Production of Monospecific Polyclonal Antibodies Against Aster Yellows Mycoplasmalike Organism-Associated Antigen

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ABSTRACT

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Proteins of mycoplasmalike organisms (MLO) associated with aster yellows were detected by Western blotting in partially purified preparations from aster yellows-infected plants. Polyclonal antiserum produced against aster yellows (AY) MLO recognized a 23-kDa protein in AY-infected plants that was not found in healthy controls. In addition, this antiserum recognized proteins common to AY-infected and healthy samples. Cross-absorption of the PA with healthy plant extracts did not completely eliminate the nonspecific antibodies. Antibodies specific for AY MLO-associated protein were purified from polyclonal antibodies by trapping them on AY MLO protein obtained by electrophoresis of infected plant extracts and transferred onto nitrocellulose. The resultant monospecific antibodies, eluted from the membrane, reacted with specific AY MLO-associated protein. The monospecific polyclonal antibodies reacted with AY isolates from Oklahoma carrot and lettuce but not with other MLO and spiroplasma isolates tested.

Aster yellows (AY), an intensively studied plant disease, affects more than 350 plant species (25). The disease causes devastating economic losses in vegetable, horticultural, and agronomic crops (25). The causal agent of AY, a noncultured mycoplasmalike organism (MLO), is transmitted by the aster leafhopper, *Macrosteles quadrilineatus* Forbes (formerly *M. fascifrons* (Stal)) (18). MLO-associated diseases were traditionally differentiated by symptomatology, host range, and vector–pathogen relationships. The presence of MLOs in the phloem tissue of infected plants can be demonstrated by fluorochromic DNA stains (3,14,15), Dienes' stain (6,8), and electron microscopy. However, none of these methods discriminate among MLOs or are suitable for epidemiological studies. Electron microscopy, in addition, is time-consuming and involves expensive equipment and reagents. In recent years, molecular probes using DNA or RNA of maize bushy stunt (3), Western X-disease (17), Eastern aster yellows (5,20), little leaf disease of periwinkle (4), witches' broom of pigeon pea (13), and others (22,24) were developed for MLO detection and identification.

Immunological methods for detecting MLOs have the potential to be highly specific and are valuable for detection (27,28). Polyclonal antisera (PA) have been produced against different strains of AY MLO in several laboratories (15,16,23,27,28). All such antisera contain antiplant antibodies that cross-react with plant material. Monoclonal antibodies (MAbs) are highly specific, but the production of a suitable MAb is tedious and expensive. Also, because the MAb recognizes only a single epitope, it may fail to detect infections with nonhomologous AY MLO strains (23). In 1986, Lin and Chen (23) compared anti-AY PA and a MAb for detection of AY MLO. Their MAb reacted specifically to AY MLO antigen and not to other MLOs, but their PA cross-reacted with plant antigen in infected samples.

AY was recently reported in 80% of lettuce plants (*Lactuca sativa* L.) and 28% of carrot plants (*Daucus carota* L.) in trap plots in Oklahoma (9,10). We report the production of PA to an AY MLO strain from Oklahoma and the affinity purification of AY MLO monospecific polyclonal antibodies from the PA with an immunoelectroblotting technique. We also report detection with these antibodies of AY-specific antigen in Western blots of partially purified preparations from infected plants. A preliminary report has been published (11).

MATERIALS AND METHODS

Diseased plants. Carrot plants showing AY symptoms were removed from field plots in southeastern Oklahoma, potted with field soil, sprayed with malathion (Ferti-Lome, Bonham, TX), and moved to the greenhouse. The AY MLO (designated AY-OC 1) was transmitted to China aster (*Callistephus chinensis* L.) by the aster leafhopper, *M. quadrilineatus* (8). The isolate was characterized as an AY MLO based on symptomatology on aster, periwinkle, and celery, Dienes' stain, and hybridization of AY-OC 1 to AY MLO DNA probes (9,21).

Infected leafhoppers as source of AY MLO. AY-OC 1 was maintained on asters by serial transfers using *M. quadrilineatus* (8,9). Late instar nymphs were allowed to feed on AY-OC 1–infected asters for 7 days and then on barley (*Hordeum vulgare* L. 'Post') for 2 wk. Representative leafhoppers from each group were caged on healthy asters to confirm that they were inoculative. The remainder were frozen (−20 C) in batches of 100. Leafhoppers from those groups that successfully inoculated asters were selected for AY purification.

