

カボチャモザイクウイルス(WMV-2)に対するモノクローナル抗体(MCA)の作製と特性

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
著者名	Somowiyarjo, S. 佐古, 宣道 野中, 福次
発行元	日本植物病理學會
巻/号	56巻4号
巻号補足	
掲載ページ	p. 541-548
発行年月	1990年10月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Production and Characterization of Monoclonal Antibodies to Watermelon Mosaic Virus 2*

Susanto SOMOWIYARJO^{***}, Nobumichi SAKO^{**},†
and Fukuji NONAKA^{**}

Abstract

Thirteen hybridoma cell lines secreting monoclonal antibodies (MCAs) to watermelon mosaic virus 2 (WMV-2) were produced by fusing spleen cells of immunized mice and mouse myeloma cell line. The MCAs recognized at least three different epitopes on the particles of WMV-2. WMV-2 and zucchini yellow mosaic virus (ZYMV) shared one epitope (cryptotope) because a group of MCAs (group 3) to WMV-2 reacted only with ZYMV treated using carbonate buffer, pH 9.6 in non-precoated indirect enzyme-linked immunosorbent assay (I-ELISA) but not in precoated I-ELISA with rabbit antiserum to WMV-2. This fact provided a basis for clarification of the serological relationship between the two viruses. ELISA using MCA WMV-2 15 was found to be useful for WMV-2 detection and/or diagnosis of field samples.

(Received April 16, 1990)

Keywords : watermelon mosaic virus 2, monoclonal antibodies, serological relationship, diagnosis.

INTRODUCTION

Watermelon mosaic virus 2 (WMV-2) is one of the most established potyviruses that has been reported to be widely distributed and to be the most prevalent virus damaging cucurbit crops in various areas of the world^{12,16}. The virus has formerly been known to be serologically related to, but distinct from, zucchini yellow mosaic virus (ZYMV)^{10,15}. More recently, however, several papers with contradictory results have been published on the extent of the relationship between these two viruses^{1,2,14,28}. Part of the contradiction may arise from the different method of assay and the heterogeneity of the antisera used in the different laboratories.

The objectives of the work were to produce and characterize the monoclonal antibodies (MCAs) to WMV-2 and to use them in enzyme-linked immunosorbent assay (ELISA) for differentiation of WMV-2 and ZYMV, and for virus diagnosis. Preliminary reports have been previously presented^{18,23}.

MATERIALS AND METHODS

Viruses and polyclonal antibodies. WMV-2 (isolate M-18) was isolated from diseased

* This study was supported by a Grant-in-Aid for Developmental Scientific Research I (No.6180005) to the second author from the Ministry of Education, Science and Culture, Japan.

** Faculty of Agriculture, Saga University, Saga 840, Japan 佐賀大学農学部

*** Present address: Faculty of Agriculture, Gadjah Mada University, Yogyakarta 55581B, Indonesia
現在: ガジャマダ大学農学部

† To whom correspondence should be addressed.

pumpkin (*Cucurbita maxima* cv. Ebisu) collected from Miyazaki Prefecture. It was then purified using a method as described previously¹⁷⁾. Polyclonal antibody (PCA) against WMV-2 was produced by injecting a total of 3.6 mg purified virus into a rabbit at monthly intervals. The virus was mixed with an equal volume of Freund's complete adjuvant at the first injection, and with incomplete adjuvant for the following injections. Booster was done at one month after the third injection. The antiserum was collected one week after booster and had a precipitin ring titer of 1,024. ZYMV and its rabbit antiserum were the same as in the previous study²⁰⁾.

Immunization of mice, cell fusion and cell culture. Two six-week-old BALB/c mice were used for immunization. Each mouse successively received intraperitoneal injection three times with three weeks between injections, using approximately 10 μ g, 30 μ g and 60 μ g of purified virus, respectively. The virus was emulsified with Freund's complete adjuvant (1:1, v/v; Difco Laboratories, Detroit, Mich.) using two syringes at the first injection and with Freund's incomplete adjuvant at the following injections. The mouse received a final immunization of virus (100 μ g) by the intravenous route in phosphate-buffered saline (PBS) 4 days before fusion.

