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Utilization of monoclonal and polyclonal antibodies to monitor the protecting and challenging strains of zucchini yellow mosaic virus in cross protection

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ABSTRACT

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A monoclonal antibody (MAb), designated F28 5H12F11, was able to differentiate a zucchini yellow mosaic virus strain (ZYMV-TW) from Connecticut, Florida, and France strains of ZYMV in plate-trapped antigen (PTA), double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy. This monoclonal antibody was determined to be an IgG1, kappa light chain class. The monoclonal antibody, in conjunction with a polyclonal antibody (ZYMV-TW/CT) which only reacted with ZYMV-TW, was used to study the interaction between the protecting mild strain of ZYMV from France and the challenging ZYMV-TW in cross protection experiments conducted on plants of zucchini squash. ELISA tests showed that ZYMV-TW was detectable at a low level after the challenge inoculation but increased rapidly just prior to severe symptom development in cross-protected plants. Conversely, the concentration of the protecting strain decreased significantly when the challenging strain increased in concentration. In cross-protected plants that did not show severe symptoms, a low concentration of ZYMV-TW was detected in ELISA tests.

Key words: zucchini yellow mosaic virus, monoclonal antibody, cross protection.

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV) is one of the most destructive pathogens infecting cucurbit crops in the United States and many other countries where it causes devastating epidemics [6,10,13,19,20]. ZYMV, a potyvirus, is transmitted nonpersistently by aphids [13]. Different strains of ZYMV have been distinguished by their reactions on indicator plants [2,6,21]. Serological comparisons revealed that ZYMV was related to watermelon mosaic virus 2 (WMV-2), pea mosaic virus (PMV), bean common mosaic virus (BCMV), bean yellow mosaic virus (BYMV), and clover yellow vein virus (CYVV) [23]. Recently, cross protection has been used successfully to control this disease in Taiwan [22] and France [11].

Monoclonal antibody (MAb) technology provides methods of producing a highly specific and unlimited supply of uniform antibodies which are useful for differentiation of virus strains [5, 16, 18]. Strain-specific MAbs can be used to monitor the interactions of viruses in host plants. They aid in cross protection studies by simultaneously monitoring the protecting and challenging strains of a virus in the same plant. Immunosorbent electron microscopy (ISEM) has been applied to the detection of viruses by virus-specific monoclonal antibodies [3]. The purpose of this study was to produce hybridomas which secrete ZYMV specific MAbs, to test the

reaction of selected MAbs against strains of ZYMV, and to characterize the interaction of the ZYMV strains that were used in cross protection experiments.

MATERIALS AND METHODS

Viruses and polyclonal antibodies

ZYMV used in this study included strains of Taiwan (ZYMV-TW), Connecticut (ZYMV-CT), Florida (ZYMV-FL), French (ZYMV-FR), and French mild (ZYMV-WK). All were maintained in plants of zucchini squash in the greenhouse. ZYMV-WK and ZYMV-FR were supplied by H. Lecoq of INRA, France and ZYMV-TW by C. H. Huang of TARI, Taiwan. The twelve potyviruses: pea mosaic (PMV), bean yellow mosaic (BYMV), pea seed-borne mosaic (PSBMV), white lupin (WLV), clover yellow vein (CYVV), bean common mosaic NY-15 and NL-8 (BCMV-NY-15 and -NL-8), tobacco etch (TEV), potato virus Y (PVY), pepper mottle (PeMV), watermelon mosaic 2 (WMV-2), and papaya ringspot watermelon strain (PRV-w) used in this study were from Dr. R. Provvidenti of Cornell University, and were maintained in squash *Cucurbita pepo* cv. Zucchini Elite, pea *Pisum sativum* cv. Ranger, bean *Phaseolus vulgaris* L. or tobacco *Nicotiana tabacum* L. in a greenhouse. Purified ZYMV-CT was used for the production of monoclonal antibodies [23]. Crude extracts from ZYMV-CT infected and healthy plants were used for screening antibody producing cells and evaluation of monoclonal antibodies produced. Fresh tissue extracts were prepared by grinding in either 0.05 M potassium phosphate buffer, pH 7.2, or coating buffer [14], pH 9.6, at a 1:20 dilution. Polyclonal antibodies to ZYMV-CT, -FL, -WK, and -TW were produced in rabbits in our laboratory [23]. An antiserum, designated as ZYMV-TW/CT, was derived by cross-absorbing antiserum to ZYMV-TW with ZYMV-CT [23]. Since ZYMV-TW/CT antiserum reacted only with ZYMV-TW, and did not react with ZYMV-WK, -CT, and -FL in direct and indirect ELISA tests, it was used for the detection of the challenge infection of ZYMV-TW in ZYMV-WK-protected plants.

