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Serological characterization of papaya ringspot virus isolates in Taiwan

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Abstract. Twenty-seven isolates of papaya ringspot virus (PRSV) were obtained from Taiwan. Differences in symptoms were observed. In addition to such isolates as necrotic severe mottle (SMN), severe mottle (SM) and mild mottle (M), another mottle type with deformation (DF) was isolated. All the isolates of PRSV and PRSV-W (PRSV type W) examined were serologically indistinguishable as determined by polyclonal antibodies-rabbit antisera to PRSV, but were consistently distinguishable with monoclonal antibodies (MAbs). Those isolates were raised against the PRSV. The virus isolates that reacted with the MAbs to PRSV were selected and applied to compare the antigenic specificity of the PRSV strain. Moreover, the immunoreactivities of the MAbs were compared with polyclonal rabbit serum to detect PRSV in foliar tissue by enzyme-linked immunosorbent assay (ELISA). Eleven selected MAbs were differentiated into five sero-groups according to sero-reaction patterns of ELISA index with 27 isolates of PRSV and PRSV-W. It was assumed that at least five antigenic epitopes were identified. Distinct results were obtained in application of MAb to ELISA for detecting PRSV strains in cucurbitaceous plants.

Keywords: Papaya ringspot disease; Plant virus; Serology.

Abbreviations: PRSV, papaya ringspot virus; **SMN,** necrotic severe mottle; **SM,** severe mottle; **M,** mild mottle; **DF,** deformation; **MAbs,** monoclonal antibodies; **ELISA,** enzyme-linked immunosorbent assay.

Introduction

Papaya ringspot virus (PRSV) causes one of the most prominent diseases in papaya (*Carica papaya* L.) and occurs wherever it is grown (Purcifull et al., 1984). PRSV, a type member of the genus potyvirus, is nonpersistently transmitted by aphids to papaya and members of the Chenopodiaceae and Cucurbitaceae families (Purcifull et al., 1984). In the late 1970s, the virus spread throughout Taiwan Island and destroyed most commercial papaya orchards (Wang et al., 1978; Wey et al., 1978). The PRSV strains that infect papaya, designated as PRSV-P, are distinct from the PRSV-W strains (formerly WMV-1), which are viruses of cucurbits that inflict serious economic damage. Papaya trees infected with PRSV-P are stunted, produce deformed fruits with some ringspots, and have decreased yields. Six distinct monoclonal antibodies (MAb) were obtained from the viral structural protein including PRSV-D-4-5-6 and PRSVN-TN-5 isolate (Chen, 1986). Several pathotypes of this virus have been described and identified as PRSV from Taiwan: SMN and SM (severe isolate), M (a mild isolate causing mosaic symptom), DF (a severe isolate causing deformed symptom) (Chang, 1979; Lin, 1980; Wang et al., 1978). In this study, we describe the production of PRSV-specific MAb and its ability to recognize other PRSV isolates.

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Materials and Methods

Virus Isolates

Each isolate of PRSV was collected from infected papaya in Taiwan and partially characterized by the apparent symptoms. All isolates were derived by serial passages of a single lesion through *Chenopodium quinoa* Wild., then cultured in a greenhouse in *Carica papaya* and in Zucchini squash *Cucurbita pepo* L. Next, single lesion isolates of PRSV, were thoroughly inoculated with the crude sap of infected tissues. The isolates of PRSV used in this study have been described earlier (Lin, 1980). The same isolate designations have been used in this paper (Table 1).

Purification of PRSV

Leaves of systemically infected zucchini, having been inoculated with leaf extracts of PRSV-infected papaya, were harvested 21~25 days after inoculation. The leaves were then homogenized with 0.5 M potassium phosphate, pH 7.5 (2 ml/g tissue), containing 0.01M EDTA and 0.1% sodium sulfite. Chloroform and carbon tetrachloride, each at 0.5 ml/g tissue, were added while the tissue was ground. After centrifuging the homogenate at 1,000 *g* for 10 min, polyethylene glycol MW 6,000 (PEG) was added to supernatant at a rate of 8 g/ 100 ml. Finally, the mixture was stirred for 1 or 2 h. The pellets were reextracted with 1 ml

Table 1. Sources and differentiated strains of papaya ring spot virus isolate cultured in papaya tree (*Carica papaya*), and Zucchini squash (*Cucurbita pepo* L.).

