Plant Pathology Bulletin 2: 161-168, 1993

Application of Monoclonal Antibodies and Cloned DNA Probes for the Detection and Differentiation of Phytopathogenic Mycoplasmalike Organisms

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Accepted for publication: August 15, 1993

ABSTRACT

Lin, C. P., Shen, W. C., Ko, H. C., Chang, F. L., Yu, Y. L., Chen, M. F., and Wu, F. Y. 1993. Application of monoclonal antibodies and cloned DNA probes for the detection and differentiation of phytopathogenic mycoplasmalike organisms. Plant Pathol. Bull. 2:161-168.

Toward mycoplasmalike organisms (MLOs) associated with sweet potato witches' broom (SPWB), peanut witches' broom (PWB), and rice yellow dwarf (RYD), hybridoma techniques and genetic cloning techniques have been applied in the production of pathogen-specific monoclonal antibodies (MAs) and cloned DNA probes. In the generation of specific MAs and DNA probes for the disease detection and pathogen differentiation, specific monoclonal antibodies for SPWB-MLO, and RYD-MLO along with cloned DNA probes for SPWB-MLO, PWB-MLO, and RYD-MLO had been successfully developed. Monoclonal antibodies against SPWB-MLO revealed the serological relatednesses among SPWB-MLO, PWB-MLO, and MLOs associated with three different witches' broomed plants found on the Penghu Islands. MAs for RYD-MLO can specifically reacted with RYD-MLO only. The antibody recognized two polypeptides, 16 kD and 41 kD, in Western blotting. Further investigations were carried out by employing cloned DNA probes for the differentiation of the serologically related MLOs associated with SPWB, PWB and witches' broom found in various weeds. When cloned DNA probes for SPWB-MLO were applied in the Southern hybridization, distinct hybridization patterns were obtained though they did cross hybridize to some serologically related MLOs in dot-hybridization. For SPWB-MLO specific DNA probes, the sizes for cloned DNA fragments ranged from 0.5 to 7 kb, and the minimal amounts of DNA detectable in dot-hybridization tests were around 0.3 to 0.7 ng toward total DNA from diseased sweet potato and 0.05 to 0.1 ng toward total DNA from SPWB-MLO affected periwinkles. For PWB-MLO specific DNA probes, the sizes of cloned DNA fragments ranged from 0.5 to 4.0 kb. Some of these PWB-MLO probe did cross react with serologically related SPWB-MLO. For RYD-MLO specific DNA probes, 12 specific probes including one plasmid-probe were obtained and reacted specifically only with RYD-MLO. The sizes of cloned DNA fragments ranged from 1.0 to 5.5 kb. The minimal detectable amounts of DNA from RYD-affected rice were around 3-13 ng when RYD-MLO DNA probes were applied in dot-hybridization.

Key words: DNA probe, monoclonal antibody, mycoplasmalike organism.

INTRODUCTION

Sweet potato witches' broom (SPWB), peanut witches' broom (PWB), and rice yellow dwarf (RYD) diseases, like most witches' broom and yellows diseases, are caused by mycoplasmalike organisms (MLOs)(44, 69). SPWB, PWB, and RYD diseases have now been discovered in many countries and areas around the world. In Taiwan, SPWB and PWB diseases were first discovered in an geographically isolated area, the Penghu Islands, in 1969 and 1975 respectively (69). Because of the particular climate, cultivation practices,

and simplicity of domestic plants on the Penghu Islands, these diseases always occur on the Islands year after year.

Like other phytopathogenic MLOs, the MLOs associated with SPWB, RYD, and PWB diseases still remain unculturable. Detection of phytopathogenic MLO is always difficult and time consuming. Host contaminants present in the MLO immunogens take the biggest responsibility for most of the frustrations in the production of specific antibodies.