Production of monoclonal antibodies

BALB/c mice were immunized initially by intraperitoneal injection with 100μg of purified ZYMV-CT in 0.05 M potassium phosphate buffer, pH 7.2, mixed with Freund's Complete Adjuvant (1:1, v/v). Second immunization was done 3 wk later with the same antigen preparation except with Freund's Incomplete Adjuvant. Four weeks after the initial immunization, sera was collected by tail-bleeding from each mouse and sera titers were determined by indirect ELISA [14]. The mouse with the highest serum titer was given an intraperitoneal injection with 100μg of purified antigen in 0.2 ml of 0.05 M potassium phosphate buffer, pH 7.2, 2 months after the initial immunization. Three days later, the spleen was harvested and gently ground through a sieve into fresh Dulbecco's Modified Eagle's Medium (DMEM) (Gibco). Cells were fused with mouse myeloma non-secreting cell line SP 2/0-Ag 14 (American Type Culture Collection). Fusion and cell maintenance protocols were adapted from that of Kohler and Milstein [8], except for the actual fusion procedure involving a 60-s exposure to 50% polyethylene glycol (MW 1450) and 0.05% dimethyl sulfoxide (DMSO) [9]. The fusion was designated as F28 for ZYMV-CT.

Cells were plated out in DMEM containing hypoxanthine, aminopterin, and thymidine for selection of hybridomas. Presence of virus-specific antibodies in culture medium was determined by indirect plate-trapped antigen (PTA)-ELISA. Antibody-secreting cell lines were cloned using the limiting dilution method. For production of ascitic fluids, BALB/c mice were primed with 0.5 ml 2,6,10,14-tetramethylpentadecane (Pristane, Sigma Chemical) 10 days prior to injection of cloned hybridoma cell lines. Hybridoma cell about 1 to 2×10^6 were injected into the peritoneal cavities of mice, and ascitic fluid was collected from each mouse with a 20 g 1 1/2" needle in 7-10 days. The ascitic fluid was centrifuged at 3000 rpm in a table-top clinical centrifuge for 10 min and supernatant aliquots were collected in small vials and stored at -80℃ with 0.01 M azide.

Isotype determination

Antibody class and subclass were determined by indirect PTA-ELISA using reagents and protocols supplied in a SBA Clonotyping Kit (Fisher Scientific, PA).

Immunoassays

Two types of indirect ELISA, plate-trapped antigen (PTA) and double antibody sandwich (DAS) ELISA, were used. In PTA-ELISA, antigens were allowed to bind directly to Immulon II plates (Dynatech Laboratories, Inc.) in 0.005 M coating buffer, pH 9.6 and incubated at 4℃ [14]. Plates were rinsed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. After washing (three times), plates were incubated with 1% bovine serum albumin (BSA)

in PBS-Tween for 1 hr at room temperature, rinsed three times with PBS-Tween, and 100µl/well of undiluted cell culture supernatants were added and incubated for additional 2.5 hr at 37°C. After another washing (three times with PBS-Tween), plates were incubated with a 1:1000 dilution (in PBS-Tween) of alkaline phosphatase-labelled goat anti-mouse IgG or IgM (Sigma Chemical Co.) at 100µl/well. Plates were washed three times in PBS-Tween before addition of substrate. Reactions were read in an MR 580 Microplate Autoreader (Dynatech Instruments Inc.) at 405 nm.