PRSV Isolates	Symptom[a]		Source[b]
	Zucchini	Papaya	
M-1-1	VC/I	SM/DF	1
M-2-1	VC/I	SM/DF	1
M-2-2	VC/M	SM/DF	1
M-2-3	VC/I	SMN	1
M-3-1	VC/M	SMN	1
M-3-2	VC/I	SM	1
M-4-1	VC/I	SM/DF	1
SM-1-1	VC/I	SMN	1
SM-1-2	VC/I	SMN	1
SM-2-1	VC/M	SMN	1
SM-2-2	VC/I	SM/DF	1
SM-2-3	VC/M	SM/DF	1
SM-3-1	VC/I	SM/DF	1
D-4-5-6	VC/S	M	2
SMN-1-1	VC/S	SM	1
SMN-1-2	VC/I	SM/DF	1
SMN-2-1	VC/I	SM/DF	1
Mnt	VC/I	SM/DF	2
TN-5	VC/S	DF	2
DF-1-1	VC/M	SM	1
DF-1-2	VC/I	DF	1
DF-2-2	VC/M	DF	1
DF-2-4	VC/I	DF	1
DF-3-1	VC/I	DF	1
WMV-1(TC-A)	VY	–	3
WMV-1(TC-C)	VY	–	3
WMV-1(F)	VY	–	4

[a]Symptom expression on Zucchini squash: VC = vein clearing; (M = mild, I = intermediate, S = severe); VY = mottle with vein yellowing; Symptom expression on papaya: SMN = severe mottle with necrosis, SM = severe mottle, SM/DF = severe mottle with part of deformation.

[b]Source origin: 1 = obtained by single lesion isolation; 2 = maintained by Su, H.-J. (Department of Plant Pathology, National Taiwan University); 3 = given by Hwang, C. -H. (Taiwan Agriculture Research Institute); 4 = given by Yeh, S. -D. (Department of Plant Pathology, National Chung Hsing University).

of extraction buffer per gram of starting tissue and centrifuged at 1,000 *g* for 5 min. The supernatant was combined and centrifuged at 10,000 *g*. Next, the pellets were reextracted with 1 ml of extraction buffer per gram of starting tissue and recentrifuged at 1,000 rpm for 5 min. The supernatants were then combined. Triton X-100 was added to a final concentration of 1% (V/V) and stirred for 30 min. Subsequently, PEG containing 0.1 M NaCl was added to a final concentration of 4% (V/V) and stirred for 1 to 2 h at 4°C. The mixture was further centrifuged in an HT rotor at 10,000 *g* for 20 min. Next, the pellets were resuspended in one-fifth the volume of the original volumes in 0.1 M potassium phosphate pH 7.2, containing 0.01 M EDTA, then centrifuged in a HT rotor at 10, 000 *g* for 10 min. The supernatants were passed through filter paper (TOYO No. 3). Next, PEG containing 0.1 M NaCl was added to a final concentration of 5% (W/V) and then stirred for 1 h at 4°C. The pellets were resuspended in 0.1 M sodium phosphate pH 7.0, containing 0.01 M EDTA, with 0.1% sodium azide (or 0.02 M Tris-HCL, pH 8.2).

Generation and Purification of Monoclonal Antibodies

Three 10-week-old female BALB/C mice were immunized intraperitoneally with 50 ug of purified PRSV emulsifed in Freund/s complete adjuvant. A second injection was given two weeks later with the same quantity of antigen in Freund's incomplete adjuvant. Fifteen days later, the mouse showing the highest titre of virus specific antibodies was boosted intraperitoneally with 50 ug purified virus. Four days later the spllenocytes were obtained from this mouse and fused with the myeloma cells (NS-1) using a standard protocol (Mirza et al., 1987). Selected hybridoma cells were cloned, expanded, and produced in vivo.