In recent years, hybridoma techniques and recombinant techniques have been broadly applied in the studies of phytobacteriology. In 1975, Kohler and Milstein developed a technique to demonstrate that somatic hybridization of myeloma cells with antibody-producing lymphocytes could be used to establish continuous culture of specific antibody-producing cells called "hybridoma" (39). The antibodies produced in such culture are fundamentally different from those in antisera or antiascites obtained by conventional means, because of virtually unlimited amount of homologous monoclonal antibodies are produced against desired antigens or haptens. Hybridoma techniques always facilitate the preparation of homogeneous antibodies of distinct specificity from complicated mixture of antigen sources such as the partially purified MLO preparations or from limited quantities of antigens. Monoclonal antibodies are powerful reagents that have been used to identify, quantitate and isolate various cellular products as well as pathogens. In plant pathology, many specific monoclonal antibodies for plant pathogens and pathogenesis related products have been produced and applied in various studies (3,23,24). In recent years, various monoclonal antibodies with great specificity against different phytopathogenic bacteria were developed and applied in the disease detection (2,14,15,16,17,31,36,50,51,52,53,54,68,70). Up to now, only few specific monoclonal antibodies to a limited number of phytopathogenic MLOs have been developed (7,29,33,50,62,63) due to the difficulties encountered in the preparation of pure MLO immunogens. We have applied the hybridoma techniques in the production of monoclonal antibodies for different phytopathogenic procaryotes. Our studies have demonstrated that the use of monoclonal antibodies could distinguish the isolates of walled bacteria (54,65,66,68) as well as the strains of wall-less spiroplasmas (51,52) and mycoplasmalike organisms (50,53,62).

Recombinant cloning techniques have also been widely applied in the studies of phytobacteriology including exploring the genetics of pathogenesis (11), and developing of DNA probes for the diagnosis purposes (56). For walled phytopathogenic bacteria, DNA probes for *Pseudomonas syringae* pv. *phascolicola* (59), *P. s.* pvs. *tomato* and *syringae* (19), *P. solanacearum* (8), *P. fluorescens* (20), *Erwinia carotovora* (67), *Xanthomonas campestris* pv. *campestris* (57), *X. c.* pv. *vesicatoria* (21), *X. c.* pv. *citri* (27), *X. c.* pv. *phaseoli* var. *fuscans* (22), *Clavibacter michiganense* subsp. *michiganense* (64), *C. m.* subsp. *sepedonicum* (35) have been produced for disease detection and pathogen differentiation. For vascular-limited fastidious procaryotes, numerous DNA probes for phytopathogenic mycoplasmalike organisms (MLOs) have been developed and successfully applied for disease detections (1,4,5,6,9,10,12,13,18,25,26,28,37,40,41,42,43,46,47,48,49,60,61). In our lab, cloned DNA probes for MLOs associated with rice yellow dwarf, peanut witches' broom (unpublished) and sweetpotato witches broom (38) were produced recently.

MATERIALS AND METHODS

Hybridoma Techniques

The standard hybridoma technique is to use a myeloma cell line that lacks the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). This enzyme enables cells to use salvage pathway to synthesize nucleotides using an extracellular source of hypoxanthine as a precursor. After fusion, the medium used for selecting fused cells contains hypoxanthine, aminopterin and thymidine (HAT medium). The aminopterin (a folic acid analog) will kill the myeloma cells because it blocks the *de novo* synthesis of purines and pyrimidines. However, the hybrids are able to grow in HAT medium because they derived the HGPRT required genes from the parental B cells and can thus operate the salvage pathway to synthesize the nucleic acid.

