

Studies on new plant phytoplasma and viruses infections and
molecular dissection of virus resistance using *Medicago*
truncatula

By

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I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institute

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ABSTRACT

The work presented in this thesis is in two areas - study of novel pathogens resulting from new encounters between crop and native species and 'mining' for plant virus resistance genes in the model legume *Medicago truncatula*.

The history of agriculture in Western Australia (WA) is less than 150 years old. All major broadacre and horticultural crops grown in WA have been introduced from overseas. These introduced horticultural and field crops potentially carry pathogens which may be transferred to infect native vegetation. Conversely, cultivated plants are vulnerable to infection by pathogens present in indigenous plants. This potential for new disease encounters is compounded by expansion of agriculture to crop new land and by predicted climate changes. These changes may provide selective advantage to a particular pest or disease, enabling infection to increase and so increase crop losses or damage native species. Global trade in agricultural produce also increases the potential for introduction of exotic pathogens. The focus of the first part of the research was to look for new pathogens of crops and native plants in WA.

A series of field trips to study diseases in horticultural crops and native vegetation were made in the agricultural regions of Carnarvon, Broome, Kununurra, Perth and the surrounding metropolitan area. Although the initial focus was on virus diseases, the work expanded to study phytoplasma-associated diseases, because of their widespread occurrence and clear symptoms.

In the agricultural region around Kununurra the potyvirus *Bean common mosaic virus* (BCMV) was found infecting *Phaseolus vulgaris* crops. Sequencing of isolates collected provided the first reliable molecular confirmation of the presence of BCMV in Australia.

In joint work with K. Bayliss three commercial Paulownia tree plantations near Perth were found exhibiting symptoms of Witches'-Broom disease. The Paulownia trees were found to be associated with '*Candidatus* Phytoplasma australiense' 16SrXII group. Chickpeas in the Kununurra region were found with symptoms of stunting, little leaf and proliferating branches and tested positive for phytoplasma. Sequencing confirmed the presence of a phytoplasma with high similarity to the 16SrII group '*Ca* Phytoplasma aurantifolia' (peanut witches broom group). This is the first molecular evidence for a phytoplasma-associated disease in chickpea. Red clover (*Trifolium pratense*), several other pasture legumes and paddy melon (*Cucumis myriocarpus*) with symptoms of diminished leaf size, pallor, rugosity, leaf deformation, shoot proliferation and stunting were observed amongst pasture plots in south-western Australia. All species with these symptoms were positive for a phytoplasma resembling '*Ca* Phytoplasma australiense, 16SrXII group. This association was confirmed for red clover and paddy melon by subsequent nested PCR and sequence analysis. This is the first time that '*Ca*. Phytoplasma australiense, 16SrXII group, has been reported infecting these hosts in southern WA. Snakebean (*Vigna unguiculata* var. *sesquipedalis*) and tomato (*Lycopersicon esculentum*) plants with phytoplasma-like symptoms were found in the horticultural region at Broome. The symptoms on snakebean were typical of phytoplasma disease. Sequence analysis identified that the agent associated

with the symptoms as a strain of sweet potato little leaf strain V4 (SPLL-V4) phytoplasma (16SrXII group, strain of '*Ca* Phytoplasma australiense'). SPLL phytoplasma has not been reported in snakebean or tomato in this isolated agricultural region. In a survey in the Gascoyne region phytoplasma-like symptoms were found in tomato, eggplant and papaya. Previously in this region plants had been found to be associated with peanut witches broom phytoplasma 16SrII group '*Ca* Phytoplasma aurantifolia'. Phytoplasma-like symptoms which included bunchy growth, witches' broom and 'little leaf' were observed in *Allocasuarina fraseriana* (Western Sheoak, Casuarina) and *Acacia saligna* (Acacia, Orange Wattle) trees in Kings Park and Botanic Garden Perth WA. Phytoplasma-associated disease was confirmed for the first time in native Australian casuarina and acacia trees in WA. Based on the identification of these phytoplasma associated diseases in WA, phytoplasma-associated diseases can be divided into two zones, because phytoplasma 16SrII group was found mostly in the north west of WA and the 16SrXII group in the south west of WA. This work has added to knowledge of the extent and distribution of phytoplasma disease in WA: it is concluded that crop-associated phytoplasma disease originated from native vegetation.