Purification of AY MLO. AY-OC 1 was partially purified from aster leafhoppers by a method similar to that of Kirkpatrick et al (17) for Western X-disease MLO. One thousand AY-OC 1–infected aster leafhopper heads (total weight approximately 0.75 g), mixed 1:4 (w/v) in extraction buffer (17), were homogenized with a pestle and clarified (1,100 *g* for 5 min). The supernatant was centrifuged (15,000 *g* for 30 min), and the pellet, containing MLO, was resuspended in 1 ml of extraction buffer without 2-mercaptoethanol. This suspension was clarified (2,500 *g* for 3 min), and the resulting supernatant was filtered (1.2 μm). Aliquots were frozen at −20 C. The procedure was completed within 1 day to minimize loss of antigen activity.

The presence of antigen was monitored after each step of the purification process by protein-A sandwich–enzyme-linked immunosorbent assay (PAS ELISA) as described (7). Reagents were applied to the microtiter plates in the following sequence: 1) protein A (1 μg/ml in carbonate coating buffer), 2) anti-AY MLO

PA (a gift from B. C. Kirkpatrick, University of California, Davis) (1:800 in phosphate-buffered saline containing 0.05% Tween 20 [PBST]), 3) partially purified plant extracts (1:100 in PBST), 4) anti-AY MLO PA (different dilutions in PBST) as detecting antiserum, 5) protein A conjugated to alkaline phosphatase (1:1000 in PBST), and 6) substrate (1 mg/ml in diethanolamine buffer; Sigma Chemical Co., St. Louis, MO). The color reaction was read in an EIA reader, model EL-307 (Bio-Tek Instruments Inc., Winooski, VT), at 405 nm.

Preparation of PA. Antiserum was produced in a white New Zealand female rabbit by injecting 1 ml of freshly purified AY-OC 1 preparation emulsified with an equal volume of Freund's incomplete adjuvant. One-half of this mixture was injected intramuscularly in the hip, and the remaining one-half was subcutaneously injected in the neck of the rabbit. Three injections were given at 1-wk intervals and then three more at biweekly intervals. Three weeks after the sixth injection, blood was collected, and the serum was clarified by low-speed centrifugation. Aliquots were stored at −20 C.

Specificity of this AY PA was evaluated by PAS ELISA. In this test, AY antiserum (provided by B. C. Kirkpatrick), diluted 1:800 in PBST, was used as coating antiserum, and fourfold serial dilutions of PA produced against the Oklahoma AY isolate were used as probing antisera. AY-OC 1 was detected in AY-infected hosts, both plants and leafhoppers. The dilution end point for the AY-OC 1 antiserum was 1:800. Thereafter, in all PAS ELISA testing, anti-AY-OC 1 serum was used for both coating and probing.

Test antigens. MLOs were transmitted from field-collected carrot, lettuce, and daisy fleabane (*Erigeron strigosus* Muhl. ex. Willd.) with "yellows"-type symptoms to aster and periwinkle by *M. quadrilineatus* in the greenhouse. MLOs were partially purified from extracts of field-collected diseased carrot, lettuce, and daisy fleabane and from greenhouse-infected aster and periwinkle. Leaf and stem tissue were homogenized and filtered through two layers of cheesecloth. The homogenate was subjected to differential centrifugation as described (18).

Separation and electroblotting of AY MLO proteins. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as reported by Laemmeli (19). Partially purified samples (400 μl at 4 mg/ml) were mixed with equal volumes of sample buffer (0.5 M Tris HCl, pH 6.8, 10% [v/v] glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.1% [w/v] bromophenol blue) and boiled for 3 min. A volume of 40 μl (16 μg) per lane was loaded onto each of 20 lanes of a 14 cm long and 3 mm thick 3/10% discontinuous polyacrylamide gel and electrophoresed 16 h at 7 mA per gel at room temperature. Standard molecular mass markers (Diversified Biotech, Newton Centre, MA) were loaded onto adjacent lanes. Electrophoretically separated proteins were transferred to nitrocellulose as described (12).

Cross-absorption of PA. Proteins of healthy periwinkle subjected to partial purification and separated by SDS-PAGE were transferred to nitrocellulose and incubated for 3 h with anti-AY MLO PA serum diluted 1:40 in TBS (Tris-buffered saline). Cross-absorption of the serum was repeated five times. After each absorption, 1 ml of the serum was removed and stored at −20 C for evaluation by Western blotting.

