Production of Monoclonal Antibodies Against a Mycoplasmalike Organism Associated with Sweetpotato Witches' Broom

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ABSTRACT

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Five stable hybridoma clones secreting specific monoclonal antibodies against the sweetpotato witches' broom (SPWB)-mycoplasmalike organism (MLO) were produced by employing an immunization scheme for the induction of the immunological tolerance of mice to plant antigens prior to the administration of MLO immunogens. Neonatal BALB/c mice were injected first with nontarget plant antigens present in an immunogen preparation and were immunized with MLO-enriched antigens prepared by Percoll density-gradient fractionation or by enzyme treatments 6 wk later. With these monoclonal antibodies, SPWB-MLO in diseased sweetpotato and periwinkle was specifically identified by indirect enzyme-linked immunosorbent assay (ELISA) and by immunofluorescent staining. Antibody titers for hybridoma-culture supernatants (measured by indirect ELISA) ranged from 320 to 5,120 for SPWB-MLO in diseased periwinkle. SPWB-MLO was differentiated serologically from MLOs associated with loofah, paulownia, and *Ipomoea obscura* witches' brooms, aster and elm yellows, and rice yellow dwarf in both ELISA and immunofluorescent staining. All monoclonal antibodies cross-reacted with peanut and asparagus bean witches' broom MLOs and MLOs associated with the witches' broom weeds, *Rhynchosia minima* and *Alysicarpus vaginalis*, found on the Penghu Islands, where there are serious occurences of SPWB.

Additional keywords: hybridoma techniques, immunodiagnostics.

Sweetpotato witches' broom (SPWB) disease, like most witches' broom diseases, is associated with a mycoplasmalike organism (MLO). SPWB-MLO-infected plants exhibit characteristic symptoms of MLO diseases, such as small leaves, shortening of internodes, stunting of plants, excessive proliferation of shoots, formation of witches' broom, sterility of flowers, and small tuberous roots that result in significant yield reduction. SPWB disease was first reported on Aguni Island of the Ryukyu Islands in 1947 (19,21). In 1970, an MLO was shown to be in constant association with SPWB (11). SPWB disease has now been recorded in many countries and areas worldwide, including Taiwan (1,3,9,10,20,22,23).

In Taiwan, SPWB disease was first discovered in a geographically isolated area, the Penghu Islands, in 1969 (23). The disease occurs annually because of climate, cultivation practices, and simplicity of domestic plants on the Penghu Islands.

Like other phytopathogenic MLOs, the MLO associated with SPWB disease remains unculturable. Detection of SPWB-MLO is always difficult and time-consuming. Only a few monoclonal antibodies against a limited number of phytopathogenic MLOs have been developed (2,5,7,15,17,18) because of the difficulties encountered in preparing pure MLO immunogens. Host contaminants present in the MLO immunogens are primarily responsible for these problems.

In this paper, we report the production of monoclonal antibodies specific to SPWB-MLO by employing an immunization scheme that included the induction of immunological tolerance to plant antigens in mice prior to the administration of the MLO immunogen. The serological relatedness among various phytopathogenic MLOs was determined by ELISA and by immunofluorescent staining with the monoclonal antibodies.

MATERIALS AND METHODS

SPWB-MLO-infected plants. Sweetpotato (*Ipomoea batatas* (L.) Lam.) naturally infected with SPWB-MLO was collected from fields on the Penghu Islands. SPWB-MLO-infected periwinkle (*Catharanthus roseus* (L.) G. Don) originally obtained by transmission through dodder (*Cuscuta australis* R. Brown) was kindly provided by I. L. Yang (Taiwan Agricultural Research Institute, Wufang, Taiwan) and was maintained and propagated by side grafting.

Antigen preparation. Threefold (1 ml of antigen suspension derived from 3 g of fresh leaf midribs) concentrated stock solutions were prepared from leaf midribs of SPWB-MLO-infected periwinkle and healthy periwinkle. Suspensions were prepared, as previously described, by an enzyme-treatment method (12) that involved the use of tissue-macerating enzymes for treating vascular tissues from leaf midribs. The stock solution of MLO-enriched antigen was used to immunize mice, screen hybridomas, and evaluate detection sensitivities and ELISA titers of antibodies. The stock solution of healthy plant extract was used as the immunogen for inducing tolerance of mice to nontarget antigens (5). Additionally, a method, described by Jiang and Chen (6), employing Percoll density-gradient fractionation also was applied to produce another type of MLO immunogen but with a modified Percoll density gradient of 20, 35, and 55%. MLO antigen was collected near the interphase between 35 and 55% Percoll layers and was used directly as immunogen for immunizing the mice.