Myeloma cell line, P3-X63-Ag8-U1, was employed in this experiment. The myeloma cells were grown using medium of Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco) supplemented with 10% fetal calf serum (FCS), 100 μ g/ml penicillin, 2 mM L-glutamine, 20 μ g/ml gentamicin sulfate, 0.2 μ M hypoxanthine and 0.01 μ M thymidine (HT medium) in water-jacketed incubator (Forma Scientific).

The fusion of spleen cells with myeloma cells was done by the method described by Galfree⁶⁾ using 45% (w/v) polyethylene glycol 4,000 (Merck) as the fusing agent. Ten days after fusion, supernatant fluid from growing culture was assayed for the presence of a specific antibody using non-precoated and precoated procedures of indirect enzyme-linked immunosorbent assay (I-ELISA) with the assay condition as used in the previous work^{20,21)}. This was done in microtitre plates (Immulon I, Nunc) with antigen of 1 μ g/ml purified WMV-2. The selected hybridomas were cloned under condition of limiting dilution using rat peritoneal cells as the feeder. Cloning was repeated three times. The hybridomas were stored in liquid nitrogen in FCS containing 10% dimethyl sulfoxide.

Production and isotype determination of MCA. Ascitic fluids were produced by intraperitoneal injection of hybridomas into mice which had been given the injection of 0.5 ml 2,6,10,14-tetramethyl pentadecane (pristane, Wako Pure Chem.) two weeks earlier.

A kit for identification of subclass specific antisera (Bio-Rad) containing rabbit anti-mouse subclass antiserum specific to mouse IgA, IgG1, IgG2a, IgG2b, IgG3, IgM, kappa light chain, and light chain was used for isotyping the MCA.

Purification of MCA. Immunoglobulins from ascitic fluids were concentrated by precipitating with 45% (v/v) saturated ammonium sulfate. The precipitates were dissolved in 0.01 M phosphate buffer, pH 8.0, containing 0.14 M NaCl and dialyzed against the same buffer. Separation of mouse IgG was done using a Protein A-Sepharose CL-4B column (Pharmacia) with the method as described by Schreier¹⁹⁾.

ELISA. Unless otherwise stated, the ELISA condition was the same as that in the earlier experiments^{20,21)}, except that the plates were Immulon II (Nunc). Three times of 3-min washing were done between each step of the assay.

RESULTS

Characterization of MCAs to WMV-2

Of the 854 wells into which the fusion products were distributed, 763 contained hybridoma colonies. The antibody-secreting activity of these hybridomas was analyzed by non-precoated and precoated I-ELISA. Of the 763 wells growing colonies, 13 produced antibodies specific to WMV-2. Some properties of 13 stable hybridomas after two cycles of cloning at limiting

dilution are given in Table 1.

The MCAs could be divided into three groups on the basis of their reactivity with WMV-2 and ZYMV in two I-ELISA procedures. The group 1 reacted specifically to WMV-2 in non-precoated I-ELISA but not in precoated I-ELISA, and group 2 reacted specifically to WMV-2 in the two I-ELISA procedures. Group 3 reacted with either WMV-2 and ZYMV in non-precoated I-ELISA but not in precoated I-ELISA (Table 2).

Epitope discrimination

Information on epitope discrimination was obtained by inhibition binding assay^{13,25)} with slight modifications. Briefly, virus was diluted in buffer containing 100 µg/ml blocking MCA to give a final concentration of 1 µg/ml. After a 120-min incubation at 37 C, 100 µl aliquots were transferred to MCA-coated microtitre plates and the level of unblocked binding sites was estimated by precoated I-ELISA using gamma-globulin (2 µg/ml) from rabbit antiserum as the intermediate antibody. The data showed that the MCAs could be divided into three groups which indicated the presence of at least three antigenic determinants (epitopes) in the particles of WMV-2 (Table 3).