Production of AY MLO monospecific polyclonal antibody. The procedure for antibody purification was modified from a protocol developed by Rybicki (26) for plant viruses. After protein electrophoresis and transfer to nitrocellulose membrane, vertical strips containing prestained standards, healthy plant extract, or AY-OC 1-infected plant extract were excised. The outermost alignment strips (infected plant extracts) were developed and aligned with the remaining blot of AY-OC 1-infected plant material. Horizontal strips, 5 mm wide, corresponding to the AY MLO-specific 23-kDa protein (present in the infected samples but not in healthy controls) were excised. A horizontal strip containing no polypeptides also was excised to serve as a control. These horizontal strips were each incubated separately with 40 ml of anti-AY MLO PA, 1:40 in TBS, for 3 h at 21 C and then washed three times in TBS buffer. The strip with its bound antibodies was agitated in 7.5 ml of 0.1 M glycine-HCl buffer, pH 2.9, for 10 min to elute the specific antibodies. The liquid was decanted and immediately neutralized with 1.4 ml of 0.1 M NaOH. The resulting solution was designated as the first eluate. A second eluate was collected from the same strips by repeating the above process, and the first and second eluates from each strip were pooled. This mixture was concentrated by lyophilization and resuspended in 1.3 ml of TBS. Protein concentration was determined by the Coomassie brilliant blue colorimetric assay (1).

The reaction of the AY monospecific polyclonal antiserum to AY-OC 1-infected and healthy carrot, periwinkle, and aster was evaluated by Western blotting and PAS ELISA.

In Western blotting, SDS polyacrylamide gels of healthy and AY-infected plant preparations were electroblotted, and vertical lanes (4.0 × 0.3 cm) of healthy and infected plant protein samples were excised. Loading prestained standards between each sample lane helped in locating a particular sample lane. The excised strips were probed with nonpurified AY PA, anti-AY monospecific polyclonal antibody mixture, or eluate from a control blot (presumably containing no antibodies).

For PAS ELISA, the AY-OC 1 antiserum (1:800 in PBST) was used for coating, and serial twofold dilutions (1:4, 1:8, and 1:16 in PBST) of anti-AY-OC 1 monospecific polyclonal antibodies or of the eluate from a control blot were used as probing sera. The absorbance values at 405 nm were subjected to analysis of variance, and means were compared by Tukey's multiple range test.

RESULTS

Detection of AY with polyclonal antiserum. Partially purified preparations from AY-OC 1-infected and healthy periwinkles were used to evaluate the specificity of the PA and the monospecific antiserum. Western blots developed with anti-AY-OC 1 PA showed an AY-specific 23-kDa protein in infected but not in healthy periwinkles (Fig. 1). Other bands appeared in the blots of both infected and healthy material, showing that the serum contained cross-reacting antibodies. In PAS ELISA, AY-infected preparations showed significantly higher A_{405nm} values than did the healthy preparations (Table 1).

The first eluate from each of the nitrocellulose strips containing AY protein had a higher yield of antibodies than did the second eluate, based on Western blotting. First and second eluates were pooled to maximize yield. To determine completeness of the antibody elution, the remaining strips were incubated with protein A-peroxidase and substrate. The colorimetric reaction in the strips was darker after the first elution than after the second elution and showed that most of the antibody was removed after two elutions.

In the Coomassie brilliant blue colorimetric assay, samples of monospecific antibody mixture contained less protein than did the controls (from nitrocellulose strips with no polypeptides). This suggested that yield was lower than the quantitation limit of the assay (1 μg/ml).

Comparison of polyclonal and monospecific polyclonal antisera. Comparison of the 1:4 dilutions of the anti-AY-OC 1 monospecific polyclonal antibody and nonpurified AY-OC 1 PA by PAS ELISA is shown in Table 1. For AY-infected plant samples, significantly higher A_{405nm} values were observed with both PA and monospecific polyclonal antibody than with preimmune serum. Both the PA and the monospecific polyclonal antibody distinguished between healthy and infected plant material, although higher absorbance values were observed with PA than with the monospecific polyclonal antibody in both healthy and infected plant extracts. The eluate from the control strips (blot with no antibodies) did not distinguish between infected and healthy plant extracts (Table 1). A_{405nm} values of the treatments with the 1:8 and 1:16 dilutions of preimmune serum, PA, monospecific polyclonal antibody, and a control blot with no antibodies were all slightly lower than those for the 1:4 dilution (data not shown), but the differences among dilutions were not

statistically significant for any of the treatments.