Production of Monoclonal Antibodies for MLOs

For the production of monoclonal antibodies for phytopathogenic MLOs, an immunization scheme employing the induction of immunological tolerance of the BALB/c mice before immunization with MLO preparations (29,30,62) was adopted and now used regularly in our lab. Neonatal BALB/c mice were first tolerized with nontarget plant antigens (29) present in immunogen preparation and then immunized with MLO-enriched antigens prepared by Percoll density-gradient fractionation (32) or by enzyme treatment of vascular tissues from leaf midribs (45) six weeks later. When immunization completed, the splenocytes were collected from the immunized mice and proceeded for cell fusion. The detail for fusing splenocytes with myeloma cells in hybridoma production was described in our previous studies (51,62,65). In practice, after fusion, one transfers small aliquots of the cells to a large number of wells in a tissue culture plates and, after 7–10 days, inspects the wells to see if they contain

growing colonies. The next step is to screen the cell supernatant in such wells for the presence of the desired antibody by the radioimmunoassay or ELISA. Because a well containing hybridoma may contain more than one hybridoma, the next step is to "clone" the cells by using limiting dilution method. The antibodies produced by such monoclonal hybridoma may now be homogeneous. Having established a monoclonal hybridoma, it can be maintained indefinitely either *in vitro* or *in vivo*. The hybridoma can always be grown in a mouse having histocompatibility antigens corresponding to those on the hybridoma cells.

Since the cloned hybridoma secretes antibodies into the culture supernatant, the monoclonal antibodies can be produced by culturing the cells in large flasks. Alternatively, larger amounts of antibodies can be obtained by growing hybridoma in histocompatible mice. Approximately $1-5 \times 10^6$ cells are injected intraperitoneally to pristane-primed mouse to induce ascitic tumors. Ten to 20 days later, the tumorous mouse may accumulate several milliliters of ascitic fluid which can be harvested by tapping the peritoneum with a 18-gauge syringe needle. Each milliliter of ascitic fluid collected may contain several milligrams of monoclonal antibodies. Lin and Chen (51) showed that anti-*Spiroplasma citri* monoclonal antibodies produced from ascitic fluid can reach a titer as high as 16 million, several thousand folds higher than those in the same volume from culture supernatant.

Generation of Cloned DNA Probes

In our lab, molecular cloning was performed by standard procedure (58). Genomic DNA for cloning and screening was prepared from procaryotes cultured in broth media or from unculturable MLO preparations purified from infected plants or insect vectors. A standard method for extraction of genomic DNA (58) was followed to extract MLO-DNA from partially purified MLO preparations. MLO-specific DNA was further purified by using CsCl-bisbenzimide density gradient centrifugation (5).

For cloning, approximately 1 μg MLO-specific DNA was digested with the suitable restriction endonuclease such as *Eco*RI that was used in the generation of DNA probes for MLOs. Resulting fragments were ligated with calf intestinal alkaline phosphatase (CIP)-treated *Eco*RI-digested pGEM3Zf(+) or other suitable cloning vectors and used to transform competent cells of *Escherichia coli* according to a standard procedure (58). White colonies of transformants that grew on Luria-Bertani medium containing ampicillin (100 μg/ml), X-Gal (5-bromo-4-chloro-3-indoyl-β-galactoside, 0.004%), and IPTG (isopropyl-β-D-thiogalactopyranoside, 0.5 mM) were subcultured. Total DNA from both healthy and MLO affected plants were nonradioactively labeled and used as hybridization probes to select the transformants that carried MLO-specific DNA fragments. The sizes of cloned inserts were estimated on the basis of agarose gel electrophoresis (0.8%) after *Eco*RI digestion of the recombinant plasmids isolated from tentative transformants by the alkaline lysis method (58). Recombinant plasmids with various sizes of inserts were randomly selected and nonradioactively labeled by a random priming procedure with digoxigenin (DIG) or by nick translation procedure with biotin. Labeled recombinant plasmids that only hybridized with the DNA preparations from MLO-affected plants but not with those from healthy plants in dot hybridizations were selected. The cloned inserts of selected recombinant plasmids were then digested with *Eco*RI, electrophoresed, eluted from agarose gel (58) and then labeled with DIG or biotin. The labeled cloned inserts were then used as DNA probes for the detection and differentiation of MLOs.

