Genetic and molecular biological studies of Annual Ryegrass resistance to *Anguina funesta*

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ABSTRACT

Annual ryegrass toxicity (ARGT) is an often fatal poisoning occurring in grazing animals following the ingestion of seedheads of the annual ryegrass, *Lolium rigidum,* infested with the corynetoxin-producing bacteria, *Rathayibacter toxicus*. Breaking the disease cycle, through the use of lines of *L. rigidum* resistant to the nematode, *Anguina funesta*, can be used to reduce the risk of ARGT outbreaks. In *L. rigidum*, resistance to *A. funesta* appears to be under the control of two unknown, but complementary genes. This study explored alternate approaches towards the allocation of genotype for lines of *L. rigidum* with respect to resistance to *A. funesta*.

A genetic approach involving the analysis of numbers of progeny, resistant and susceptible to *A. funesta,* from factorial crosses to infer parental genotypes was employed. Allocations of the resistance genotypes were possible for a number of *L. rigidum* lines.

Two alternate molecular approaches were taken, in an attempt to isolate molecular markers linked to the regions of the *L. rigidum* genome responsible for resistance to *A. funesta*. A total of 62 arbitrary 10-mer oligonucleotide primers were used to screen a pair of putative near isogenic lines (NILs), differing in resistance to *A. funesta* (R799 and S1150), for RAPD markers linked to the genes conferring resistance. RAPD reactions with the primers OPAM-1 and OPAM-08 yielded products with genomic DNA from R799 but not S1150 as template. However, the association of these products with resistance was not maintained across an extended range of *L. rigidum* lines. A more targeted molecular approach used degenerate oligonucleotide primers, designed on highly conserved motifs of the nucleotide binding site (NBS) region from the proteins encoded by many cloned plant resistance genes, to amplify resistance gene analogues (RGAs) in *L. rigidum*. This is the first record of the presence to RGA sequences in *L. rigidum*. A total of 91 cloned, amplified products were analysed, from which 22 were sequenced and assigned to one of

four classes, each exhibiting high levels of similarity to previously cloned RGA sequences in other plant species. Each class was detected in low or moderate copy number in the *L. rigidum* genome. *L. rigidum* genomic sequences hybridised by class 2 and class 3 RGA sequences are presented as potential markers of resistance.

Statement of Originality

This thesis contains no material that has been accepted for the award of any degree or diploma by any other university. To the best of my knowledge it contains no material that has previously been published by any other persons, except where due reference has been made in the text. I consent to this thesis, when deposited in the university library, being available for photocopying and loan.

Aaron Thomas Mitchell

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ABBREVIATIONS

λ	bacteriophage Lambda
μCi	microcurie
°C	degrees Celsius
A	adenosine
AFLP	amplified fragment length polymorphism
ARGT	annual ryegrass toxicity
ATP	adenosine triphosphate
bp	base pair
BSA	bulk segregant analysis
CTAB	hexadecyltrimethyl ammonium bromide
C	cytidine
CC	coiled-coil
cm	centimeter
cM	centi Morgan
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	2'-deoxy-thymidine-5'-triphosphate
EDTA	ethylenediamine tetra acetic acid
EST	expressed sequence tag
F_p	genetic similarity between parental lines
F_s	genetic similarity between siblings
F_{sp}	genetic similarity between parent and sibling
g	force of gravity
g	gram
G	guanosine
h	hour
HPLC	high pressure liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside

Kb	kilobase
KOAc	potassium acetate
LB	Luria-Bertani
LRR	leucine rich repeat
LZ	leucine zipper
M	moles per litre
MD	minimum genetic distance
MES	2[N-morpholino]ethanesulfonic acid
mg	milligram
min	minute
MR	moderately resistant to *A. funesta*
mRNA	messenger ribonucleic acid
M_p	genetic similarity between parental lines
M_s	genetic similarity between siblings
M_{sp}	genetic similarity between parent and sibling
Na_2EDTA	ethylenediaminetetra acetic acid disodium salt
NaMOPS	3-[N-morpholino]propanesulfonic acid
NaOAc	sodium acetate
NBS	nucleotide binding site
NIL	near-isogenic line
OD	optical density
p	probability
PCR	polymerase chain reaction
PEG-8000	polyethylene glycol 8,000
PEG	polyethylene glycol
PVP-360	polyvinylpyrrolidine-360
R	resistant to *A. funesta*
RACE	random amplification of cDNA ends
RAPD	random amplified polymorphic DNA
RGA	resistance gene analogue
RNase	ribonuclease
rpm	revolutions per minute
S	susceptible to *A. funesta*
s	second