Immunization. The immunization scheme for hybridoma production was similar to that described by Hsu et al (5). Five neonatal BALB/c mice were each injected intraperitoneally with 20 μl of normal healthy plant antigens within 24 h after birth. The second intraperitoneal injection was made 7 days later with 20 μl of the same preparations. When the mice were 6 wk old, three mice were injected intraperitoneally with 200 μl of MLO-enriched antigen prepared by the enzyme-treatment method, and the other two were injected intraperitoneally with 200 μl of the

partially purified MLO antigen prepared by the Percoll density-gradient method. Their spleens were removed for cell fusions 3 days after the final injection.

Monoclonal antibody production. The procedure for splenic cell collection and fusion with murine myeloma cells (P3-NS1/1-Ag4-1) was similar to those described by Lin and Chen (13–15). RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Laboratories; complete medium) was used for routine cultivation of myeloma and hybridoma cells. After fusion, hybridomas grown in HAT selective medium (complete medium supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) were identified with the aid of an inverted microscope. The culture fluids from wells containing hybridomas were screened for the presence of SPWB-MLO-specific antibodies by indirect ELISA with the peroxidase enzyme system after the procedure described previously (16). ELISA plates were first coated with antigen preparations from SPWB-MLO-infected and healthy periwinkles, respectively. The coating antigens used were prepared by the enzyme-treatment method, as described in antigen preparations, but were diluted 24-fold from the threefold concentrated stock solutions in 0.05 M carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$ adjusted to pH 9.6 with HCl as needed) to 0.125 g/ml (fresh weight of leaf midribs per milliliter of antigen preparation). Hybridomas that secreted antibodies reacting with diseased but not with healthy plant preparations were selected for further cloning by limiting dilution (4). Monocloned hybridoma cells were subcultured for antibody production and stored in liquid nitrogen for further use.

Antibody isotype determination. Antibody class was determined by indirect ELISA with the mouse monoclonal subisotyping kit (Hyclone Laboratories, Inc., Logan, Utah), according to the vendor's instructions. Monoclonal antibodies used in this study were harvested from hybridoma-culture supernatants when cell titers reached 1×10^7 cells/ml and were used undiluted.

ELISA titer of antibody. Indirect ELISA with a peroxidase system was used in the ELISA titer tests of monoclonal antibodies. Monoclonal antibodies used in the study also were harvested from hybridoma-culture supernatants, as in antibody isotype determinations. A 1:2 dilution series of culture supernatants prepared in 0.02 M phosphate-buffered saline (PBS, pH 7.4) was tested with a minimum dilution of 1:20. Coating antigens used in the tests were prepared from the leaf midribs of both SPWB-MLO-infected and healthy periwinkles by the enzyme-treatment method, and the concentration of coating antigens applied in the test was diluted from the threefold concentrated stock solutions to 0.125 g/ml in 0.05 M carbonate buffer (pH 9.6). ELISA titer of antibody was determined as the greatest dilution of antibody that gave a positive ELISA reaction in which the absorbance value at 490 nm was greater than 0.1.

Antibody sensitivity test. Indirect ELISA also was used to determine the detection sensitivity of the SPWB-MLO monoclonal antibodies for SPWB-MLO in diseased periwinkle and sweetpotato. Monoclonal antibodies were harvested from hybridoma-culture supernatants and were used undiluted. Three types of SPWB-MLO antigen preparations at various dilutions were prepared from diseased periwinkle and sweetpotato. The antigen preparations from the midrib tissues of both SPWB-MLO-infected and healthy periwinkles were obtained by the enzyme-treatment method (12). The antigen preparations from whole leaves of SPWB-MLO-infected and healthy periwinkles and sweetpotatoes were obtained by following the method of Jiang and Chen (6), except these preparations were not subjected to density-gradient centrifugation. For each of the three antigen preparations, a 1:2 dilution series prepared in carbonate buffer (pH 9.6) was used in the tests, with a minimum dilution at a concentration of 1 g/ml. The results of the antibody sensitivity test were compared as the dilution endpoint of antigen preparations. The values were expressed as the reciprocal of the greatest dilution of antigen that produced an A_{490nm} value greater than 0.1.

