ins, a reddish colour and a stiff

DETECTION OF APPLE PROLIFERATION AND OTHER MLOs BY IMMUNO-CAPTURE PCR (IC-PCR)

J. Rajan and M.F. Clark
Horticulture Research International
East Malling, West Malling
Kent ME19 6BJ
England

Abstract

We have developed a sensitive immunocapture-PCR detection method in which the target MLO is selectively captured by specific antibody adsorbed on thermostable microtitre plates, following which MLO DNA is released and amplified using either specific or universal primers. The method has been applied successfully to several MLOs in Catharanthus roseus, including the MLOs associated with European aster yellows, peach yellow leafroll, Molieres disease and apple proliferation. Differentiation of these MLOs was achieved using either polyclonal or monoclonal antibodies as the capture agent.

We have also successfully detected and identified apple proliferation MLO in apple bark using rabbit polyclonal antibodies to capture the MLO, followed by amplification with universal primers. All samples from infected trees gave a product which was characteristic of apple proliferation MLO by RFLP analysis. No product was obtained from healthy trees or from infected bark samples added to microplate wells coated with antibodies against other MLOs.

1. Introduction

Serological methods for detecting mycoplasmalike organisms (MLOs) can exhibit a high degree of specificity in identifying and differentiating among MLOs (Clark et al., 1988; Clark 1992), even those with similar base sequences in the highly conserved 16-23S ribosomal DNA regions. PCR detection methods, although potentially much more sensitive than those based on serology, require the production of specific primers to achieve a similar differentiation and may give confusing results if more than one MLO is present. The combination of serology and PCR exploits antibody specificity to selectively capture a target MLO which can then be detected by PCR amplification of a DNA fragment using either universal or specific MLO primer pairs. As well as permitting selective MLO capture prior to PCR detection, immunocapture-PCR (IC-PCR) also enables amplification of MLO DNA without the need for extensive extraction and clean-up procedures. Polyclonal antibodies are particularly successful due to their generally high affinity compared with monoclonal antibodies, while amplification of MLO DNA as the ultimate target component of the MLO is possible even using polyclonal antibody preparations containing antibodies to plant antigens.

2. Materials and Methods

Antibodies were raised against the MLOs of European aster yellows (EAY), peach yellow leafroll isolate of western X (PYLR) and Molières disease (MOL) using published methods (Clark et al., 1989; Davies and Clark, 1992). Source materials for these preparations were extracts of petioles and of midribs of infected C. roseus plants. Preparations were enriched for MLO membranes by a combination of molecular exclusion chromatography, differential

centrifugation and affinity absorption of healthy plant antigens. Balb/C mice, or rabbits, were immunised at 3- or 4-week intervals with preparations emulsified with Freunds adjuvant or Imject alum (Pierce Chemicals). In addition to the polyclonal antibodies raised and evaluated, several monoclonal antibodies were obtained, of which EMA1 (primula yellows isolate of aster yellows), EMA4 (PYLR) and EMA15 (MOL) were also evaluated as capture antibodies.

The above method of immunogen preparation was unsuccessful with apple proliferation (AP) MLO (strain AT from Germany) for which a different protocol was developed. Tip leaves and stems of C. roseus, newly colonised by MLO, were extracted, and an MLO-enriched preparation obtained by simple differential centrifugation. Immunising injections were made with Imject alum at weekly intervals for 4 weeks, followed by a rest period of 6 weeks, followed by a further series of three 1-weekly injections. Evaluation of all antibodies was carried out by reciprocal indirect $F(ab')_2$ ELISA tests (Barbara and Clark, 1982) and by Western blot.

3. Results

All antibodies were highly specific for their homologous antigens and no evidence of cross-reactivity was obtained for any heterologous antigen-antibody combination.