The aim of the second part of the research was to screen and map a virus resistance gene in the model legume *M. truncatula* to better understand host/pathogen interactions of legume-infecting viruses. Natural resistance genes found in *M. truncatula* could then be used to locate similar genes in grain legumes (e.g. chickpea and lupins) for practical applications. *M. truncatula* is a model legume which has a relatively small genome. International consortia have been established to develop genomic resources for *M. truncatula*. The *M. truncatula* core collection (from SARDI, South Australia) totalling 230 accessions was screened for resistance/susceptibility to four legume-infecting viruses: *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Bean yellow mosaic virus* (BYMV) and *Subterranean clover mottle virus* (SCMoV). Five plants from each of the 230 phenotypically distinct members of the *M. truncatula* core collection were challenged with one isolate of each virus using infectious sap together with five uninoculated control plants for each accession. The symptoms that developed were recorded and virus presence was confirmed by ELISA for inoculated and systemic leaves. Accessions that were potentially resistant were retested to check for escapes. The result from this screen was that 5 accessions were potentially resistant to AMV, 56 to BYMV, 21 to CMV and 42 to SCMoV. The remaining accessions were susceptible to all four viruses with symptoms which ranged from no apparent symptoms (symptomless systemic infection) to highly susceptible and plant death. In continuing work with DAFWA (Dr R. Jones) accessions potentially resistant to AMV, BYMV and CMV are being challenged with additional isolates to check for the presence of genes providing broader resistance.

The Sobemovirus SCMoV was chosen for further study because it is the most widespread viral pathogen of subterranean clover pastures in Australia. It is also a high titre, mechanically transmitted virus which gave the least escapes on infection. SCMoV has a linear, single-stranded positive-sense RNA genome of 4.25 Kb. Making use of natural resistance is an effective means to reduce pasture losses caused by SCMoV. From the screen of the core collection of *M. truncatula*, amongst the lines resistant to SCMoV a single dominant hypersensitive resistance was detected in line DZA-315. To accelerate mapping of the SCMoV resistance gene, an F₈ RIL population of a cross between the

resistant line (DZA-315) and a susceptible line (Jemalong-J6, A-17) was sourced and obtained from INRA Toulouse. A total of 166 RILs were phenotyped for resistance and susceptibility to SCMoV. Resistant and susceptible lines showed parental phenotypic symptoms with 84 being susceptible and 82 being resistant. This indicated the presence of a single resistance (*R*) gene. This phenotypic data was combined with genotypic data (76 polymorphic molecular markers) already available for this RIL population to provide a framework map. Mapmaker and Mapmanager mapping programs were used to locate the position of the resistance gene. This framework map indicated a position for the resistance gene on the long arm of chromosome 6.

Additional polymorphic SSR markers flanking the *R* gene locus on chromosome 6 were used to map the position of the *R* gene more closely. These SSR markers were developed from a parental cross of *M. truncatula* line A17 and A20 at UC Davis and from a parental cross between line A17 and DZA 315 developed at INRA Toulouse. Ten new polymorphic SSR markers were identified and located on the long arm of chromosome 6 after analysis of the F₈ RIL population. When combined with the other phenotypic and genotypic data a more accurate map position for the SCMoV *R* gene was obtained. The results indicate that the *R* gene to SCMoV is located on the long arm of *M. truncatula* chromosome 6 between position 35 to 38 centimorgans (cM). The closest marker to the SCMoV *R* gene is marker mtic153 which is about 2.3 cM away. From existing maps of *M. truncatula* most of the *R* genes located in this region are of the TIR-NBS-LRR type and occur in *R* gene clusters. A series of BACs that span the region of interest have been identified in which SCMoV *R* gene should be present.