Table 1. Some properties of selected monoclonal antibodies (MCAs) against watermelon mosaic virus 2 (WMV-2)

MCA	Subclass	Titre (—log) ^{a)}	
		Culture medium	Ascitic fluid
WMV-2 1	IgM	2-3	5-6
WMV-2 2	IgG2a	2-3	6-7
WMV-2 3	IgG2a	2-3	6-7
WMV-2 4	IgG2a	2-3	6-7
WMV-2 6	IgG2a	3-4	6-7
WMV-2 7	IgM	3-4	6-7
WMV-2 8	IgG2a	4-5	8-9
WMV-2 12	IgG2a	4-5	n.t. ^{b)}
WMV-2 14	IgG2a	3-4	7-9
WMV-2 15	IgG2a	4-5	8-9
WMV-2 16	IgG2a	2-3	6-7
WMV-2 17	IgG2a	4-5	8-9
WMV-2 18	IgG2a	3-4	6-7

a) The titre was determined using non-precoated indirect enzyme-linked immunosorbent assay with 1 µg/ml of purified watermelon mosaic virus 2 as the antigen.

b) n.t.=not tested.

Table 2. Reactivity of 13 monoclonal antibodies to watermelon mosaic virus 2 (WMV-2) with WMV-2 and zucchini yellow mosaic virus (ZYMV) in two indirect-ELISA procedures

Hybridoma		Non-precoated I-ELISA ^{a)}		Precoated I-ELISA ^{b)}	
Group	Number	WMV-2	ZYMV	WMV-2	ZYMV
1	1, 7	+	—	—	—
2	2, 3, 4, 6, 8, 12, 15, 17	+	—	+	—
3	14, 16, 18	+	+	—	—

a) Non-precoated indirect enzyme-linked immunosorbent assay (I-ELISA) was done using 1 µg/ml of purified WMV-2 or ZYMV as the antigen.

b) Precoated I-ELISA was done using 2 µg/ml of gamma-globulin from rabbit antiserum to WMV-2 as the coating antibody.

Table 3. Inhibition binding assay with monoclonal antibodies (MCAs) to watermelon mosaic virus 2 (WMV-2)

Test MCA	Decreasing rate of ELISA absorbance when test MCAs were blocked using a)				
	None	MCA 7	MCA 14	MCA 15	MCA 17
MCA 1	0	+++	+	0	0
MCA 7	0	+++	+	0	0
MCA 2	0	0	0	+++	+++
MCA 3	0	0	0	+++	+++
MCA 4	0	0	0	+++	+++
MCA 6	0	0	0	+++	+++
MCA 8	0	0	0	+++	+++
MCA 12	0	0	0	+++	+++
MCA 15	0	0	0	+++	+++
MCA 17	0	0	0	+++	+++
MCA 14	0	+	+++	0	0
MCA 16	0	+	+++	0	0
MCA 18	0	+	+++	0	0

a) The proportion by which ELISA absorbance values decreased when the test MCA was blocked using the blocking MCA; 0: 0-15%; +: 15-30%; ++: 30-85%; +++: 85-100%.

Cross-reactivity of WMV-2 and ZYMV in I-ELISA using MCAs

In the tests using PCA, it was found that WMV-2 was separated from ZYMV by a serological differentiation index (SDI) of 3, which indicated that the two viruses were serologically related (data not shown). However, differences in the cross-reactivity between WMV-2 and ZYMV in different ELISA procedures using PCA²²⁾ have created several interesting questions. For example, it was not clear why the viruses did not cross-react each other in PCA-based DAS-ELISA whereas they cross-reacted strongly in I-ELISA. This phenomenon was clarified by the following experiments using common epitope-specific MCAs.