The monospecific polyclonal antibody reacted in Western blots with an AY-specific 23-kDa protein (Fig. 2, lower arrow) in extracts of plants infected with an AY isolate from Oklahoma carrot (AY-OC 1) in periwinkle (Fig. 2) and in aster leafhoppers (data not shown) and with an AY isolate from Oklahoma lettuce (AY-OL; Fig. 2). Minor cross-reactivity with healthy samples (Fig. 2) occurred. One of these bands, at 44 kDa (Fig. 2, upper arrow), was more prevalent in AY-infected than in healthy samples. No reaction was observed in the extracts of healthy leafhoppers (data not shown) or in plants infected with other MLOs (MLO-OC from carrot and MLO-OF from daisy fleabane; Fig. 2). No bands appeared in preparations of cultured *Spiroplasma citri* Saglio or *S. kunkelii* Whitcomb et al.

DISCUSSION

Although the inability to isolate and culture MLOs hinders the development of efficient detection methods for MLO diseases, both nucleic acid probes and serological tests have been used to detect and differentiate these phytopathogens. Not all laboratories are equipped to use nucleic acid probes, however, and serology is limited by the lack of highly specific PA. In one case (28), a PA distinguished the AY MLO from the peach and clover yellow edge MLOs but not from clover phyllody MLO. A report on partial purification of AY from infected plants suggested the need for highly purified antigens for antiserum production (27,28).

In this study, AY-specific antibodies were purified from PA, using an affinity absorption method developed for detection of low molecular weight virus proteins that are present in low concentrations (26). In this method, an immobilized antigen (29) is used to remove unwanted antibodies from serum or to purify monospecific antibodies (24,26). We adapted the method to purify antibodies that were specific for AY-associated protein. The monospecific polyclonal antibody mixture reacted with a 23-kDa protein in AY-infected plants and leafhoppers but not in healthy controls.

In Western blots developed with the monospecific polyclonal antibody (Fig. 2), the 23-kDa AY-specific protein appeared only in AY-OC 1– and AY-OL–infected periwinkles (Fig. 2) and in AY-OC 1–exposed leafhoppers (data not shown). Numerous and varied strains of AY MLO have been described, for which the taxonomic and evolutionary relationships have recently been reexamined with molecular tools. AY-OC 1 and AY-OL (referred to as OKAY 3 and OKAY 1, respectively) were shown by restriction fragment length polymorphism analysis to fall into two different taxonomic "AY clusters" (21), with AY-OC 1 belonging to a cluster including several AY strains from California, New York, and Maryland and AY-OL included in a cluster with an AY strain from Canada, periwinkle little leaf from Connecticut, and tomato big bud from Arkansas. The monospecific antibody, thus, is capable of recognizing MLOs in at least two AY clusters. The specificity of the monospecific antibody to AY was demonstrated by its failure to react with two MLOs other than AY (8,9), from carrot (MLO-OC) and daisy fleabane (MLO-OF), or with two phytopathogenic spiroplasmas, *S. citri* and *S. kunkelii*.

In our experiments, a 23-kDa protein was recognized by the antibodies produced against an Oklahoma strain of AY MLO. A PA and MAb made against primula yellows MLO-associated

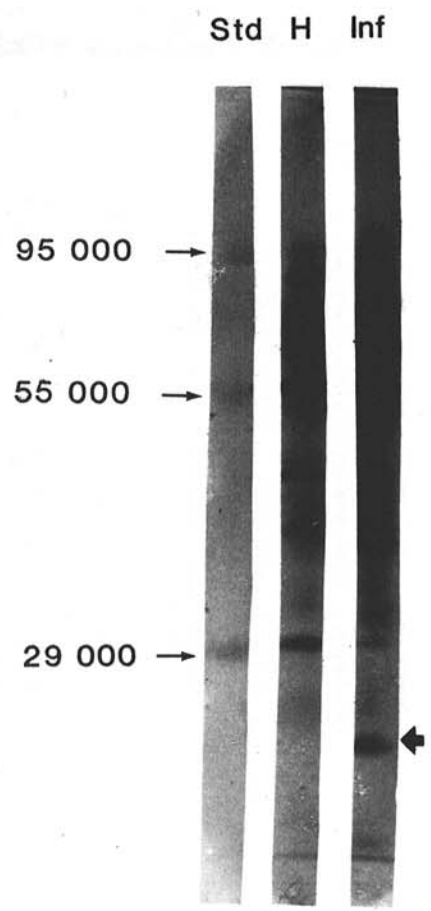

Fig. 1. Western blot developed with AY OC-1 polyclonal antiserum. Std = molecular weight standards, molecular weights indicated on left; H = healthy periwinkle plant; Inf = periwinkle plant infected with AY OC-1. Arrow on the right indicates AY OC-1–specific band at 23 kDa.