In DAS-ELISA, plates were coated with purified immuno-globulin of rabbit antisera to four strains of ZYMV (1µl/ml) for 2 hr at 37°C [1]. Infected plant tissues were ground in 0.05 M potassium phosphate buffer, pH 7.2, and incubated overnight at 4°C. The remaining steps were the same as those in the PTA-ELISA after antigen incubation.

Immunosorbent electron microscopy

The immunogold-labelling technique [3] was used to verify binding of the MAb F28 5H12F11 to ZYMV strains. Preparations of partially purified strains of ZYMV were absorbed directly to the grids. The immobilized virions were decorated with undiluted culture supernatant of F28 5H12F11 mAb for 10 min, and then labelled with the Protein A-gold (1:20) [3] in PBS for additional 10 min. Grids were washed with 20 drops of distilled water each time between steps, and stained with 2.5% aqueous phosphotungstic acid for 30 sec. Grids were examined under a JEM-100 SX electron microscope.

Detection of ZYMV strains within a plant

Zucchini plants, grown in 24 cm pots, were used in this experiment. Twelve of the 22 plants were inoculated with the mild strain ZYMV-WK. Ten days after inoculation, these 12 plants were tested by ELISA with antiserum to ZYMV-WK for the presence of ZYMV-WK. At the 11th day, 6 of 12 ZYMV-WK-infected plants were inoculated with severe strain ZYMV-TW and the remaining four plants were mock-inoculated with 0.05 M potassium phosphate buffer, pH 7.2. Six healthy zucchini plants, grown under the same conditions were inoculated with ZYMV-TW at the same time as the challenge inoculation of the protected plants. Four healthy zucchini plants were used as negative controls. After the challenge inoculations, leaf specimens were collected from the topmost expanded leaf of each of 22 tested plants at 5 day intervals. Four discs (0.012 g/disc) were sampled from four evenly dispersed positions on the leaf. Samples were tested using the polyclonal antibody ZYMV-TW/CT, which allowed the monitoring of ZYMV-TW alone, and the monoclonal antibody F28 5H112F11, which allowed the monitoring of ZYMV-WK alone, in indirect PTA-ELISA tests. The ZYMV-TW antiserum was cross-absorbed with crude antigens from ZYMV-CT and ZYMV-FL according to the method of C. H. Huang [7]. One ml of antiserum was mixed with a aliquot of 20 ml from a crude extract prepared from 10 g fresh infected leaf tissue ground in 0.1 M potassium phosphate buffer, pH 7.0. The preparation was incubated at 37°C for 1 hr, then cooled in ice for one additional hour. Precipitates were discarded after a low-speed centrifugation at 3000 g for 10 min. Immunoglobulins were purified using a DEAE sephacel column (Pharmacia Co.) from non-cross-absorbed and cross-absorbed ZYMV-TW antisera. Symptom development in ZYMV-WK-infected, ZYMV-TW-infected, ZYMV-WK-challenge inoculated with ZYMV-TW, and healthy plants were recorded.

RESULTS

Production of monoclonal antibodies

A total of 504 wells from Fusion 28 (ZYMV-CT) were tested against extracts from healthy and ZYMV-CT infected plants in indirect PTA-ELISA. Eighty-five cultures producing anti-ZYMV-CT antibody were obtained. These 85 primary hybridoma cultures were retested with extracts from healthy tissue and tissues infected with ZYMV-CT, -FL, -FR, -WK or -TW. A culture that reacted to ZYMV-CT, -FL, -FR, -WK but not to ZYMV-TW was cloned once. Another culture that reacted to all tested strains was cloned twice. These two cell lines, designated F28 5H12F11 and F28 3F1C4B2, produced IgG1 immunoglobulins of kappa light chain, and were used in ascites fluid production. The two cell lines are described in more detail in later sections.