M. truncatula has been used as a model legume to study a number of symbiotic (e.g. rhizobium) and pathogenic interactions (e.g. fungal and nematode), but this is the only example of its use to study legume-virus interactions. The results obtained indicate the potential of using *M. truncatula* as a model to study resistance response to other legume viruses and provide a firm basis for identifying the hypersensitive *R* gene that confers resistance to SCMoV.

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DEDICATION

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PUBLISHED WORK AND ORAL PRESENTATIONS

The following papers and conference presentations have been published from work described in this thesis.

Papers from this research

Saqib M, Jones RAC, Cayford B and Jones MGK (2005) First report of *Bean common mosaic virus* in Western Australia. *Plant Pathology* **54**, 563.

Saqib M, Bayliss KL, Dell B and Jones MGK (2005) First record of a phytoplasma-associated disease of chickpea (*Cicer arietinum*) in Australia. *Australasian Plant Pathology* **34**, 425-426.

Bayliss KL, **Saqib M**, Dell B, Jones MGK and Hardy GESTJ (2005) First record of ‘*Candidatus* Phytoplasma australiense’ in *Paulownia* trees. *Australasian Plant Pathology* **34**, 123-124.

Saqib M, Bayliss KL and Jones MGK (2006) Identification of Sweet Potato Little leaf phytoplasma in *Vigna unguiculata* var *sesquipedalis* and *Lycopersicon esculentum*. *Australasian Plant Pathology* **35**, 293-296.

Saqib M, Jones MGK and Jones RAC (2006) *Candidatus* Phytoplasma australiense is associated with diseases of red clover and paddy melon in south-western Australia. *Australasian Plant Pathology* **35**, 283-285.

Saqib M, Smith BD, Parrish JL, Ramsdale RA and Jones MGK (2007) Detection of phytoplasma in *Allocasuarina fraseriana* and *Acacia saligna* in Kings Park. *Journal of the Royal Society of Western Australia* **90**, 175-178.

Manuscripts in preparation

Saqib M, Fosu-Nyarko JN, Jones MGK and Jones RAC. Screening of the *Medicago truncatula* core collection for responses to four legume viruses: AMV, CMV, SCMoV and BYMV.

Saqib M, Ellwood S, Jones RAC and Jones MGK. Mapping a SCMoV resistance gene in *Medicago truncatula*.

Saqib M, Jones RAC and Jones MGK. Identification of phytoplasma associated diseases of papaya, tomato and eggplant in the remote isolated Gascoyne region of Western Australia.

Conference Oral Presentations

Saqib M, Fosu-Nyarko JN, Smith T, Wylie S, Jones RAC and Jones MGK (2005) Screening of the core *Medicago truncatula* collection against four viruses: AMV, CMV, SCMoV and BYMV 6th *Australasian Plant Virology Workshop* Gold Coast, Queensland from Monday, 30 August-2 September 2004.

Saqib M, Fosu-Nyarko JN, Jones RAC and Jones MGK (2005) Screening of the core *Medicago*

truncatula collection against four viruses: AMV, CMV, SCMoV and BYMV 2nd Australian Model Legume Workshop 5-8 April. Perth Western Australia.

Saqib M, Ellwood S, Jones RAC and Jones MGK (2006) Mapping a SCMoV resistance gene in *Medicago truncatula*: 7th Australasian Plant Virology Workshop (8-11 November) Perth Western Australia.

Saqib M, Ramsdale RA, Jones RAC and Jones MGK (2006) Identification of phytoplasma-associated disease in native and cultivated plants from Western Australia: 7th Australasian Plant Virology Workshop (8-11 November) Perth Western Australia.

Saqib M, Ellwood S, Jones RAC and Jones MGK (2008) Mapping a SCMoV resistance gene in *Medicago truncatula*. International conference on Molecular Mapping and Marker Assisted Selection in Plants (3-6 February) Vienna, Austria.