SDS	sodium dodecyl sulphate
T	thymidine
Taq	*Thermus aquaticus*
TAE	tris acetic acid EDTA
TBE	tris-boric acid EDTA
TIR	Toll and interleukin-1 receptor
UC	University of California
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

TABLE OF CONTENTS

Chapter 3
Genetics of Disease Resistance

Chapter 4
RAPD Analysis of *L. rigidum* Lines

Chapter 5
Resistance Gene Analogues of *L. rigidum*

Chapter 1

Introduction

1.1 PLANT PATHOGEN RESISTANCE

1.1.1 Plant Defences to Pathogen Attack

Specific plant defences to pathogen attack are described in Flor's (1971) gene-for-gene model of plant pathogen resistance. In this model, a specific pathogen signal molecule produced directly or indirectly by a dominant avirulence gene, *avr,* interacts with the product of a resistance gene, R, of the host to trigger the defence response, arresting pathogen growth. The interaction of the pathogen signal molecule with the host *R* gene product is thought to trigger signal transduction within the host plant, leading to the activation of a defence response mechanism by the transcriptional activation of defence response genes (Gabriel and Rolfe, 1990; Keen, 1990).

Defence responses may include a hypersensitive response involving rapid localised cell death at the site of infection, which may be observed as tissue necrosis, limiting the spread of the pathogen (reviewed by Keen *et al.,* 1993). Cellular events involved in this response include the production of reactive oxygen intermediates (O_2, OH^- and H_2O_2), lipid peroxidation and electrolyte leakage due to the disruption of cellular vacuoles and membranes (Lamb *et al.*, 1989; Levine *et al.,* 1994). Other defence responses may include the production of antimicrobial compounds (phytoalexins) and lytic enzymes and the reinforcement of cell walls surrounding infected areas by the cross-linkage of cell wall bound phenolics (reviewed by Dixon *et al.,* 1994; Hammerschmid, 1999). A hypersensitive response may trigger nonspecific systemic acquired resistance, through the entire plant to decrease the severity of the pathogen attack on the entire plant (Ryals *et al.,* 1996).

1.1.2 Genetic Control of Disease Resistance

1.1.2.1 Resistance Genes

In the past decade a variety of plant resistance genes (R-genes) from a range of plants have been cloned and analysed. Conserved nucleotide binding site (NBS) and leucine -rich repeat (LRR) motifs were present in the putative proteins of a number of the first R-genes to be cloned; the tobacco *N* gene, the flax *L6* gene and the *Arabadopsis thaliana RPS2* gene (Staskawicz *et al.,* 1995). Alternative conserved motifs have been identified in subsequently cloned R-genes (Dangl and Jones, 2001). There appears to be no relationship between R-protein structure and the target pathogen.

In many genomes, it is common for R-genes to be mapped in clusters. Examples of these complex loci include the *Rp*1 and *Rp*3 loci in maize containing 14 and 6 rust resistance genes, respectively (Sudupak *et al.,* 1993; Richter *et al.*, 1995), the *Dm* locus in lettuce containing 12 resistance genes (Witsenboer *et al.*, 1995) and the *pca* crown rust resistance cluster in diploid *Avena* species that contains a least 5 resistance genes (Yu *et al.,* 2001). R-genes are often multiallelic, such as the barley *Mla* locus with 28 alleles (Jorgensen, 1994).

1.1.2.2 NBS-LRR Type R-genes

The largest class of R-genes cloned encode proteins with a putative nucleotide binding site (NBS) domain and a C-terminally located block of leucine rich repeats (LRR) (Staskawicz *et al.,* 1995). Deduced proteins of this type of R-gene contain either a coiled-coil (CC)/Leucine zipper (LZ) domain or a region with homology to the cytoplasmic domains of the *Drosophila* Toll protein and interleukin-1 receptor (TIR) at their amino terminus (Meyers, 1999; Pan *et al.*, 2000a).

