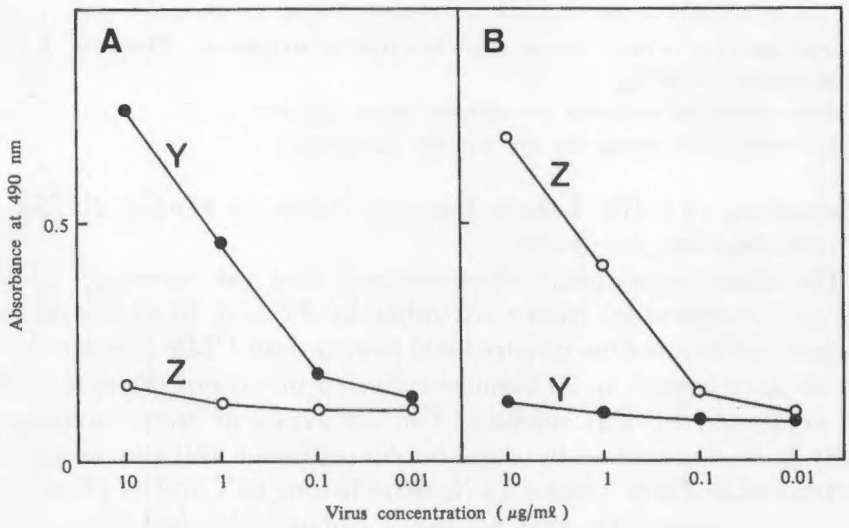

FIG. 2. Selective detection of two serotypes of CMV in $F(ab')_2$ ELISA with cross-absorbed antibody. Purified CMV-Y (serotype Y) and CMV-Z (serotype P) were added to the wells of microplates precoated with $F(ab')_2$ prepared from antiserum to CMV-Y (A) or CMV-Z (B) and then homologous IgG (10 μg/ml) absorbed with heterologous serotype was reacted. Other details as in Fig. 1.

TABLE 1. Reaction of some CMV isolates in $F(ab')_2$ ELISA with cross - absorbed antibodies*

Serotype	Isolate	*Cross - absorbed antibodies to*	
		Y type	P type
Y	CMV - Y	0.87**	0.09
	1	0.76	0.12
	2	0.98	0.18
	3	0.73	0.11
P	CMV - Z	0.12	1.02
	4	0.09	0.89
	5	0.11	0.79
	6	0.12	0.69

* Crude sap from infected tobacco plants was used as antigens at a dilution of 1 : 100. Other details as in Fig. 2.

** Absorbance values at 490nm for undiluted reacted substrate. Each value is the average for two separate experiments.

3. *Serotyping of CMV Isolates Found in Japan by $F(ab')_2$ ELISA with Cross-absorbed Antibodies*

The above experiments demonstrated that two serotypes of CMV could be differentiated from each other by $F(ab')_2$ ELISA with cross-absorbed antibodies. One hundred and twenty-four CMV isolates obtained from 38 plant species in 20 families collected in various districts in Japan were serotyped by this method. The serotypes of some isolates have already been determined by a gel double-diffusion test previously[5, 6, 9, 11]. As listed in Table 2 and 3, 115 isolates belong to Y and 9 to P serotypes. It was very interesting that all the 3 isolates obtained from *Petasites japonicus* were P type. Tochihara and Tamura[15] also reported an isolate of P type from this species. This fact may strongly suggest that the P serotype of CMV has an affinity to *P. japonicus*.