TABLE 1. Evaluation of aster yellows (AY) polyclonal antiserum and monospecific polyclonal antibody on healthy and AY-infected periwinkles by protein-A sandwich–enzyme-linked immunosorbent assay (PAS ELISA)

	Mean absorbance at A_{405nm}[y]			
Samples	PI	PA	MPA	Control
Healthy plant	0.120 b[z]	0.405 d	0.139 b	0.078 a
AY-infected plant	0.119 b	1.237 e	0.198 c	0.097 ab
Buffer control	0.118 b	0.062 a	0.062 a	0.063 a

[y] Values are means of three experiments. Antiserum: PI = preimmune serum, PA = nonpurified polyclonal AY antiserum, MPA = monospecific polyclonal antibody, Control = the eluate from the blot with no antibodies. All sera or antibody mixtures were diluted 1:4 in PBST (phosphate-buffered saline containing 0.05% Tween 20).

[z] Values followed by the same letters are not significantly different according to Tukey's multiple range test ($P = 0.05$).

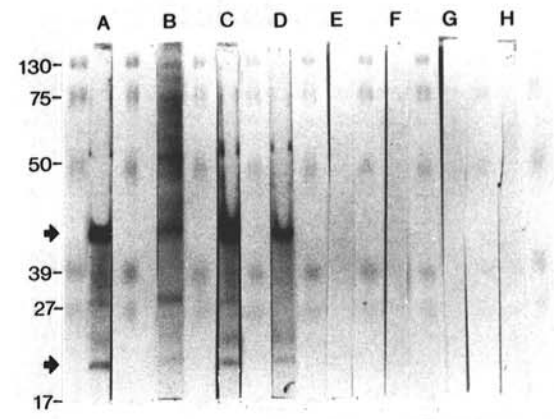

Fig. 2. Western blot developed with AY OC-1 monospecific polyclonal antiserum. Lanes A and C, periwinkle plants infected with AY OC-1; lane B, healthy periwinkle plant; lane D, periwinkle plant infected with AY-OL; lane E, periwinkle plant infected with MLO-OC; lane F, periwinkle plant infected with MLO-OF; lane G, *Spiroplasma citri* culture; lane H, *S. kunkelii* culture. Unlabeled lanes between sample lanes were loaded with standard molecular weight markers; molecular weights (kiloDaltons) indicated on left.

antigens both detected a single major antigen of 22.4 kDa in plant tissues with primula yellows and European aster yellows (2). A major 18.5-kDa protein was detected in AY MLO antigen from infected lettuce by Jiang and Chen (15) with PA and MAb. The 23-kDa protein of OK AY MLO may be similar to the 22.4-kDa protein of primula yellows and to the 18.5-kDa protein of the lettuce MLO.

In PAS ELISA with PA as probing serum, a high level of cross-reactivity occurred with proteins of both healthy and AY MLO-infected plants. Cross-reaction was less obvious with the monospecific antibody mixture, and a significant difference was detected by PAS ELISA between infected and healthy plant material (Table 1). Western blotting was more sensitive than PAS ELISA in detecting AY MLO proteins, although minor cross-reactivity was still detected with healthy plant preparations. A band of 44 kDa appeared much darker in infected than in healthy samples. It is possible that this is a plant protein that is over-produced in the presence of AY MLO or that there is a 44-kDa AY protein that comigrates with a plant protein.

The sensitivity of the monospecific polyclonal antibody was tested with a twofold dilution series of the antibody. This method was preferable to a determination of the lowest detectable concentration of the sample protein because the proportion of the target (MLO) protein relative to the plant-host protein was unknown. In these assays, the antibody dilution end-point was less than 1:16.

The production of monospecific polyclonal antibodies by affinity purification is time consuming and may not be suitable for large-scale purifications. Very few micrograms (less than 1 μg/ml per Western blot) of specific antibodies were obtained from the electroblots of many gels. This method is useful in conducting experiments that require small amounts of antisera, such as Western blotting and ELISA. Such purified antibodies can further help in the study of specific AY MLO proteins and their receptors in plant and insect hosts.

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