Sequences Submitted to Genbank

AY850005 *Bean common mosaic virus* strain MS1 from Australia polyprotein gene, partial cds
gi|56967594|gb|AY850005.1|[56967594]

EF059538 Uncultured bacterium isolate 5KPBG 16S ribosomal RNA gene, partial sequence
gi|117574659|gb|EF059538.1|[117574659]

DQ777761 Unidentified mollicute isolate 222 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic spacer and tRNA-Ile gene, complete sequence; and 23S ribosomal RNA gene, partial sequence
gi|111182615|gb|DQ777761.2|[111182615]

DQ777760 Sweet potato witches'-broom phytoplasma isolate 111 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic spacer and tRNA-Ile gene, complete sequence; and 23S ribosomal RNA gene, partial sequence
gi|111182614|gb|DQ777760.2|[111182614]

DQ777762 Sweet potato witches'-broom phytoplasma 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic spacer and tRNA-Ile gene, complete sequence; and 23S ribosomal RNA gene, partial sequence
gi|111054796|gb|DQ777762.2|[111054796]

DQ777763 *Candidatus* Phytoplasma aurantifolia isolate 19e5 16S ribosomal RNA gene, partial sequence
gi|110592204|gb|DQ777763.1|[110592204]

DQ665841 Red tassel witches'-broom phytoplasma 16S ribosomal RNA gene, partial sequence
gi|110083968|gb|DQ665841.1|[110083968]

DQ310885 *Candidatus* Phytoplasma australiense from *Cucumis myriocarpus* 16S ribosomal RNA gene, partial sequence
gi|83033894|gb|DQ310885.1|[83033894]

DQ269471 *Candidatus* Phytoplasma australiense from *Trifolium pratense* 16S ribosomal RNA gene, partial sequence
gi|83033893|gb|DQ269471.1|[83033893]

DQ375778 Sweet potato little leaf phytoplasma 16S ribosomal RNA gene, partial sequence
gi|87241731|gb|DQ375778.1|[87241731]

DQ375777 Sweet potato little leaf phytoplasma 16S ribosomal RNA gene, partial sequence
gi|87241730|gb|DQ375777.1|[87241730]

DQ321672 Uncultured bacterium clone Maano_carrot47 16S ribosomal RNA gene, partial sequence
gi|84310210|gb|DQ321672.1|[84310210]

AY858053 *Candidatus* Phytoplasma aurantifolia 16S ribosomal RNA gene, partial sequence
gi|57341402|gb|AY858053.1|[57341402]

LIST OF ABBREVIATIONS

AFLPs	amplified fragment length polymorphisms
ALIRU	Australian Legume Inoculant Research Unit
ALPs	amplicon length polymorphisms
AQIS	Australian Quarantine and Inspection Service
ATP-	adenosine 5'-triphosphate
<i>Avr</i>	avirulence gene
BAC	bacterial artificial chromosome
cM	centimorgan
dpi	days post inoculation
dsDNA	double stranded DNA
<i>Eds1</i>	enhanced disease susceptibility gene
EF-Tu (tuf)	elongation factor
GTP	guanosine-5'-triphosphate
HR	hypersensitive response
I	intercellular
INRA	Institut National de la Recherche Agronomique Toulouse/French National Institute for Agricultural Research
IRPCM	International Research Programme on Comparative Mycoplasmaology
ISA	Inter-simple sequence repeat amplification
KAPP	kinase-associated protein phosphatase
KDa	Kilo Dalton
LRR	leucine-rich repeat
LZ	leucine zipper
MAS	marker assisted selection
NADPH	nicotinamide adenine dinucleotide phosphate hydrogenase
NBS	nucleotide binding site
<i>Ndr1</i>	no disease resistance
ORFs	open reading frames
<i>npr1</i>	No PR gene induction
PCD	programmed cell death
PCR	polymerase chain reaction
PR	pathogenesis-related
QTLs	quantitative trait loci
<i>R</i>	resistance gene
Rb	retinoblastoma
Rcr	required for <i>C. fulvum</i> resistance
RAPD	random amplified polymorphic DNA
RDR	Required for Disease Resistance
RFLP	restriction fragment length polymorphism