TABLE 2. Detection of Y serotypes of CMV from various plants in Japan

Host plant		Number of isolates	Locality
Family	Species		
Amaryllidaceae	*Narcissus* spp. (narcissus)	2	Mie, Ooita
	Hippeastrum sp. (amaryllis)	1	Okayama
Apocynaceae	*Nerium indicum*(seet - scented oleander)	1	Okayama
Balsaminaceae	*Impatiens balsamina* (garden balsam)	1	Tokyo
Caryophyllaceae	*Stellaria media* (chickweed)	2	Fukuoka, Mie
Chenopodiaceae	*Spinacia oleracea* (spinach)	3	Mie, Miyazaki, Okayama
Commelinaceae	*Commelina communis* (dayflower)	20	Fukuoka, Gunma, Ishikawa, Kagawa, Mie (5)*, Miyagi (4), Miyazaki, Nagano, Nara, Okayama (4)
Compositae	*Chrysanthemum morifolium* (chrysanthemum)	2	Mie (2)
	Dahlia sp. (dahlia)	1	Fukuoka
	Zinnia elegans (zinnia)	3	Mie, Nara, Okayama
Cruciferae	*Brassica campestris* (chinensis group)	1	Okayama
	B. oleracea (cabbage)	1	Mie
	Capsella bursa - pastoris	1	Okayama
	Raphanus sativus (Japanese radish)	14	Fukuoka (2), Kumamoto (4), Mie (3), Okayama (2), Saitama, Shimane (2)
Cucurbitaceae	*Cucurbita maxima* (pumpkin)	1	Okayama
	Cucumis sativus (cucumber)	6	Mie (2), Miyagi, Okayama, Shimane, Yamaguchi
	Trichosanthes cucumeroides	1	Fukuoka
Gentianaceae	*Gentiana scabra* var. *buergeri*	2	Niigata (2)
Graminineae	*Zea mays* (corn)	1	Okayama
Leguminosae	*Glycine max* (soybean)	2	Nagano (2)
	Phaseolus angularis (adzuki bean)	4	Mie, Miyagi, Okayama (2)
	P. vulgaris (snap bean)	2	Fukuoka, Gunma
	Vigna sinensis (cowpea)	4	Mie (2), Miyagi, Okayama
Liliaceae	*Lilium* × *elegans* (Thunberg lily)	3	Mie, Okayama, Tokushima
	L. longiflorum (Easter lily)	14	Fukuoka (5), Kagoshima (4), Okayama (2), Saga (3)
	Tricytis spp. (hairly tod lily)	3	Mie, Okayama (2)
Plumbaginaceae	*Limonium sinuatum* (statice)	1	Okayama
Scrophulariaceae	*Antirrhinum majus* (snapdragon)	3	Miyazaki, Okayama, Tokyo
	Paulownia tomentosa (paulownia)	1	Okayama
Solanaceae	*Capsicum annuum* (sweet pepper)	4	Fukuoka, Mie (2), Okayama
	Lycopersicon esculentum (tomato)	3	Fukuoka, Miyagi, Okayama
	Petunia × *hybrida* (petunia)	2	Fukuoka, Okayama
	Solanum melongena (eggplant)	2	Fukuoka, Saga
Thymelaeaceae	*Daphne odora* (winter daphne)	1	Fukuoka
Zingiberaceae	*Zingiber mioga*	2	Fukuoka, Mie

* Figures in parentheses indicate number of isolates.

TABLE 3. Detection of P serotyses of CMV from various plants in Japan

Host plant		Number of isolates	Locality
Family	Species		
Compositae	*Petasites japonicus* (Japanese butterbur)	3	Gunma, Okayama, Saitama
	Zinnia elegans (zinnia)	1	Nagano
Cucurbitaceae	*Cucumis sativus* (cucumber)	1	Mie
Leguminosae	*Phaseolus angularis* (adzuki bean)	1	Miyagi
Orchidaceae	*Calanthe discolor*	1	Okayama
Solanaceae	*Lycopersicon esculentum* (tomato)	1	Miyagi
Violaceae	*Viola* × *wittrockiana* (garden pansy)	1	Okayama

SUMMARY

Two serotypes of cucumber mosaic virus (CMV) designated Y and P types in Japan were tested against homologous and heterologous antibodies in $F(ab')_2$ ELISA. The ELISA values of homologous reactions were higher than those in heterologous combinations. The use of cross-absorbed antibodies with heterologous serotypes as detecting antibodies was demonstrated to be valuable in differentiating two serotyes of CMV in $F(ab')_2$ ELISA using crude sap from infected plants. One hundred and twenty-four isolates of CMV obtained from 38 plant species in 20 families collected in Japan were serotyped by this method. One hundred and fifteen isolates belonged to Y and 9 to P serotypes. All 3 strains isolated from *Petasites japonicus* were P type strains.

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