Production of Antisera

Purified antigen in 1.0 ml of 0.02 M Tris-HCl, pH 8.2 was emulsified with 1.0 ml of Freund complete adjuvant. A portion of the emulsion was injected into the subcutaneous area of New Zealand rabbit; the remaining emulsion was

injected intramuscularly with 2 mg virus preparation each time over a period of three weeks. Next, blood was collected weekly after the final injection.

Purification of Antibodies from Ascites and Polyclonal Sera

The IgGs were purified by affinity chromatography (Schots et al., 1992) and stored at -80°C at a concentration of 1 mg/ml. The IgGs from polyclonal anti-PRSV antibodies were purified according to the protocol of Clark and Adams (1977).

Characterization of MAbs

The hybridomas secreting antibodies that reacted with extracts of infected foliar tissues with PRSV were recloned in microtiter plates by limiting dilution (one cell per well) until reaching stability (Schots et al., 1992). The stable clones were cultured in flasks or ascites cultured in Balb/C mice to large-scale production of MAbs. The isotypes of heavy and light chains of each MAb produced were then determined by indirect ELISA using alkaline phosphatase-conjugated goat anti-mouse IgG antibodies specific for different mouse heavy- and light-chain polypeptides (Miles Scientific, Naperville, IL). MAbs were isotyped as described by Delaunay and Manouvriez (1990) as shown in Table 2.

ELISA Procedures

The double-antibody sandwich (DAS) method of ELISA described by Clark and Adams (Clark and Adams, 1977) was used to detect PRSV; however, the DAS test was not further evaluated because of a nonspecific background. Nevertheless, this problem could be averted by using the indirect method of ELISA (I-ELISA) (Chen, 1986).

Purification and Conjugation of MAbs

The MAbs were purified using a Protein-A column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Bound antibodies were eluted with a 0.1 M citric acid-NaOH buffer, pH 4.0. The purified antibodies were conjugated with AP as described by Tijssen (Tijssen, 1985).

Results

Detection of Viruses by I-ELISA

A newly prepared polyclonal antiserum and the monoclonal antibody (MAb) clones were applied to compare the antigenic specificity of PRSV strains. Also, a field inspection of PRSV-infected papaya and cucurbitaceous plants was performed. Distinct results were obtained in application of monoclonal antibodies to ELISA tests for detecting PRSV isolates in cucurbitaceous plants.

Sensitivity of MAb by I-ELISA

Indirect ELISA was used to titer supernatant and to ascites test its specificity against PRSV isolates. Two wells were coated with one of a series of twofold dilutions of MAbs in culture supernatant ranging in concentration from 1 to 3,200 reciprocal times dilution. For two hours after adding the substrate, the plate was read. Next, average absorbance readings at 405 nm were performed for each of the twofold series dilutions. According to those results, a nonspecific background markedly decreased, as indicated in the ELISA test with polyclonal antiserum to healthy antigen. The titer of antiserum from ascited fluid reached as high as 167,772,160 X; that of MAB-14 in ascited fluid without background was 327,680 X.

Table 2. Properties of monoclonal antibodies selected for this study and ELISA index showing the antigenic specificity of monoclonal of antibodies differentiated by different PRSV strains in indirect ELISA.

Monoclonal antibldy no.	Immunogen origin [a]	Isotype	PRSV isolates[c]									
			SMN-1	SMN-2	SM-1	M-1	DF-1	DF-2	DF-3	WMV-1	H	MAbs group[d]
3	PRSV-D-4-5-6	IgG2a	–	–	+++	–	–	–	–	–	–	I
4	PRSV-D-4-5-6	IgG2a	+++	+	+++	++	+++	+	++	+	–	II
12	PRSV-D-4-5-6	IgG2b	–	+++	++	+	–	+	–		–	I
13	PRSV-D-4-5-6	IgG2b	++++	++++	+++	+	+++	++++	+	+++	–	III
14	PRSV-D-4-5-6	IgG2b	++++	+	+	+	+++	+	+++++	++	–	V
33	PRSV-TN-5	IgG2b	+	++	++	–	++++	++++	–		–	III
41	PRSV-TN-5	IgG2b	++++	++	+++	+	++++	++	+		–	II
43	PRSV-TN-5	IgG1	+	+	+++++	–	+++++	+	+	–	–	IV
46	PRSV-TN-5	ND[b]	++++	++	++	++	+++	++	++		–	II
53	PRSV-TN-5	ND	–	–	–	+	+	–	–		–	I
63	PRSV-TN-5	IgG2a	+++++	+++++	–	+	+++++	–	+		–	II

