'LUCERNE YELLOWS' DISEASE – TEM AND MOLECULAR STUDIES

L.J. Pilkington[a], K.S. Gibb[b], G.M. Gurr[a], M.J. Fletcher[c], A. Nikandrow[c], E. Elliott[d], R. van de Ven[c] and D.M.Y. Read[a]

[a] University of Sydney Orange, P.O. Box 883, Orange, 2800, New South Wales
[b] Faculty of Science, Northern Territory University, Darwin, 0909, Northern Territory
[c] Orange Agricultural Institute, NSW Agriculture, Forest Road, Orange, 2800, New South Wales
[d] New South Wales Agriculture, P. O. Box 369, Forbes, 2871, New South Wales

Introduction

Lucerne yellows (LY) is a major lucerne disease in New South Wales and has a particularly severe impact on seed production. It causes reductions in seed yield leading to an overall economic impact estimated at $7M per annum (1). The aim of this work was to use transmission electron microscopy (TEM) and molecular biology techniques to identify the agent that causes LY.

Materials and Methods

DNA Extraction DNA was extracted from the leaf midribs and stems of field collected LY symptomatic plants using standard methods (2).

Primer pairs and PCR protocols Eight generic primers based on the phytoplasma 16s region rDNA were used. Each DNA sample was diluted to 1:1, 1:10, 1:50 and 1:100. PCR cycling protocols were manipulated to maximise reliability. Samples were amplified by nested PCR using the P1/P7 primers followed by amplification with the primers M23sr/fU5. To test for inhibition in LY DNA preparations, LY DNA was combined with DNA of the phytoplasma responsible for tomato big bud (TBB) and subjected to PCR.

RFLP Five µl of LY and TBB PCR products were digested with enzymes MseI, AluI, RsaI and HpaII following the manufacturer's instructions (New England Biolabs, Inc., MA 01915-5599, USA). These products were then run on a 5% polyacrylamide gel that was then stained with ethidium bromide.

TEM Leaf midrib tissue containing phloem was fixed in 3% glutaraldehyde/0.1M phosphate buffer, post-fixed in 1% OsO_4/buffer and embedded in 100% resin. Specimens were then cut into ultra-thin (80nm) sections and viewed with a transmission electron microscope.

Results

Detection of phytoplasmas in lucerne In nested PCR tests, the TBB positive controls gave a product of 1600 bp and negative controls gave no amplified products. Some of the LY samples gave the expected sized product but no sample gave consistent results in repeat trials. No positive signal was observed from DNA extracted from healthy plant tissue. Combining LY and TBB DNA consistently yielded a positive phytoplasma signal.

RFLP When the PCR product obtained from amplified DNA was digested with the restriction enzymes AluI, HpaII, RsaI and MseI, differences were observed between LY and TBB (Figure 1 left). Several repetitions of RFLP assays yielded consistent restriction patterns for both LY and TBB DNA.

TEM Numerous phytoplasma bodies of 200-400nm in diameter were present in symptomatic lucerne phloem (Figure 1 right). The bodies were bound by a single unit membrane and lacked cell walls. No phytoplasma bodies were found in the phloem of sections prepared from non-symptomatic plants.

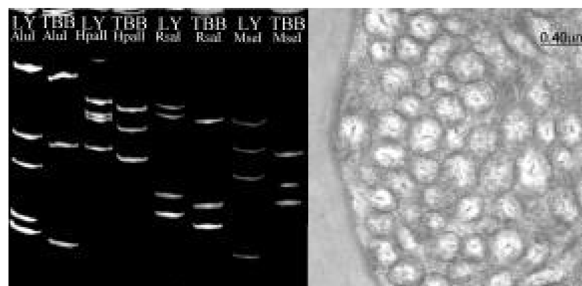

Figure 1. (left) RFLP profiles of 16s rDNA amplified by nested PCR from LY and TBB DNA. (right) Lucerne phloem section containing numerous phytoplasma bodies.

Discussion

The structures visualised in LY affected plant tissue by EM were consistent in size, shape and structure with phytoplasmas that are responsible for other plant diseases (3). Molecular biology techniques also showed a phytoplasma to be associated with LY symptomatic plants suggesting it may be the causal agent.

Although no consistent positive signal was achieved from DNA extracted from LY affected plants, a consistent difference between LY and TBB restriction profiles was observed. This result suggests that the LY DNA amplified is different to that of TBB (which is associated with little leaf and phyllody symptoms in lucerne) and is not the result of contamination.

TBB DNA amplified successfully in the presence of LY DNA eliminating the possibility that inconsistent amplification of LY DNA was caused by PCR inhibitors. Current development of a LY-specific primer may improve detection and reliability and allow applied issues, such as identity of vectors, to be addressed.

Acknowledgements

We thank Rural Industries Research and Development Corporation for providing funding for this work and Mr Don Gowanlock (University of Queensland) for his aid in interpretation of the electron micrographs.

References

1. Pilkington, L., Gurr, G.M., Fletcher, M.J., Nikandrow, A. and Elliott, E. (1999). Occurrence and severity of lucerne yellows disease in Australian lucerne seed crops. *Australasian Plant Pathology* **28:** 235 - 39.
2. Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983). A Plant DNA Minipreparation: Version II. *Plant Molecular Biology Reporter* **1:** 19-21.
3. Cronje, C.P.R., Tymon, A.M., Jones, P. and Bailey, R.A. (1998). Association of a phytoplasma with a yellow leaf syndrome of sugarcane in Africa. *Annals of Applied Biology* **133:** 177-186.