Immunofluorescent staining. Cross sections of leaf midribs, stems, and roots were prepared from healthy and SPWB-MLO-infected periwinkle and sweetpotato by freehand sectioning. These sections were fixed in cold acetone for 40 min, air-dried at room temperature, and rinsed with three changes of PBS (pH 7.4) at 30-min intervals. Tissue sections were incubated 4 h at room temperature in 2 ml of undiluted hybridoma-culture supernatants. RPMI-1640 complete medium was used in place of hybridoma-culture supernatant as an antibody control. The sections were washed three times in PBS and incubated in PBS at 4 C overnight. The sections were incubated in 2 ml of fluorescein isothiocyanate (FITC)-conjugated antimouse IgG + IgA + IgM (Caltag Laboratories, San Francisco, CA) diluted 75-fold in PBS for an additional 4 h at room temperature.

Samples were mounted on microslides with glucose PBS (9:1, v/v) and examined with an Olympus epifluorescence microscope with an HBO 100-W high-pressure mercury lamp and a combination of filters (a BP-495 excitation filter, a DM 505 dichroic mirror, and a 515 IF barrier filter) to generate an excitation with the main wavelength at 495 nm. Photomicrographs were made with a 35-mm Olympus automatic photomicrographic system (PM-10ADS) with ASA 400 film (Kodak Tri-X Pan).

Antibody specificity. Indirect ELISA and immunofluorescent staining were used to test the specificity of SPWB-MLO monoclonal antibodies and to reveal the serological relatedness among various phytopathogenic MLOs. Twelve MLO preparations tested in this study were prepared from diseased plants of sweetpotato, *Ipomoea obscura*, loofah, paulownia, peanut, and asparagus bean witches' brooms, aster (western and New Jersey strains) and elm yellows, rice yellow dwarf (kindly provided by C. C. Chen, Taichung District Agricultural Improvement Station, Changhua, Taiwan), and witches' broom diseases of *Rhynchosia minima* (L.) DC. and *Alysicarpus vaginalis* (L.) DC. Among these, MLO preparations from diseased plants of rice yellow dwarf and peanut, asparagus bean, *R. minima*, and *A. vaginalis* witches' brooms were prepared from original hosts; all the others were prepared from infected periwinkle. Test antigens for ELISA were prepared from healthy and MLO-infected plants in the same manner, as the preparation of partially purified coating antigens from whole leaves; the concentration of coating antigens was 0.25 g/ml diluted in carbonate buffer (pH 9.6). Cross sections of leaf midribs were prepared for the immunofluorescent staining from healthy and MLO-infected plants.

RESULTS

Five stable hybridoma clones that secreted monoclonal antibodies specific for SPWB-MLO, MA6, MA16, MA21, MA35, and MA40, from five independent fusions were selected. Two were obtained from mice immunized with immunogen prepared by Percoll density-gradient fractionation, and three were from mice immunized with immunogen prepared by enzyme treatments of leaf midribs. The monoclonal antibodies were all of the IgM

TABLE 1. Dilution endpoints of various sweetpotato witches' broom-mycoplasmalike organism (SPWB-MLO)-enriched antigen preparations reacted with SPWB-MLO monoclonal antibodies in indirect ELISA[a]

Hybridoma clone[b]	Antigen preparation[c]		
	Midrib, periwinkle	Whole leaf, periwinkle	Whole leaf, sweetpotato
MA6	1,024	512	128
MA16	1,024	256	128
MA21	128	64	32
MA35	1,024	512	256
MA40	2,048	1,024	512

[a] Dilution endpoint is the reciprocal of the greatest dilution of antigen diluted from the original antigen preparation at a concentration of 1 g/ml (fresh weight of indicated plant tissue per milliliter of antigen preparation) that gave an $A_{490\,nm} > 0.1$.

[b] Monoclonal antibodies tested were harvested from culture supernatants of hybridoma clones when cell titers reached 1×10^7 cells/ml and were used undiluted.

[c] Antigen was prepared and diluted in 0.05 M carbonate buffer, pH 9.6.