Since MCA WMV-2 14 was found to have the highest titer among the common epitope-specific MCAs (Table 1), it was used for further experiments. The first experiment was done to know the effect of conjugation with alkaline phosphatase on the reactivity of the MCA. In this experiment the partially purified MCA was further purified by using Protein A-Sepharose CL-4B column and was conjugated with alkaline phosphatase. The conjugate was then reacted with antigens which were immobilized on microtiter plates directly (two-step direct ELISA), and which were trapped on the plates previously coated with the unconjugated MCA (DAS-ELISA). The results showed that the conjugate reacted with WMV-2 and ZYMV which were immobilized directly on the plates, but not with which were immobilized on plates previously coated with the MCA (Fig. 1). These data suggested that the reactivity of the common epitope-specific MCA was not destroyed by conjugation with alkaline phosphatase.

Since in non-precoated I-ELISA and two-step direct ELISA, antigens were diluted in carbonate buffer, pH 9.6, it was necessary to know the effect of this buffer on the antigenicity of these viruses. In this test, viruses in crude extracts of leaves were diluted (1:10) in carbonate buffer, pH 9.6 or PBS, pH 7.4, and were incubated for 2 hr at 37 C. They were then used as antigen in I-ELISA on plates coated with gamma-globulin (2 µg/ml) from rabbit antiserum and intermediate antibody of MCA WMV-2 14. It was found that the MCA reacted with the viruses previously treated using carbonate buffer, but not with those treated using PBS (Fig. 2). Thus, it was concluded that the common epitope became active under alkaline condition.

The use in indirect-ELISA for virus detection

Although I-ELISA using PCA has provided a suitable method for rapid assay of several viruses^{3,7-9)}, the assay is not suitable for diagnosis of WMV-2 because of the presence of cross-

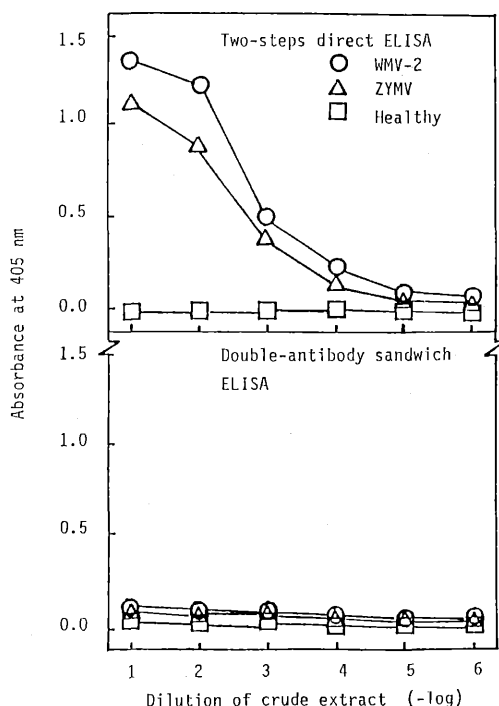


Fig. 1. Reactivity of alkaline phosphatase-labeled monoclonal antibody specific to watermelon mosaic virus 2 (WMV-2) (MCA WMV-2 14) and zucchini yellow mosaic virus (ZYMV) with antigens in crude extracts of pumpkin leaves in two enzyme-linked immunosorbent assay (ELISA) procedures.

reaction between WMV-2 and ZYMV²²). In order to develop an I-ELISA that can be used for diagnostic works, MCA WMV-2 15 was selected, because it had high titer and reacted specifically to WMV-2. It was found that the MCA could be successfully used in I-ELISA to detect WMV-2 at minimum detectable concentration of 0.1–0.01 $\mu\text{g/ml}$. The assay was also suitable to detect virus in crude extracts of pumpkin leaves at dilution end point of 10^{-6} – 10^{-7} times (Fig. 3).