[a]Cell lines are designated by the orignal immunogen used to produced and screen the line (PRSV-D-4-5-6 and PRSV-TN-5).
[b]ND = not determination.
[c]PRSV isolates used as differential virus strains for antigenic specificity of MAbs. SMN = severe mottle with necrosis type; SM = severe mottle type; M = mild mottle type; DF = leaf deformation; WMV-1 = Watermelon mosaic virus (PRSV-W); H = healthy.
[d]MAbs were differentiated into 5 groups according to serological reaction patterns.
[e]ELISA index: –, <0.1; +, 0.1~0.5; ++, 0.6~1.0; +++, 1.1~1.5; ++++, 1.6~2.0; +++++, >2.0.

Characterization of MAbs

Eleven MAbs produced by hybridoma cell lines 3, 4, 12, 13, 14, 33, 41, 43, 46, 53 and 63 were then selected and tested by indirect ELISA against PRSV isolates with respect to their specificity in reacting with mild mottle and severe mottle disease caused by PRSV (Table 1). The subclass of the immunoglobulins produced by the hybridomas was determined with an ELISA. Briefly, each hybridoma producing antibodies (Table 2) was tested in ELISA with rat-anti-mouse IgG1, IgG2a, IgG2b, and Kappa. Furthermore, the reaction of the antibodies with rat-anti-mouse isotype antibodies was visualized with a rat-anti-mouse immunoglobulin alkaline phosphatase.

The results presented herein demonstrate that the symptom expression of PRSV-P isolates and of PRSV-W on zucchini plants are similar. PRSV-infected papaya induces similar mottle symptoms and displays a yellow mosaic structure with leaf distortion (Table 1).

The results of using either rabbit anti-PRSV serum alone, MAb alone, or the two in combination were compared to detect PRSV. All assays readily distinguished between healthy and infected materials. The assay employing the rabbit source antibody as both the coating antibody and probe MAb antibody had the highest background reading among the other assays. The indirect double sandwich ELISA, in which plates were coated with antibodies from rabbit serum and samples probed with unfractionated MAb culture supernatant fluid followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibodies, gave quick detection and did not require the isolation of MAb from the culture supernatant fluid. The reactivity of MAbs raised to PRSV were evaluated in DAS-ELISA against a panel of PRSV isolates (Table 2) representative of the ones in Taiwan. Eleven hybridoma cell lines were selected for their specificity in reacting with PRSV isolates collected; each type was differentiated by its symptoms. Two MAbs, 13 and 14, were further selected for their sensitivity in reacting with PRSV crude extract preparation. Likewise, MAbs 4, 13, and 46 had the greatest reactivity to SMN, SM, and DF isolates. In this system, MAbs were differentiated into five sero-groups. MAb-I, recognizing a specific epitope, reacted with SM isolates strongly; meanwhile, it did not react with SMN, DF or PRSV isolates. MAb-II and III, recognizing a common epitope, reacted with the all isolates; however MAb-II reacted weakly with M and PRSV-W isolates. MAb-IV, which recognized another specific epitope, reacted strongly with SM and DF isolates while not reacting with M and PRSV-W isolates and only weakly reacting with SMN isolates. Meanwhile, these MAbs did not react with M isolates, only weakly reacted with SM isolates, and moderately reacted with PRSV-W isolate. In the ELISA test, SM-1 isolates of this virus were recognized with all MAbs; meanwhile, in the system, MAbs I reacted only with SM-1 and did not detect DF virus type or else reacted weakly to the other isolates. However, polyclonal antisera are not able to specifically distinguish each PRSV we isolated.