RIL	Recombinant inbred lines
RNAi	RNA interference
RGAs	Resistance gene analogue
ROS	reactive oxygen species
SAR	systemic acquired resistance
SARDI	South Australian Research and Development Institute
SNP	single nucleotide polymorphism
SCARs	sequence characterized amplified regions
SSCP	single strand conformation polymorphism
SSR	simple sequence repeats
ssDNA	single stranded DNA
SSRP	simple sequence repeat polymorphism
STS	sequence-tagged sites
TILLING	Targeting Induced Local Lesions in Genomes
TIR-NBS-LRR	Toll-interleukin-1 homology region/nucleotide binding site/leucine-rich repeat
TIR	Toll and the interleukin-1 receptor
tRNA. ile	isoleucine transfer RNA
USDA	United States Department of Agriculture
WA	Western Australia
WA (SABC)	Western Australian State Agricultural Biotechnology Centre
YAC	yeast artificial chromosome

Viruses/Phytoplasma:

AGY	Australian grapevine yellows phytoplasma
AluY	Australian Lucern yellow
AMV	<i>alfalfa mosaic virus</i>
AY	aster yellows
BCTV	<i>beet curly top virus</i>
BLRV	<i>bean leaf roll virus</i>
BLTV	<i>beet leafhopper-transmitted virescence</i>
BYMV	<i>bean yellow mosaic virus</i>
BWYV	<i>beet western yellows virus</i>
Ca	<i>candidatus</i>
CCDV	<i>chickpea chlorotic dwarf virus</i>
CMV	<i>cucumber mosaic virus</i>
OYDV	<i>onion yellow dwarf virus</i>
OVP	oenothera virescence phytoplasma
PSbMV	<i>pea seed-borne mosaic virus</i>
PVY	<i>potato virus Y</i>
PVX	<i>potato virus X</i>
SCMoV	<i>subterranean clover mottle virus</i>

SMV	<i>soyabean mosaic virus</i>
SPFMV	<i>sweet potato feathery mottle virus</i>
SPLL-V4	sweet potato little leaf strain V4
TBB	tomato big bud
TEV	<i>tobacco etch virus</i>
TGMV	<i>tomato golden mosaic virus</i>
TMV	<i>tobacco mosaic virus</i>
TSWV	<i>tomato spotted wilt virus</i>
TMV	<i>tobacco mosaic virus</i>
ToMV	<i>tomato mosaic virus</i>
TYLCV	<i>tomato yellow leaf curl virus</i>
TuMV	<i>turnip mosaic virus</i>
TYLCV	<i>tomato yellow leaf curl virus</i>
WSMV	<i>wheat streak mosaic virus</i>
WDV	<i>wheat dwarf virus</i>
MSV	<i>maize streak virus</i>
PVX	<i>potato virus X</i>

Other abbreviations used in the text:

3'	hydroxyl-terminus of DNA molecule
5'	phosphate-terminus of DNA molecule
α	alpha
Amp	ampicillin
bp	base pairs
CaCl ₂	calcium chloride
CIAP	calf intestinal alkaline phosphatase
CTAB	cetyltrimethylammonium bromide
cm	centimeter
cv	cultivar
cDNA	complementary deoxyribonucleic acid
DEPC	diethylpyrocarbonate
DNA	deoxyribose nucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra-acetic acid
g	gram
HCl	hydrochloric acid
h	hour
k	kilo
kb	kilobase
kbp	kilobase pair
L	litre
LB medium	Luria-Bertani medium

μ	micro
M	molar
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mol	mole
MW	molecular weight
μ g	microgram
μ L	microlitre
μ m	micrometer
oligo	oligonucleotide
n	nano
NaAC	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
$^{\circ}$ C	degrees celsius
TAE buffer	tris-acetate acid-EDTA electrophoresis buffer
<i>Taq</i>	<i>Thermus aquaticus DNA polymerase</i>
Tris	tris (hydroxymethyl) aminomethane
UV	ultra violet