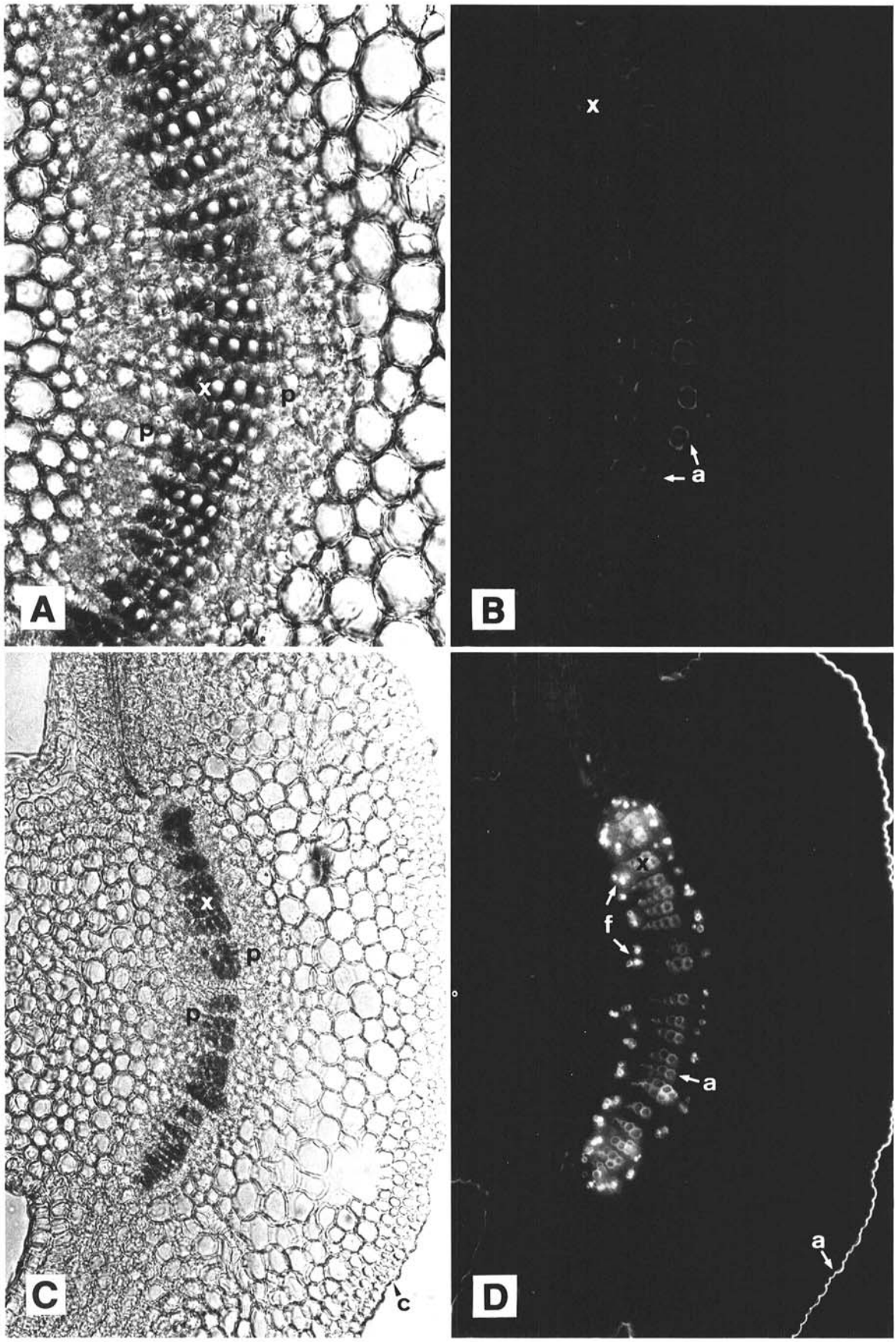

Fig. 1. Immunofluorescent staining on cross sections of leaf midribs from healthy and diseased periwinkles reacted with the monoclonal antibody for sweetpotato witches' broom-mycoplasmalike organism from undiluted culture supernatant of hybridoma clone MA40. **A and B,** cross sections of healthy periwinkle; **C and D,** cross sections of diseased periwinkle. **A and C,** bright-field micrographs showing the locations of cuticle (c), xylem (x), and phloem (p) areas and revealing the size difference between healthy and diseased midrib tissues under the same magnification; **B and D,** fluorescent micrographs of the same microscopic fields as those of **A and C**, respectively, showing yellowish autofluorescence (a) in cuticle and xylem areas of both healthy and diseased sections, and the fluorescein isothiocyanate-specific apple-green fluorescence (f) in phloem tissues of the diseased section. (×100).

class and reacted specifically with the SPWB-MLO preparations from diseased periwinkle and sweetpotato.