Immunoassay

ZYMV-TW did not react with F28 5H12F11 in either PTA-ELISA or DAS-ELISA; whereas in both types of ELISA tests, ZYMV-CT, -FL, -FR, and -WK reacted with this MAb (Table 1). F28 3F1C4B2 reacted with all strains including ZYMV-TW. Monoclonal antibodies F28 5H12F11 and F28 3F1C4B2 were tested against other potyviruses in indirect PTA-ELISA and against strains of ZYMV in PTA-and DAS-ELISA tests. The two monoclonal antibodies did not react

with uninfected plant tissues of pea, bean, tobacco and zucchini or plant materials individually infected with BYMV, CYVV, BCMV, WLV, PSBMV, PRV-w, WMV-2, TEV, PVY, and PeMV.

Immunosorbent electron microscopy

Comparison of protein A-gold labelling in electron microscopy using ZYMV-WK and ZYMV-TW revealed that ZYMV-WK virions were decorated by protein A-gold particles (Fig. 1A), whereas ZYMV-TW virions were not decorated (Fig. 1B). Results of immunosorbent electron microscopy indicated that F28 5H12F11 did not bind to partially purified ZYMV-TW virions. However, it did bind to virions of the preparation of ZYMV-CT, -FR, -WK, and -FL.

Interactions of ZYMV strains in a host plant

The six zucchini plants infected only with ZYMV-WK did not show severe symptoms during the tested period. ELISA reactions of extracts from these plants, tested with F28 5H12F11, gave an O.D. value above 0.5 at 405 nm (Fig. 2B). The six zucchini plants infected with ZYMV-TW showed typical severe symptoms 8 days after inoculation. The ELISA reaction of these plants, tested with polyclonal antiserum

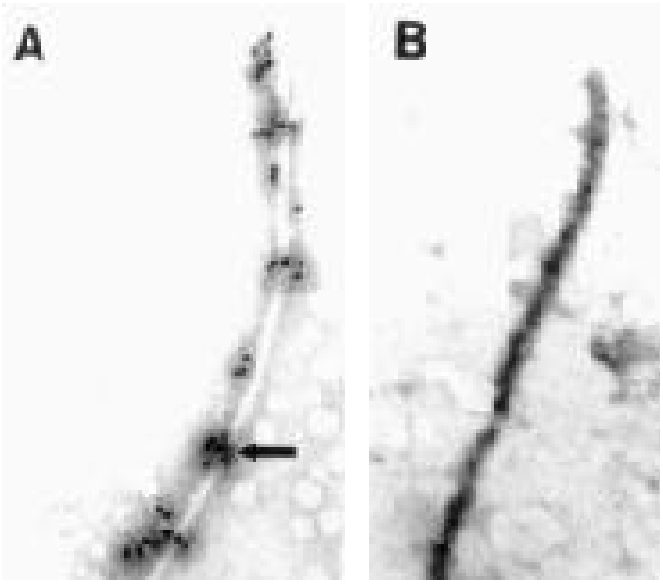

Fig. 1. Immunosorbent electron microscopy of virions of partially purified zucchini yellow mosaic virus (ZYMV) decorated with protein A-gold particles. The virions were absorbed to the grids, treated with monoclonal antibody, and labelled with protein A-gold. (A), a virion of the mild strain ZYMV-WK was decorated by protein A-gold particles (arrows). (B), a virion of Taiwan strain ZYMV-TW was not decorated by protein A-gold particles.