DNA Hybridization

Dot hybridizations were applied in the evaluation of specificities and sensitivities of DNA probes. Southern hybridization was applied to differentiate the serologically related MLO species. For dot hybridization, DNA preparations from diseased sample sources were denatured by boiling for 10 min, immediately cooled on ice for 5 min and then applied directly to nylon membranes using micropipette. For Southern hybridization, two microgram MLO-specific DNA from diseased plants or insect vectors was digested with suitable restriction endonuclease, electrophoresed in agarose gels, alkaline-denatured (0.5 M NaOH in 1.5 M NaCl for 10 min), and transferred to nylon membranes. Membranes were air-dried and cross-linked with the DNA using a UV-crosslinker or a vacuum oven. Filters were prehybridized and hybridized with labeled DNA probes. Blots were then subjected to either high- or moderate-stringency washes for sensitivity and specificity test respectively. Regularly, under high stringency, filters were washed twice (30 min each wash) at room temperature with $2 \times$ SSC containing 0.1% SDS, twice at 65 C with $0.1 \times$ SSC containing 0.1% SDS. Under moderate stringency, the washes with $0.1 \times$ SSC containing 0.1% SDS were performed under 55 C instead of 65 C. After washing, the filters were air-dried and processed for signal detection. For DIG-labeling and detection system, the blots were visualized by incubating the membranes in the dark in a solution containing colorimetric substrate, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Monoclonal Antibodies against MLOs

In our studies, five stable hybridoma clones secreting specific monoclonal antibodies, of the IgM class, against sweet potato witches' broom (SPWB) mycoplasmalike organism (MLO) were produced by employing an immunization scheme for the induction of the immunological tolerance of the mice before immunization (62). With these monoclonal antibodies, SPWB-MLO in diseased sweet potatoes and periwinkles were specifically identified by indirect enzyme-linked immunosorbent assay (ELISA) and immunofluorescent staining. Antibody titers for hybridoma culture supernatants (measured by indirect ELISA) ranged from 320–5120 toward SPWB-MLO from diseased periwinkles. The SPWB-MLO were serologically differentiated from the MLOs associated with loofah witches' broom, paulownia witches' broom, *Ipomoea obscura* witches' broom, aster yellows, elm yellows and rice yellow dwarf in both ELISA and immunofluorescent staining. All the monoclonal antibodies developed cross reacted with peanut witches' broom MLO, asparagus bean witches' broom MLO, and MLOs associated with the witches' broomed weeds, *Rhynchosia minima* and *Alysicarpus vaginalis*, found in the Penghu Islands.

Recently, monoclonal antibody for rice yellow dwarf (RYD)-MLO was developed also by employing the immunization scheme involved the induction of immunological tolerance of the mice. The antibody reacted with RYD-MLO specifically. In Western blotting using the MAs for SPWB-MLO and RYD-MLO, two polypeptide, 17 kD and 44 kD, were recognized by the SPWB-MLO specific monocloal antibodies and two polypeptides with similar sizes, 16 kD and 41 kD, were recognized by RYD-MLO monoclonal antibody. The polypeptide bands were excised from blots and used as immunogens for the development of RYD-MLO or SPWB-MLO specific monoclonal and polyclonal antibodies.