Antibody ELISA titers of hybridoma-culture supernatants for MA6, MA16, MA21, MA35, and MA40 were 640, 1,280, 80, 1,280, and 5,120, respectively. The monoclonal antibody from MA40 had the highest ELISA titer when measured against SPWB-MLO-enriched diseased preparations at a concentration of 0.125 g/ml and also produced the highest ELISA absorbance value among these five monoclonal antibodies at the same antibody dilutions.

Dilution endpoints of SPWB-MLO antigens that reacted with these five monoclonal antibodies for SPWB-MLO also were determined in three different antigen preparations (Table 1). The dilution endpoints of the midrib preparations from diseased periwinkle ranged from 128 to 2,048. Dilution endpoints ranged from 64 to 1,024 and from 32 to 512 when coating antigens were prepared from whole leaves of either SPWB-MLO-infected periwinkle or sweetpotato, respectively. Both of the sample sources prepared from periwinkles produced higher dilution endpoints for all five monoclonal antibodies when compared with samples prepared from sweetpotato. The supernatant from clone MA40 was consistently more sensitive than from other monoclonal antibodies (Table 1).

Representative micrographs showing the results of immunofluorescence tests on the cross sections of midribs from both healthy and SPWB-MLO-infected periwinkles stained with monoclonal antibodies from undiluted hybridoma-culture supernatant of clone MA40 are shown in Figure 1. Only phloem elements of the diseased section packed with SPWB-MLO showed FITC-specific fluorescence. All five monoclonal antibodies reacted positively to the SPWB-MLO in the cross sections of midribs, stems, and roots of infected periwinkle and sweetpotato in immunofluorescent staining. The phloem elements of diseased sections exhibited FITC-specific, bright apple-green fluorescence, whereas those of healthy sections did not fluoresce. The cuticle and xylem areas from both healthy and diseased plants exhibited greenish- or brownish-yellow autofluorescence that could be easily differentiated from specific apple-green immunofluorescence. Neither healthy nor diseased sections treated with RPMI complete medium, instead of monoclonal antibody, in the antibody treatment step exhibited any FITC-specific fluorescence.

In the antibody specificity test, all five monoclonal antibodies reacted specifically in ELISA and in immunofluorescent staining with SPWB-MLO-infected but not with healthy periwinkle tissues. In addition, the SPWB-MLO monoclonal antibodies used in ELISA and in immunofluorescent staining recognized preparations from plants infected with MLOs that cause peanut, asparagus bean, *R. minima*, and *A. vaginalis* witches' brooms collected from the Penghu Islands (Table 2). The antibodies did not react either in ELISA or in immunofluorescent staining with preparations from plants infected with the diseased agents that cause *I. obscura*, loofah, and paulownia witches' brooms, aster (both western and New Jersey strains) and elm yellows, and rice yellow dwarf.

DISCUSSION

A standard immunization scheme to efficiently produce monoclonal antibodies requires relatively pure immunogens. MLOs are wall-less procaryotes and are restricted to phloem tissues in infected plants. All attempts to culture phytopathogenic MLOs have been unsuccessful. Some efforts have been made to enrich MLO preparations from infected plants, but host contaminants present in the MLO-enriched preparations make the production of monoclonal antibodies very difficult. Procedures employing Percoll density-gradient centrifugation (6) and enzyme treatments (12) are the most promising methods available for purification of MLOs from diseased plants. Modified Percoll density combinations of 20, 35, and 55% have been carried out in our lab for a better fractionation of SPWB-MLO. The enriched MLO immunogens prepared by either method, along with the conventional immunization scheme were applied in our lab initially for the production of monoclonal antibodies for SPWB-, peanut witches' broom-, and rice yellow dwarf-MLOs. More than 10 independent fusions for each organism were conducted without success because of contamination of plant antigens. After employing the tolerance-inducing procedure described by Hsu et al (5), we successfully developed the SPWB-MLO monoclonal antibodies with the immunogen prepared either by Percoll density-gradient fractionation or by the enzyme-treatment method with tissues from the leaf midrib. This immunization strategy, which induces immunological tolerance to plant antigen in mice prior to immunization with the target antigen (5), is now practiced routinely in our lab to facilitate the recent production of monoclonal antibodies against rice yellow dwarf-MLO (F. L. Chang and C. P. Lin, *unpublished data*).