The suitability of I-ELISA using MCA WMV-2 15 for detecting virus in naturally infected plants in Saga Prefecture was also tested. It was found that of 60 field samples tested, 23 reacted with the MCA in I-ELISA as well as with PCA in DAS-ELISA, but not with the MCA specific for ZYMV (MCA ZYMV 45)²²).

DISCUSSION

Thirteen MCAs to WMV-2 have been produced which could be divided into three groups based on their reactivities with WMV-2 and ZYMV in two I-ELISA procedures (Table 2) as well as on their reaction pattern in inhibition binding assay (Table 3). It indicated that WMV-2 had at least three antigenic determinants (epitopes). In the view of the type of epitopes, MCA group 1 and 3 were specific for a hidden epitope or cryptotope, *i.e.* determinant that become antigenically active only after breakage, depolymerization or denaturation of antigen²³). This group of MCA could easily react with antigen in non-precoated I-ELISA, because the use of carbonate buffer, pH 9.6, and direct adsorption of virus particles to the microtitre plates lead to considerable particle breakdown^{3,4,6,10,27}). Alternatively, the MCA could react with antigen

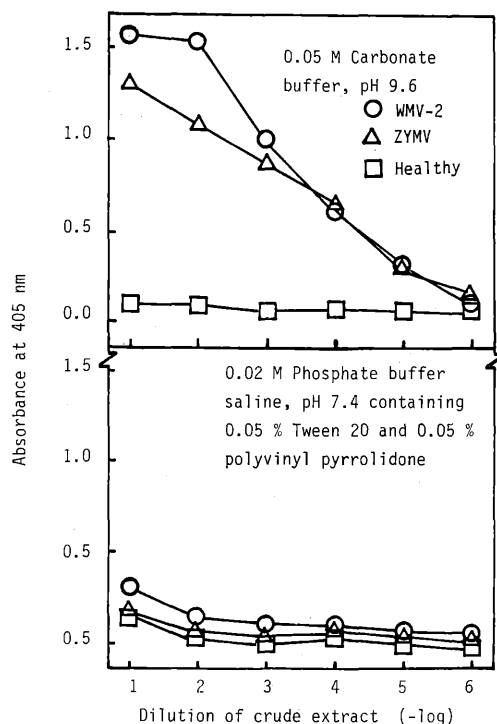


Fig. 2. Effect of antigen buffer on the reactivity of watermelon mosaic virus 2 (WMV-2) and zucchini yellow mosaic virus (ZYMV) in crude extracts of pumpkin leaves with monoclonal antibody against WMV-2 14 (MCA WMV-2 14) in precoated indirect enzyme-linked immunosorbent assay using rabbit anti-WMV-2 gamma-globulin as coating antibody. Each point is the mean of three experiments using three wells in each experiment.

in precoated I-ELISA when the antigen was previously treated with carbonate buffer, pH 9.6 before tested (Fig. 2). MCA group 2 was specific for a metatope, *i.e.* epitope that present either in degraded protein or in intact virion²⁶⁾, thus they could be recognized by MCA in non-precoated I-ELISA as well as in precoated I-ELISA.

The results obtained using either PCA or MCAs support the observation of Lisa¹¹⁾ and others^{2,7,15)}, who reported that WMV-2 and ZYMV were serologically related, but contrast with the results of Nameth¹⁴⁾ and Chala¹⁾, who reported the lack of cross reactivity between the viruses. The results also contrast with the absence of serological relationship between Japanese isolates of 80 and 8E of WMV reported by previous workers^{28,29)} in leaf-dip serology and immunodiffusion tests. The WMV isolates of 80 and 8E were considered to be WMV-2 and ZYMV, respectively²⁴⁾. The failure of several workers to detect cross-reaction of these viruses was probably derived from the characteristics of antibodies, but also from the low sensitivity of the methods used. Inability of precipitin test to recognize cross-reactivity between the viruses that could be detected by I-ELISA, has been previously reported with other viruses⁹⁾. In connection with the characteristics of antibody, Purcifull¹⁵⁾ reported that antiserum against WMV-2 collected 7–9 weeks after immunization did not cross-react with ZYMV, while antiserum collected 5–8 months later from the same rabbit did. However, it cannot be excluded that the different reactions were also probably due to the different strains of virus tested.