Cloned DNA probes for MLOs

In addition to monoclonal antibodies, DNA probes for SPWB-MLO, PWB-MLO, and RYD-MLO were developed in our lab. For SPWB-MLO DNA probes, *Eco*RI-digested genomic DNA from sweet potato witches' broom mycoplasmalike organism (SPWB-MLO) was ligated with pGEM3Zf(+) and cloned in *Escherichia coli* JM109. The cloned inserts from 12 SPWB-MLO specific recombinants selected were labeled with digoxigenin. Dot and Southern hybridization analyses were applied in the studies. Two of the 12 probes, SPWB16 (4.1 kb) and SPWB27 (3.5 kb) hybridized positively with DNAs from SPWB-MLO affected periwinkle and sweet potato specifically but not with DNAs from healthy plants and plants infected with MLOs associated with peanut witches' broom (PWB), loofah witches' broom, paulownia witches' broom, *Ipomoea obscura* witches' broom, elm yellows, rice yellow dwarf and aster yellows. Ten other probes SPWB8 (2.5 kb), SPWB10 (4.3 kb), SPWB15 (1.6 kb), SPWB20 (0.2 kb), SPWB28 (1.1 kb), SPWB59 (3.0 kb), SPWB62 (5.2 kb), SPWB80 (2.0 kb), SPWB89 (4.0 kb) and SPWB99 (2.3 kb), hybridized positively both with DNAs from periwinkles affected with SPWB-MLO and PWB-MLO. Probes SPWB16 and SPWB27 can detect the DNA from SPWB-MLO affected periwinkle to 0.05–0.1 ng and the DNA from SPWB-MLO affected sweet potato to 0.3–0.7 ng effectively in dot-hybridizations. All 12 probes hybridized positively to DNA extracted from witches'-broomed field-grown weed *Rhynchosia minima*. In the Southern hybridization analyses, except SPWB59, all can differentiate the SPWB-MLO from PWB-MLO readily according to the distinct band patterns of Southern hybridization. Two oligomers derived from the cloned fragment were now used to amplify SPWB-MLO specific DNA by employing polymerase chain reaction (PCR) analysis for the detection and differentiation purposes (38).

For PWB-MLO specific DNA probes, the sizes of cloned DNA fragments ranged from 0.5 to 4.0 kb. Some of these PWB-MLO probe did cross reacted with serologically related SPWB-MLO. For RYD-MLO specific DNA probes, 12 specific probes including one plasmid-probe (4.3 kb) were obtained and reacted specifically only with RYD-MLO. The sizes of cloned DNA fragments ranged from 1.0 to 5.5 kb. The minimal detectable amounts of DNA from RYD-affected rice were around 3–13 ng when RYD-MLO DNA-probes were applied in dot hybridization.

DNA probes for peanut witches' broom MLO and SPWB-MLO developed in our lab were now applied in the identification and differentiation of the serologically related MLOs. Similar Southern hybridization patterns were obtained among DNAs from SPWB-MLO and other phytopathogenic MLOs found on the Penghu Islands in the preliminary studies (unpublished data). The ecosystem for SPWB and other MLO diseases can be further explored using the DNA probes along with the monoclonal antibodies.

DISCUSSION

An efficient and successful scheme for the production of monoclonal antibodies for MLOs requires pure immunogens for the immunization. MLOs are wall-less procaryotes and restricted to phloem tissues in infected plants. Until now, all attempts to culture phytopathogenic MLOs remain unsuccessful. Some efforts had been tried to enrich MLO preparations

from infected plants, but the host contaminants present in the MLO-enriched preparations still make the production of monoclonal antibodies very difficult. The procedures employing Percoll density-gradient centrifugation (32) and enzyme treatment (45) seems to be the most promising approaches in the purification of MLOs from various diseased plants. Besides, various approaches such as the intrasplenic immunization method and the immunization strategies involved the induction of immunological tolerance of experimental mice (29,30) have been regularly practiced in our lab in order to facilitate the production of specific monoclonal antibodies for MLOs.

As a result of the high specificity of the monoclonal antibodies, they will provide a powerful tool for detection and identification of minute quantities of MLO *in vitro* and *in situ* by ELISA, immunofluorescent staining, and tissue blotting techniques (7,33,50,55). In the antibody specificity tests using ELISA and immunofluorescent staining, the SPWB-MLO specific monoclonal antibodies cross reacted with preparations from witches'-broomed peanut, asparagus bean, and two different weeds collected on the Penghu Islands. This findings strongly suggested that very close serological relatedness might exist among different MLOs that coevolved in the diseased crops, weeds, and even the insect vectors on the Penhu Islands.