This class of R-genes confers resistance to a wide range of pathogens including insects, nematodes, fungi, bacteria and viruses. NBS-LRR R-genes include the *A. thaliana* genes *RPS2,* conferring resistance to pathogenic strains of *Pseudomonas syringae* expressing the avirulence gene *avrRpt2* (Bent *et al.*, 1994) and *RPP5*, conferring resistance to powdery mildew (Parker *et al.,* 1997). The flax rust resistance genes L6 and M (Lawrence *et al.*, 1995; Anderson *et al.*, 1997) and the tobacco *N* gene conferring resistance to the tobacco mosaic virus (Whitham *et al.,* 1994) are also members of this class of R-gene.

The deduced protein structure suggests most R-proteins are located in the cytoplasm (Dangl and Jones, 2001), with the exceptions of the plasma membrane RPM1 protein of *A. thaliana* (Boyes *et al.*, 1998) and L6 from flax, which contains a putative signal peptide (Lawrence *et al.,* 1995).

1.1.2.3 Potential Functions of NBS-LRR Resistance Proteins

The NBS in R-proteins is distinct from those found in other protein kinases (Taylor *et al.*, 1993). NBS regions were first identified within the ATPase domain of proteins involved in programmed cell death (Aravind *et al.*, 1994; van der Biezen *et al.*, 1998) and mediate the oligomerisation required for the activation of apoptotic proteins (Yang *et al.*, 1998). The NBS region collectively consists of three major domains; the kinase 1a or phosphate binding loop (P-loop), the kinase 2 and the kinase 3a domains. Site-directed mutagenesis of amino acids within the NBS domain encoded by the R-gene *Prf* abolished resistance to *Pseudomonas syringae* pv. *tomato* expressing the avirulence gene *avrPto* in tomato, indicating this region is essential for resistance to this pathogenic strain of *P. syringae* (Salmeron *et al.*, 1996).

There is evidence to indicate the LRR domain has a major role in determining the specificity of pathogen recognition (Ellis *et al.,* 1999). In non-R-proteins LRR domains participate in protein-protein interactions in a range of organisms (Kobe and Deisenhofer, 1994; Jones and Jones, 1996). Exposed residues within the β-strand/β-turn structual motif of LRR may be involved in the specificity of pathogen ligand binding (Parniske *et al.,* 1997; Wang *et al.*, 1998). Through site-directed mutagenesis, both the NBS and LRR motifs of the tobacco *N* gene have been shown have an indispensable role in the induction of a response against the tobacco mosaic virus (Dinesh-Kumar *et al.*, 2000).

Based on their structure and function in related proteins, the CC/LZ or TIR motifs located at the amino terminus of R-proteins are thought to play a role in signal transduction to trigger a resistance response within the plant cell (Dinesh-Kumar *et al.*, 1995; Ellis *et al.*, 1999).

A key feature of Flor's gene-for-gene model of resistance is the interaction of the R-proteins with the pathogen *avr* product to trigger signaling pathways to result in resistance. Direct interaction of the *Arabadopsis RPS*2 protein with the AvrRpt2 protein expressed by *P. syringae* carrying the *avrRpt2* gene has been detected *in vivo* (Leister and Katagiri, 2000). A number of distinct signalling pathways appear to operate in *Arabadopsis.* In lines mutant for the *ndr*1 gene, resistance mediated by *RPM*1, *RPS*2 and *RPS*5 was supressed (Century *et al.,* 1997; Aarts *et al.*, 1998). In contrast, in lines mutant for *eds*1 *RPS*4 mediated resistance was supressed and *RPM*1, *RPS*2 and *RPS*5 mediated resistance remained active (Aarts *et al.*, 1998). The two, distinct signalling pathways correlated with the subtype of R-gene as resistance mediated by CC/LZ-NBS-LRR family members were *ndr*1 dependent and resistance mediated by TIR-NBS-LRR family members was *eds*1 dependent. The *RPP13-Nd* gene, which prevents parasitism by isolates of the downy mildew *Peronspora parasitica* (At)

in *Arabadopsis,* functioned independently of both *NDR1* and *EDS1* genes (Bittner-Eddy and Beynon, 2001). Therefore, in *Arabadopsis,* there appear to be at least three operational signalling pathways. Each distinct pathway may be triggered following the interaction of a specific R-protein with the pathogen *avr* gene product.