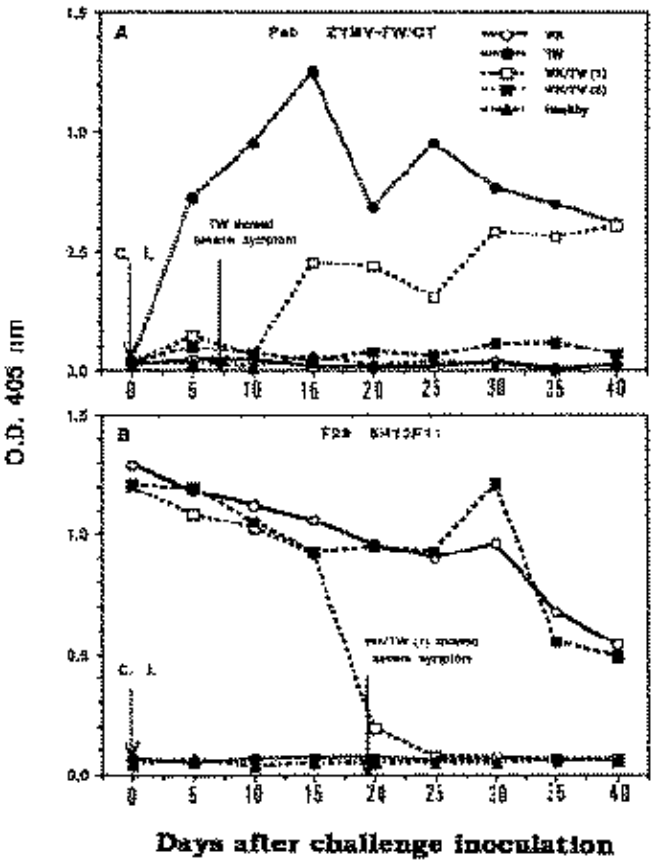

Fig. 2. Differential detection of mild strain (WK) and Taiwan strain (TW) of zucchini yellow mosaic virus (ZYMV) in zucchini plants. Antigens were tested with polyclonal antibody ZYMV-TW/CT (Pab ZYMV-TW/CT) (A), and monoclonal antibody F28 5H12F11 (B). WK/TW (1) and WK/TW (2) represent zucchini plants that were first inoculated with the mild strain, followed by challenge inoculation with ZYMV-TW. WK and TW represent zucchini plants that were inoculated only with mild and Taiwan strains, respectively. In WK/TW (1), cross-protected plants showed severe symptoms 20 days after challenge inoculation. In WK/TW (2), cross-protected plants did not show severe symptoms during the course of the experiment. C. I. represents challenge inoculation. The O. D. 405nm values were recorded 30 min after addition of substrate.

ZYMV-TW/CT, showed an O.D. of 0.9 ten days after inoculation (Fig. 2A). Severe symptoms developed 20 days after the challenge inoculation with ZYMV-TW on four of the 6 ZYMV-WK-protected plants. Moreover, ELISA reactions of these plants, when tested with F28 5H12F11, decreased from 0.9 on 15th day to 0.05 on 25th day after the challenge inoculation (Fig. 2B). At the same time, the ZYMV-TW antigen levels in these plants, measured by ELISA using the antibody to ZYMV-TW/CT, increased from 0.02 to 0.6 (Fig. 2A). Two of the 6 ZYMV-WK-protected plants did not show

severe symptoms during the experiment. The ELISA reaction of these plants when tested with F28 5H12F11 was above 0.5 (Fig. 2B). Conversely, the ZYMV-TW antigen levels in these plants, when tested with antibody (ZYMV-TW/CT) were below 0.1 (Fig. 2A).

DISCCUSION

A monoclonal antibody that reacted to ZYMV-CT, -FL, -FR, and - WK but not to ZYMV-TW was produced using mice immunized with ZYMV-CT. The specificity of the reaction was demonstrated through various ELISA tests and immunosorbent electron microscopy. These results supported recent investigation that polyclonal antibodies to ZYMV-TW, after absorption with ZYMV-CT or -FL antigens, reacted only with ZYMV-TW, but not with ZYMV-CT, -FL and -WK [23].

Virions of ZYMV-CT, -FL, -FR, and -WK contain a surface epitope which is not present on ZYMV-TW. This observation is supported by protein A-gold labelling (Fig. 1) and the DAS-ELISA test (Table 1). The reactivity of the MAb in PTA-ELISA test (Table 1) is probably because the epitope was not destroyed when intact virus particles became dissociated in the test.