The monoclonal antibodies specific to SPWB-MLO were now applied for the investigation of the distribution of SPWB-MLO in various parts of infected plants, and insect vectors with immunofluorescent staining. We have also applied the antibodies as antigen-specific ligands and coupled them onto the chromatography matrix for the isolation of SPWB-MLO. MLO immunogens purified by the affinity chromatography procedure (34) or excised from the MLO-specific polypeptide bands in Western blotting membranes will be valuable for the production of MLO-specific polyclonal antibodies and also for the generation of the whole arrays of monoclonal antibodies specific to various epitopes of SPWB-MLO. Close evaluation of the serological relatedness will then become possible by using the MLO-specific polyclonal or monoclonal antibodies thus prepared.

Since the MLO-specific DNA can be effectively separated from host plant DNA simply by an CsCl-bisbenzimide centrifugation (5), it is now more efficient for us to generate a MLO-specific DNA probe than to develop a MLO-specific monoclonal antibody. By sequencing the MLO-specific DNA inserts from the recombinant plasmids will now provide us many useful oligomer pairs for the polymerase chain reaction (PCR). One can now detect a very minute amount of MLO DNA from plants or insect vectors very easily and accurately simply by taking advantage of the DNA amplification effects of the PCR. To identify the PCR-synthesized target MLO-DNA fragments in agarose or polyacrylamide gel electrophoresis can be accomplished in 2–3 h after the crude extraction of total DNA from suspected plants or insect vectors. DNA probes also provide us a very valuable tools for the identification of serological related MLOs and to reveal the genetic relatednesses among MLOs (47).

ACKNOWLEDGEMENTS

We would like to express our most sincere gratitude to Dr. Ren-Jong Chiu for his sustained encouragement and valuable advice right from the beginning stage of our setting up plant bacteriology lab at National Taiwan University. This research was supported in part by Council of Agriculture, R.O.C. Development of DNA probes for SPWB-MLO was supported in part by a grant NSC82-0409-B002-083 from National Science Council, R.O.C.

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摘　　要

林長平、沈偉強、柯曉芝、張富林、游燕伶、陳妙帆、吳鳳儀．1993．單元抗體及 DNA 探針在植物病原似菌質體之鑑定及偵測上之應用．植病會刊 2:161-168．(台北市　國立台灣大學植物病蟲害學系)

針對甘藷簇葉病、花生簇葉病與水稻黃萎病利用融合瘤技術及 DNA 選殖技術，成功地研製出對抗甘藷簇葉病與水稻黃萎病病原似菌質體之單元抗體，以及對抗甘藷簇葉病、花生簇葉病與水稻黃萎病病原似菌質體之 DNA 探針。對抗甘藷簇葉病病原似菌質體之單元抗體顯示了甘藷簇葉病病原似菌質體、花生簇葉病病原似菌質體及在澎湖島上發現的一種雜草簇葉病之病原似菌質體間具有血清相關性。而對抗水稻黃萎病病原似菌質體之單元抗體則只專一地對水稻黃萎病病原似菌質體具血清反應，此單元抗體可在西方漬染反應中認識二條大小爲 16 kD 及 41 kD 屬於水稻黃萎病病原似菌質體之多胜肽。在 DNA 探針之研製上，利用對抗甘藷簇葉病病原似菌質體之 DNA 探針在南方漬染反應中可依其雜配反應圖譜而明確地鑑別出上述具血清相關性之似菌質體。此些甘藷簇葉病病原似菌質體之 DNA 探針，其大小介於 0.5–7 kb，其對病甘藷之全 DNA 的偵測能力爲 0.3–0.7 ng，而對日日春病株全 DNA 則可偵測至 0.05–0.1 ng。對抗花生簇葉病病原似菌質體之 DNA 探針其大小介於 0.5–4 kb，亦能明確地鑑別不同病原似菌質體。而在對抗水稻黃萎病病原似菌質體之 DNA 探針之研製上，共獲得十二株特異之探針，其大小介於 1.0–5.5 kb，其中並包括一大小爲 4.3 kb 的質體探針，此些探針對病水稻株之全 DNA 偵測能力爲 3–13 ng。

關鍵詞：DNA 探針、單元抗體、似菌質體。