1.1.2.4 Other R-gene Classes

The *A. thaliana* recessive resistance gene *RRS1-R,* which confers resistance to several strains of *Ralstonia solanacearum* causing bacterial wilt, is a novel NBS-LRR type R-gene (Deslandes *et al.,* 2002). In addition to containing sequences encoding TIR, NBS and LRR motifs, the *RRS1-R* gene also encodes a potential nuclear localisation signal at the carboxyl terminus and a 60 amino acid WRKY motif found in many activator plant transcription factors.

R-genes not classified as NBS-LRR type proteins have been cloned from a variety of plants (Baker *et al.,* 1997). As the encoded proteins differ in structure and cellular location, they may represent alternative resistance mechanisms and signalling pathways. The tomato *Cf9* gene encodes proteins with putative LRR, but not NBS domains (Jones *et al.*, 1994). They are predicted to be extracytoplasmic with a carboxy terminal membrane anchor. The tomato R-gene *Pto* encodes a putative serine-theronine kinase that may play a role in signal transduction (Martin *et al.*, 1993). *Pto* cannot function without a functional *Prf* gene that encodes an NBS-LRR containing protein (Salmeron *et al.*, 1996). The rice *Xa*21 gene, conferring resistance to *Xanthomanos oryzae* pv. *oryzae*, encodes a putative extracellular LRR and an intracellular serine-threonine kinase (Song *et al.,* 1995). The sugar beet $Hs1^{pro-1}$ encodes a putative membrane spanning protein containing a leucine rich region at the amino

terminal that is not homologous to the LRR domain (Cai *et al.*, 1997). The barley *Mlo* gene encodes a protein with 7 putative transmembrane helices in the plasma membrane with the amino terminal located extracellularly and the carboxy terminal located intracellularly (Buschges *et al.*, 1997; Devoto *et al.*, 1999). The *Hm*1 gene product in maize is functionally distinct from other R-gene products as it achieves resistance by inactivating a toxin produced by race 1 isolates of the fungus *Cochliobolus carbonum* (Johal and Briggs, 1992).

1.1.2.5 Evolution of NBS-LRR Type Resistance Genes

Many copies of NBS-LRR sequences exist in plant genomes, but not all encode known resistance genes. In *Arabadopsis*, for example, approximately 10% of NBS-LRR sequences are pseudogenes (Pan *et al.*, 2000a). Both pseudogenes and NBS-LRR sequences not encoding characterised resistance genes are classified as resistance gene analogues (RGAs). Phylogenetic analysis of RGA sequences from different plant species indicates RGA sequences existed in the plant genome prior to species divergence (Michelmore and Meyers, 1998). The high sequence diversity observed at R-gene clusters such as rice (Song *et al.*, 1997), tomato (Parniske *et al.*, 1997) and lettuce (Meyers *et al.*, 1998) has evolved through gene duplication followed by point mutation, deletion and duplication of intragenic DNA repeats between related genes leading to the observed diversity (Ellis *et al.*, 2000). Proteins encoded by these sequences are proposed to function as adaptable surveillance molecules for rapidly evolving pathogen avr proteins (Hammond-Kosack and Jones, 1997).

Sequence comparisons of the NBS domains encoded from both R-genes and RGAs indicated these sequences could be divided into two main groups (Meyers *et al.*, 1999; Pan *et al.*, 2000a). Group 1 sequences, which included the R-genes *N, M, L6, RPP*1 *and Rpp*5, were

found only in dicotyledonous plants. In contrast, group 2 sequences were found in both monocotyledonous and dicotyledonous plants and included the R-genes *RPS2, RPM1, Mi, Dm3, Xa1, RPP3, RPS5 and Prf.* The two groups could also be distinguished by the conserved motifs located at the amino terminal of the encoded protein, with group 1 R-genes containing sequence encoding for the TIR motif and group 2 R-genes containing sequence encoding for the CC/LZ motif.