The fact that WMV-2 and ZYMV shared a common cryptotope provided a basis for clarification on the serological relationship between WMV-2 and ZYMV, and this solved the long

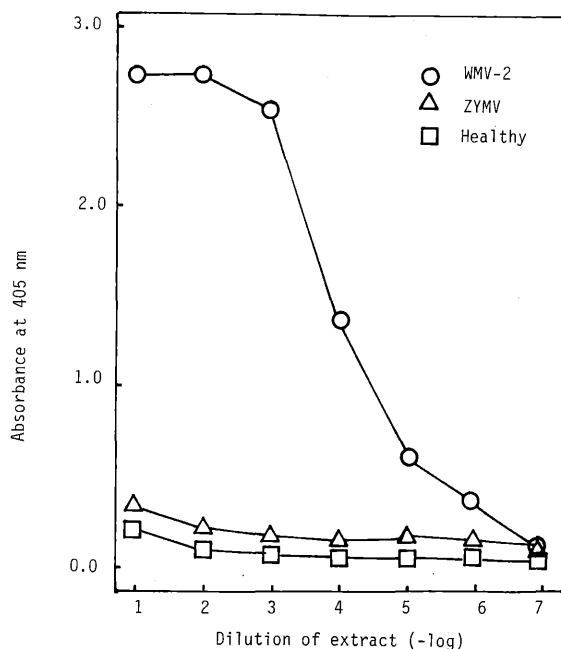


Fig. 3. Detection of watermelon mosaic virus 2 (WMV-2) and zucchini yellow mosaic virus (ZYMV) in crude extracts of pumpkin by non-precoated indirect enzyme-linked immunosorbent assay using MCA WMV-2 15 as intermediate antibody. Each point is the mean of the three experiments using three wells in each experiment.

unresolved phenomenon of the cross-reactivity between WMV-2 and ZYMV in PCA-based I-ELISA but in DAS-ELISA.

Results obtained so far in diagnosis of WMV-2 in field samples indicated that any samples reacted with MCA in I-ELISA also gave a positive reaction with PCA in DAS-ELISA. However, since I-ELISA offered several advantages over DAS-ELISA for plant virus assay²⁶⁾, the availability of MCA specific to WMV-2 should facilitate improvement of diagnostic works in the future.

Thank is due to the Hitachi Scholarship Foundation for providing a scholarship to the first author for his study at Saga University.

Literature cited

1. Chala, V.V., Harrison, C.W. and Halliwell, R.S. (1987). *Plant Dis.* 71: 750-752.
2. Davis, R.F. (1986). *Ibid.* 70: 735-738.
3. Diaco, R., Lister, R.M., Hill, J.H. and Durand, D.P. (1986). *J. gen. Virol.* 67: 353-362.
4. Dore, I., Weiss, E., Altschuch, D. and Van Regenmortel, M.H.V. (1988). *Virology* 162: 279-289.
5. Galfree, G., Howe, S.C., Milstein, C., Butcher, G.W. and Howard, J.C. (1977). *Nature* 266: 550-552.
6. Halk, E.L., Hsu, H.T., Aebig, J. and Franke, J. (1984). *Phytopathology* 74: 367-372.
7. Huang, C.H. and Hseu, S.H. (1985). *FFTC Book Series No. 33*: 43-50.
8. Jaegle, M. and Van Regenmortel, M.H.V. (1985). *J. Virol. Methods* 11: 189-198.
9. Koenig, R. (1981). *J. gen. Virol.* 55: 53-62.
10. Koenig, R. and Torrance, L. (1986). *Ibid.* 67: 2145-2151.
11. Lisa, V., Boccardo, G., D'Agostino, G., Dellavalle, G. and d'Aquilio, M. (1981). *Phytopathology* 71: 667-672.