The mechanism (s) of cross protection is various [17]. However, our data showed that the concentration of the challenging antigens remained very low, until 5 days before the appearance of severe symptoms when it increased in the protected plants. The concentrations of the ZYMV-TW in the protected plants showing severe symptoms were much lower than those of the unprotected plants during the period from 5 to 25 days after inoculation. Conversely, ZYMV-TW was detected only at a low titer (0.1 O.D. at 405 nm) in the protected plants not showing severe symptoms after challenge inoculation. These results indicated that ZYMV-TW was present in the protected plants, but only increased in those plants which showed severe symptoms. The concentration of the protecting antigen decreased before appearance of symptoms and remained very low during the onset of symptoms. This study demonstrated the value and utility of a discriminating monoclonal antibody F28 5H12F11 and a cross-absorbed polyclonal antibody ZYMV-TW/CT in quantitative evaluation of replication and distribution of the protecting and challenging strains within a host plant.

Table 1. Reactions of monoclonal antibody (MAb) to different strains of zucchini yellow mosaic virus (ZYMV) in indirect double-antibody sandwich (DAS) ELISA and plate-trapped (PTA) ELISA tests.

Samples [1]	PTA-ELISA [2]		DAS-ELISA [3]	
	F28 5H12F11	F28 3F1C4B2	F28 5H12F11	F28 3F1C4B2
ZYMV-CT	0.85[4]	0.84	1.25	1.04
ZYMV-FL	0.70	1.15	1.21	0.92
ZYMV-FR	0.71	1.20	1.11	1.31
ZYMV-WK	0.64	0.71	1.02	0.69
ZYMV-TW	0.08	1.24	0.06	1.20
Healthy zucchini	0.01	0.02	0.04	0.03
PBS[5]	0.001	0.002	0.002	0.001

1. ZYMV-CT, -FL, -FR, -WK, and -TW denote zucchini yellow mosaic virus Connecticut, Florida, French, French mild, and Taiwan strains, respectively.
2. Plates were coated with antigens at a 1:20 dilution. Samples were probed with undiluted culture supernatants followed by alkaline-phosphatase labelled goat anti-mouse IgG.
3. Plates were coated with rabbit polyclonal anti-ZYMV homologous strain IgG at 1 μg/ml. Samples were ground in 0.05M potassium phosphate buffer, pH 7.2, at a 1:20 dilution. The remaining steps were the same as those in PTA-ELISA test.
4. Values were the averages of the two experiments, and were read 30 min after addition of substrate.
5. PBS: phosphate-buffered saline.

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摘　要

王惠亮[1,2]、Dennis Gonsalves [3]. 1999 利用可區別矮南瓜黃化嵌紋病毒不同系統之單元及多元抗體在交互保護之研究. 植病會刊8:111-116. ([1.] 高雄市　國立高雄師範大學生物科學研究所；[2.] 聯絡作者：電子郵件hlwang@nknucc.nknu.edu.tw，傳真07-7169030；[3.] 美國紐約州　康乃爾大學植物病理系)

單元抗體F28 5H12F11用在直接與間接酵素連結抗體檢定法 (ELISA) 和抗體電子顯微鏡檢定法 (ISEM) 中，可區別矮南瓜黃化嵌紋病毒之台灣系統和其他系統包括Connecticut, Florida和法國系統。F28 5H12F11為一具IgG1重鏈和Kappa輕鏈之單元抗體。本研究應用此單元抗體以及只和台灣系統反應之多元抗體 (ZYMV-TW/CT)，在交互保護試驗中，探討輕症系統和重症系統之交互影響。ELISA結果顯示交互保護植株，在重症系統 (ZYMV-TW) 挑戰接種後，初期ZYMV-TW之病毒濃度不高，但如果交互保護不佳，則在嚴重病徵出現前ZYMV-TW之病毒濃度會增高；相反地，ZYMV-WK之病毒濃度則會明顯下降。在交互保護效果良好的植株，沒有嚴重病徵會出現，而ZYMV-TW之病毒濃度亦均維持在很低的程度。

關鍵字：矮南瓜黃化嵌紋病毒 單株抗體 交互保護