Discussion

Although differences occur in the severity of symptoms, as measured by host reactions of PRSV isolates (Lin, 1980; Wang et al., 1978), the PRSV-P isolates from Hawaii and Florida are considered serologically indistinguishable from PRSV-W (Purcifull and Hiebert, 1979; Quiot-Douime et al., 1986; Yeh et al., 1984; Yeh and Gonsalves, 1984). This study has attempted to detect each isolate and differentiate it by serology. The results presented herein confirm that our MAbs can be used to raise specific type, SM, and M type isolates. To distinguish curcurbit potyvirus, all the MAbs also tested failed to react with WMV-2 and ZYMV. Herein, the infected plant included zucchini squashes or papaya trees have been used to raise antibodies detect PRSV with varying extraction buffer to raise optimal results. In previous trials, papaya strains of PRSV have proved to be extremely complicated and unstable (Mieko, 1986). In addition to the three isolates formally found—SMN, SM and M—another mottle isolate (DF) was more stable than the isolates of SMN or SM type.

There is a serological difference of at least one epitope in the capsid proteins of PRSV-W and PRSV-P (Baker et al., 1991). However, this is not surprising in light of the fact that there are eight amino acid differences in these capsid proteins (Quemada et al., 1990). Experimental results demonstrate that the monoclones of MAbs fell into five sero-groups according to sero-reaction patterns of the ELISA index with twenty-seven papaya strain isolates and PRSV-W strain of PRSV. It was assumed that five distinct antigenic epitopes at least were identified.

Serological studies with the complicated PRSV isolates must take into account that PRSV is variable in symptom. A previous report demonstrated that, heretofore unknown, serological and biological differences exist in the Florida population of PRSV-W (Baker et al., 1991). The production of monoclonal antibodies and polyclonal anti-sera in rabbit is an efficient means of using long-term maintenance to produce antibodies. The monoclonal antibody from ascites can yield high amounts and titers of antibody comparable to cell cultured supernatant, with varying buffer of antigen extraction, thereby improving detection by ELISA. The fact that the reactions of these PRSV isolates were serologically very homogenous with polyclonal antiserum accounts for why a MAb array consisting of MAb-I, II, III and IV, could be applied to identify PRSV isolates according to seroreaction pattern. Comparison was made of the two PRSV genomes. Taiwan and Hawaii isolates share an overall nucleotide identity of 83.4%. These data also indicate that evidence of variability exists at the amino acid and nucleotide levels, particularly between Asian isolates (Bateson et al., 1994). The comparison of the whole genome can only be done after the nucleotide sequences of the viruses have been completely elucidated. The differences among PRSV isolates (SMN, SM, M and DF) also provide the opportunity to analyze the biological proper-

ties-strain variation interaction for infecting papaya. These MAbs will be selected in the future to develop a diagnostic kit for PRSV in the field.

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木瓜輪點病毒系統之血清學特異性

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木瓜輪點病毒由國內分離出27個不同型系統分離株，發現木瓜輪點病毒病徵型系統之變異，包括壞疽嵌紋型、重嵌紋型、輕嵌紋型外，尚有畸型嵌紋型等系統，這些木瓜病毒與另一非木瓜系統之西瓜嵌紋病毒第一型，同時在矮南瓜病徵表現做比較。利用製備之多元抗體，無法區分所有木瓜輪點病毒分離株和西瓜嵌紋病毒，而以製備之單元抗體系列則可區分。一些病徵型與單元抗體血清型反應型不儘吻合，由各單元抗體與 27 個不同型系統分離株利用酵連抗體免疫標誌法之反應型可歸納 11 株單元抗體爲 5 群，推測此病毒至少具5種判讀抗原基，使用單元抗體有利於鑑定瓜類中病毒系統。

關鍵詞：植物病毒；木瓜輪點病；血清學。