1.1.2.6 Complex Versus Simple R-loci

Complex resistance loci (R-loci) contain clusters of R-gene families and closely related sequences. As an example, the *Dm* cluster contains R-genes coding for resistance to different isolates of the lettuce downy mildew, *Bremia lactucae,* and the lettuce root aphid, with each R-gene product directed towards an individual pathogen *avr* product (Sicard *et al.,* 1999). The number of R-genes in the lettuce *Dm* major R-gene cluster varies between cultivars. In contrast, the simple R-locus *RPM1* in *A. thaliana* encodes a versatile protein with specificities for both *avr* products avrRpm1 and avrB of *P. syringae* (Bisgrove *et al.*, 1994; Grant *et al.*, 1995). Complex R-loci are able to promote structual divergence through recombination, accelerating the evolution of novel R-genes in response to co-evolving pathogens. Simple R-loci are restricted in their ability to evolve new *avr* specificities and therefore limited in their rate of evolution (Grant *et al.,* 1998).

1.1.3 Approaches to Cloning Plant Resistance Genes

1.1.3.1 Disruption of Resistance Genes

A number of R-genes including the tomato *Cf-9* gene (Jones *et al.*, 1994), the tobacco *N* gene (Whitham *et al.*, 1994) and the flax *L6* gene (Lawrence *et al.*, 1994) have been isolated with the use of transposon insertion. Initially, regions of the genome encoding resistance were identified by the insertion of transposons into target genes in plants carrying the target resistance gene to disrupt the resistance phenotype. Candidate R-genes were isolated by cloning sequences surrounding the transposon insertion site and were screened for their ability to complement a phenotypically susceptible line when transformed. Such an approach does not require the physical or genetic location of the resistance gene of interest to be known.

1.1.3.2 Map/Linkage Based Cloning Approaches

Plant R genes cloned by map based approaches include the tomato genes *Pto* and *Prf* (Martin *et al.*, 1993), *RPS2* and *RPM1* in *A. thaliana* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995) and the rice *Xa21* gene (Song *et al.*, 1995a). The success of map based cloning approaches relies on the availability of high resolution maps. The high resolution mapping of plant genomes has been assisted by the use of molecular markers generated by PCR-based techniques such as random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) and amplified fragment length polymorphisms (AFLP) (Zabeau and Vos, 1993). Markers closely linked to resistance genes are used to isolate large inserts of genomic DNA contained within genomic libraries of yeast or bacterial artificial chromosome (YAC/BAC) or cosmid clones. Isolated R-gene candidates must be confirmed as R-genes by

the genetic complementation of a susceptible phenotype or disruption of the gene in a resistant line to cause susceptibility. Alternatively, support may be provided for R-gene confirmation if sequencing of known loss of function alleles detects premature termination or amino acid substitution. As an example, both premature termination codons and amino acid substitutions were present in alternative loss of function alleles of the *RPM1* gene (Grant *et al.,* 1995).

The type of approach taken is dependent on the physical proximity of the marker to the target R-gene. Chromosome walking requires a "walk" from the marker through overlapping DNA clones to the R-gene (Rommens *et al.*, 1989). Chromosome landing in which the marker and target R-gene are contained within the same clone requires the identification of a marker at a physical distance less than the average size of the inserts within the genomic library (Tanksley *et al.*, 1995). Chromosome walking is often not possible in plant genomes due to the high levels of repetitive DNA.

Near Isogenic Lines (NILs) and Bulk Segregant Ananlysis (BSA) can aid the isolation of R-genes. BSA makes use of a segregating population originating from a single cross to screen a large number of loci for linkage to targeted R-genes (Michelmore *et al.,* 1991). Each bulk contains individuals selected to be identical for the trait or gene of interest, but with random genetic backgrounds at loci unlinked to the selected gene of interest. NILs are created by backcrossing programs to introgress resistance into cultivars. They facilitate the isolation of sequences that differ between the two lines as, due to 'linkage drag', the sequences surrounding the target gene diminish at a slower rate than any unlinked sequence (Stam and Zeven, 1981). In the case of NILs differing in resistance to pathogen infection, any marker or gene differing between the two lines has a high chance of being linked to the resistance gene. NILs screened with RAPD markers were first used by Martin *et al.* (1991) to isolate markers linked to resistance.