The monoclonal antibodies produced in this study were all of the IgM class. This may due to the early collection of splenocytes three days after the only immunization shot. Antibodies of the IgG class (a more efficient serological ligand for specific chromatography matrix) can be obtained by applying a booster injection 1 wk after the immunization shot.

In addition, DNA probes for peanut witches' broom- and SPWB-MLOs recently have been developed in our lab and applied in the identification and differentiation of these serologically related MLOs. Similar southern hybridization patterns were obtained among DNA from SPWB-MLO and other phytopathogenic MLOs found on the Penghu Islands in preliminary studies (H. C. Ko and C. P. Lin, *unpublished data*). The serological and genetic relatedness among SPWB-MLO and other MLOs can be further explored by the DNA probes along with the monoclonal antibodies.

The monoclonal antibodies produced in this study are currently used to investigate the distribution of SPWB-MLO in various parts of infected plants and in insect vectors with immunofluorescent staining. MLO purified by the affinity chromatography procedure was reported to be valuable for the production

TABLE 2. Specificity of sweetpotato witches' broom-mycoplasmalike organism (SPWB-MLO) monoclonal antibodies[a] for MLOs associated with witches' broom diseases in various plants, determined by immunofluorescent staining and enzyme-linked immunosorbent assay (ELISA)

MLO-infected or healthy plant preparation[b]	Hybridoma clone				
	MA6	MA16	MA21	MA35	MA40
Sweetpotato wb	F[c]/2+[d]	F/2+	F/1+	F/3+	F/4+
Ipomoea obscura wb	NF/−	NF/−	NF/−	NF/−	NF/−
Loofah wb	NF/−	NF/−	NF/−	NF/−	NF/−
Paulownia wb	NF/−	NF/−	NF/−	NF/−	NF/−
Aster yellows (western)	NF/−	NF/−	NF/−	NF/−	NF/−
Aster yellows (NJ)	NF/−	NF/−	NF/−	NF/−	NF/−
Elm yellows	NF/−	NF/−	NF/−	NF/−	NF/−
Healthy periwinkle	NF/−	NF/−	NF/−	NF/−	NF/−
Rice yellow dwarf	NF/−	NF/−	NF/−	NF/−	NF/−
Healthy rice	NF/−	NF/−	NF/−	NF/−	NF/−
Peanut wb	F/2+	F/2+	F/1+	F/2+	F/3+
Healthy peanut	NF/−	NF/−	NF/−	NF/−	NF/−
Asparagus bean wb	F/2+	F/1+	F/1+	F/2+	F/3+
Healthy asparagus bean	NF/−	NF/−	NF/−	NF/−	NF/−
Rhynchosia minima wb	F/1+	F/1+	F/1+	F/2+	F/2+
Healthy *R. minima*	NF/−	NF/−	NF/−	NF/−	NF/−
Alysicarpus vaginalis wb	F/1+	F/1+	NT/1+	NT/2+	F/2+
Healthy *A. vaginalis*	NF/−	NF/−	NT/−	NT/−	NF/−

[a] Undiluted hybridoma-culture supernatants after cultures reached 1×10^7 cells/ml.

[b] Wb = witches' broom. Rice yellow dwarf and peanut, asparagus bean, *R. minima*, and *A. vaginalis* witches' brooms were prepared from original hosts; all the others were prepared from infected periwinkles. The coating antigens for ELISA were prepared from whole leaves and diluted to 0.25 g/ml (fresh weight of leaves per milliliter of antigen preparation) in carbonate buffer, pH 9.6.

[c] Results of immunofluorescent staining: F = specific immunofluorescent staining in phloem tissue; NF = no fluorescent staining observed; NT = not tested.

[d] Results of ELISA (A_{490nm}): 0.0–0.1 (−); 0.1–0.2 (1+); 0.2–0.4 (2+); 0.4–0.6 (3+); 0.6–0.8 (4+); 0.8–1.0 (5+); 1.0–1.2 (6+); 1.2–1.4 (7+); 1.4–1.6 (8+); 1.6–1.8 (9+).

of MLO-specific polyclonal antibodies and also for the generation of whole arrays of monoclonal antibodies specific for various MLO antigens (8). These antibodies are now used as antigen-specific ligands coupled to a chromatographic matrix to isolate SPWB-MLO. Close evaluation of serological relatedness among various MLOs will become possible with these MLO-specific polyclonal or monoclonal antibodies.

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