IC-PCR was carried out both with thermostable polycarbonate microtitre plates and with polypropylene microcentrifuge tubes. The plates allowed greater sensitivity and more reproducible results, and were quicker and easier to handle. Optimal antibody coating concentrations were in the order 10 ug ml^{-1}, about 10 x greater than for ELISA. 'Blocking' of adsorption sites on the plates could be achieved by post-coat treatment with a solution of protein or skim milk powder, but addition of 10 g/L ovalbumin to the antigen preparation was equally effective. Antigens comprised clarified buffer extracts of plant tissues prepared without detergent, which were added to the coated plates and incubated overnight at 4^0C. Thorough washing of the plates, rinsing with distilled water and air-drying immediately before addition of PCR components was essential in order to prevent carry-over of immunocapture components and to obtain consistent and sensitive results. The presence of Tween 20 in the washing solutions completely prevented the formation of any detectable amplification product, presumably through destruction of the MLO membrane and subsequent leaching of DNA. Several 'universal' primer pairs were evaluated but most consistent results were obtained with the primer pairs U5/U3 and U1/U4 (generously supplied by Dr. E. Seemüller, Dossenheim).

Using the universal primers we could selectively detect each of the four MLOs in 30 ul samples of aqueous buffer extracts of C. roseus. As controls, reciprocal tests were carried out using all of the MLOs with their monoclonal or polyclonal antibodies. Homologous combinations of MLO and antibody gave amplification products from as little as 1:1000 (g/ml) dilution of infected plant extract (Fig. 1). At the highest concentrations of antigen tested (1:4 dilution) non-homologous MLOs sometimes gave a weak amplification product (Fig. 1), but such products were uncommon at dilutions greater than 1:20 g/ml. Restriction digestion of the amplification products with Alu I, Hinf I and Rsa I produced fragment patterns characteristic for the individual MLOs.

We have also successfully detected and identified apple proliferation MLO in crude extracts of apple bark using rabbit polyclonal antibodies, followed by amplification with the primer pairs U1/U4 (Fig. 2) or U5/U3. All samples from infected trees gave a product which was characteristic of apple proliferation by RFLP analysis. The highest dilution of

bark extract which gave detectable product was 1:32 (g/ml). No product was obtained from healthy trees or from infected bark samples added to microplate wells coated with antibodies against other MLOs. These tests were done during November and December when MLO titres in apple bark appeared to be very low as few MLOs were observed in bark sections stained with DAPI and viewed under a fluorescence microscope. Additional confirmation of the validity of the IC-PCR method for detecting AP-MLO was obtained in tests conducted with samples collected from orchard trees in Germany during April and May. For these samples there was complete agreement between the results of IC-PCR tests and those of standard PCR tests made using AP-specific primers.

Acknowledgements

We wish to thank Dr. E. Seemüller for supplying details of primer sequences; Ms H. Kison, Dr. G. Krczal and Dr. E. Refatti for collecting and providing infected and healthy apple samples.

This investigation was jointly funded by The Commission of European Communities, Project No. PL900064, and the UK Ministry of Agriculture, Food and Fisheries, Contract No. HH0112STF. The work was carried out under Plant Pests (Great Britain) Order, 1980, licence no. PHF 1532/536/53.

References

Barbara, D.J., and Clark, M.F. 1982. A simple indirect ELISA using $F(ab')_2$ fragments of immunoglobulin. J. Gen. Virology 58:315–322.

Clark, M.F. 1992. Immunodiagnostic techniques for plant mycoplasma-like organisms. p. 34–45. In: Duncan, J.M. and Torrance, L., eds., Techniques for the Rapid Detection of Plant Pathogens, Blackwell Scientific Publications, Oxford.

Clark, M.F., Davies, D.L., Buss, S.L., and Morton, A. 1988. Serological discrimination among mycoplasma-like organisms using polyclonal and monoclonal antibodies. Acta Horticulturae 235:107–113.

Clark, M.F., Morton, A., and Buss, S.L. 1989. Preparation of mycoplasma immunogens from plants and a comparison of polyclonal and monoclonal antibodies made against primula yellows MLO-associated antigens. Annals of Applied Biology 114:111–124.