12. Lovisolo, O. (1980). *Acta Hort.* 88: 33-82.
13. Molday, R.S. and MacKenzie, D. (1983). *Biochemistry* 22: 653-660.
14. Nameth, S.T., Dodds, J.A., Paulus, A.O. and Kishaba, A. (1985). *Plant Dis.* 69: 785-788.
15. Purcifull, D., Adlerz, W.C., Simone, G.W., Hiebert, E. and Christie, S.R. (1984). *Ibid.* 68: 230-233.
16. Purcifull, D., Hiebert, E. and Edwardson, J. (1984). *CMI/AAB Description of Plant Viruses No.* 293.
17. Sako, N., Matsuo, K. and Nonaka, F. (1980). *Ann. Phytopath. Soc. Japan* 46: 639-646.
18. Sako, N. and Somowiyarjo, S. (1988). *Abstr. 5th Int. Congr. Pl. Pathol., Kyoto.* p. 460.
19. Schreier, M., Kohler, G., Hengartner, H., Berek, C., Trucco, M., Forni, L., Staehelin, T., Stocker, J. and Takacs, B. (1980). *Hybridoma Techniques.* Cold Spring Harbor, New York. pp. 36-37.
20. Somowiyarjo, S., Sako, N. and Nonaka, F. (1985). *Ann. Phytopath. Soc. Japan* 51: 569-575.
21. Somowiyarjo, S., Sako, N. and Nonaka, F. (1986). *Ibid.* 52: 653-659.
22. Somowiyarjo, S., Sako, N. and Nonaka, F. (1988). *Ibid.* 54: 436-443.
23. Somowiyarjo, S., Sako, N. and Nonaka, F. (1989). *Ibid.* 55: 535 (Abstr. in Japanese).
24. Terami, F., Yamamoto, T. and Inouye, T. (1985). *Ibid.* 51: 83 (Abstr. in Japanese).
25. Thomas, J.E., Massalski, P.R. and Harrison, B.D. (1986). *J. gen. Virol.* 67: 2739-2748.
26. Van Regenmortel, M.H.V. (1982). *Serology and Immunochemistry of Plant Viruses.* Academic Press, London. pp. 135-137.
27. Van Regenmortel, M.H.V. and Buckard, J. (1980). *Virology* 106: 327-334.
28. Yamamoto, T. (1986). *In Transmission of Plant and Animal Viruses by Vectors* (Hidaka, Z. and Sako, N. eds.). Nishinippon Printing Ind. Co., Fukuoka. pp. 71-84.
29. Yamamoto, T., Ishii, M. and Katsube, T. (1982). *Ann. Phytopath. Soc. Japan* 48: 613-619.

和 文 摘 要

Susanto SOMOWIYARJO・佐古宣道・野中福次：カボチャモザイクウイルス (WMV-2) に対するモノクローナル抗体(MCA) の作製と特性

WMV-2 で免疫したマウス脾細胞とミエローマ細胞を用いて、MCA を産生する 13 個の融合細胞株を作製した。これらの MCA により WMV-2 には少なくとも 3 種類の抗原決定基の存在が明らかになった。このウイルスに対する第 3 グループの MCA は、non-precoated 間接エリザ法により、炭酸水素ナトリウム・炭酸ナトリウム緩衝液、pH 9.6 で処理したズッキーニ黄斑モザイクウイルス (ZYMV) と反応し、ウサギ抗 WMV-2 抗体を用いる precoated 間接エリザ法では反応しなかったことから、両ウイルスは 1 種類の抗原決定基 (cryptotope) を共有していることが判明した。このことは両ウイルス間の血清学的関係についてより明確な実証を示したものと考えた。MCA WMV-2 15 は圃場試料からの本ウイルスの検出および診断に利用できる」と結論した。