Davies, D.L., and Clark, M.F. 1992. Production and characterization of polyclonal and monoclonal antibodies against peach yellows leafroll MLO-associated antigens. Acta Horticulturae 309:275–283.

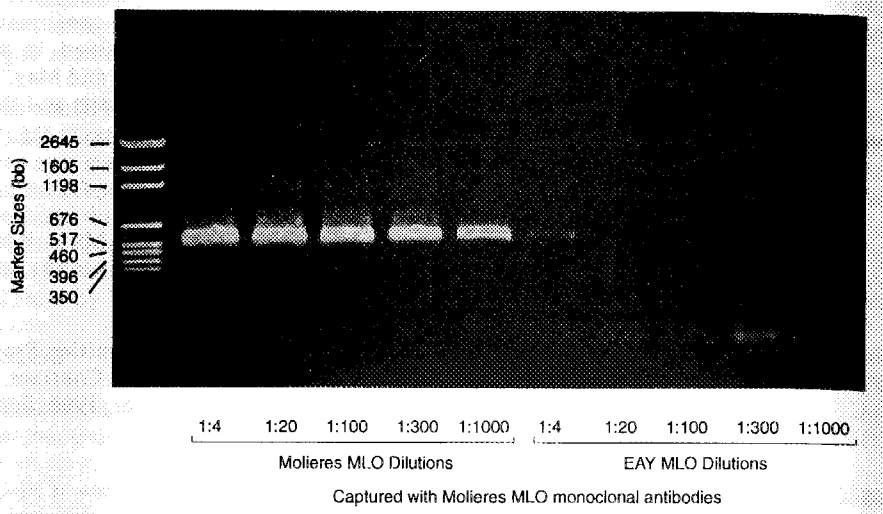

Figure 1 - Specificity of immunocapture PCR for homologous combinations of MLO and antibody. Diluted extracts of C. roseus plants infected with MOL MLO or with EAY MLO were applied to thermostable microtitre plates coated with MOL MLO monoclonal antibodies. DNA was released by heating to 95^0C for 5 min. PCR amplification was for 35 cycles using 'universal' primers U1/U4.

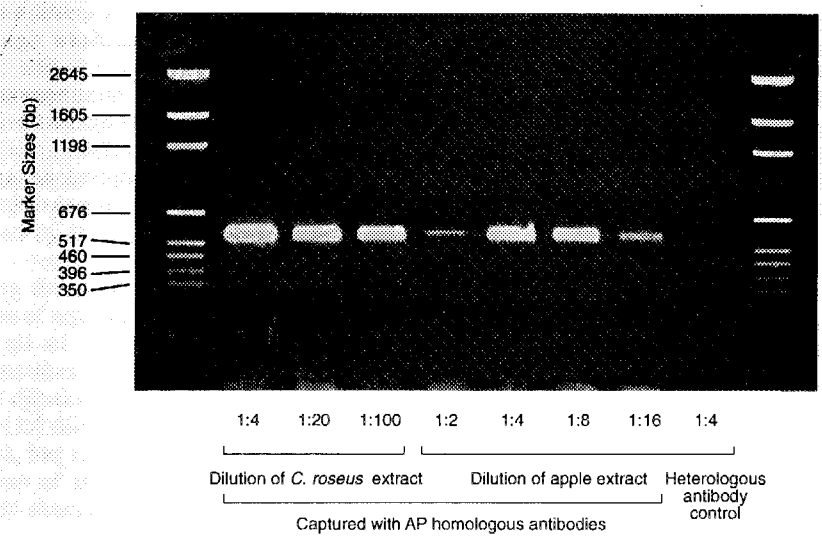

Figure 2 - Immunocapture PCR detection of AP MLO in extracts of infected apple or of infected C. roseus. MLOs were captured on thermostable microtitre plates coated with polyclonal antibodies prepared against AP MLO (homologous) or against MOL MLO. DNA was released by heating to 95^0C for 5 min. PCR amplification was for 35 cycles using 'universal' primers U1/U4.