1.1.3.3 PCR-Based Cloning Strategies

The observation of conserved motifs encoded by R-genes cloned in a range of plant species prompted several groups to use a PCR-based strategy to isolate R-gene candidates. Degenerate primers designed to conserved regions within NBS-LRR type R-genes were used in PCRs to amplify resistance gene analogues (RGA). RGA sequences have been isolated by this approach in soybean (Kanazin *et al.,* 1996; Yu *et al.*, 1996), maize (Collins *et al.,* 1998), rice (Mago *et al.,* 1999), wheat and barley (Seah *et al.*, 1998), pepper (Pflieger *et al.,* 1999) and coffee (Noir, *et al.*, 2001). R-gene candidates are evaluated on their linkage to target R-gene loci. RGA sequences that co-segregate with resistance loci may be identified as R-genes if supported by transgenic experiments to either disrupt the resistant allele to cause susceptibility or complement a susceptible phenotype or by sequence analysis of mutant alleles. Such an approach allowed the successful cloning of the flax R-gene *N* (Dodds *et al.*, 2001). Alternatively, if transgenic complementation analysis does not support the identification of the co-segregating RGA sequence as the R-gene of interest, the RGA can still be useful in the isolation of BAC or YAC clones that may potentially contain the R-gene of interest. Analysis of RGA sequences in rice, identified a RGA sequence co-segregating with the R-gene *Xa4* (Wang *et al.,* 2001). This RGA was used to screen for clones that upon further testing may contain the *Xa4* R-gene.

1.1.4 Nematode Resistance Genes

A number of R-genes responsible for resistance to nematode infection have been cloned. Example of these include the tomato *Mi* gene (Milligan *et al.*, 1998), the potato *Gpa*-2 gene (van der Vossen *et al.,* 2000) and the sugar beet $Hs1^{pro-1}$ gene (Cai *et al.,* 1997). Both the

Mi gene, responsible for resistance to several strains of root knot nematode, including *Meloidogyne incognita*, and the *Gpa*-2 gene, responsible for resistance to some isolates of the potato cyst nematode, *Globodera pallida,* encode putative NBS-LRR containing proteins. The *Gpa*-2 gene encodes a protein with a putative LZ domain near its amino terminal. In contrast the $Hs1^{pro-1}$ gene, responsible for resistance to the sugar beet cyst nematode, *Heterodera schachtii*, encodes a putative membrane spanning region with a leucine rich region that does not fit the pattern of an LRR.

1.2 ANNUAL RYEGRASS TOXICITY (ARGT)

1.2.1 The Biology of ARGT

Annual ryegrass toxicity (ARGT) is an often fatal poisoning occurring in grazing animals following the ingestion of seed heads of the annual ryegrass, *Lolium rigidum,* infested with the corynetoxin producing bacterium, *Rathyibacter toxicus*. The first reported livestock deaths due to ARGT in Australia occurred in 1955 in the mid-North region of South Australia (Fisher, 1977). By 1985, ARGT had been found to occur in most cropping regions of South Australia and parts of Western Australia (McKay 1986, Stynes and Wise, 1980).

The mortality rate for livestock feeding in infested pasture can exceed 90% if livestock are not relocated immediately following the first signs of animal toxicity (McKay and Ophel, 1992). The economic cost to farmers due to ARGT is not limited to the direct stock losses due to toxicity. Ingestion of the corynetoxin causes ewes to abort resulting in a reduced lambing rate (Berry and Wise, 1975, McIntosh *et al.*, 1967 and Schneider, 1981). Additional costs are also incurred through pasture treatments and daily inspections of stock for symptoms of toxicity (Robert and Baxter, 1991).

An outbreak of ARGT is dependent on a number of biological interactions and environmental conditions (Figure 1.1). The toxin producing bacteria is carried into developing annual ryegrass seed heads by the seed gall nematode, *Anguina funesta.* Nematode galls containing both nematodes and toxin producing bacteria are formed within the seed heads. Infected seed heads are most toxic when ingested by grazing animals as the pasture dries off. Consequently, the majority of stock losses occur in late spring to summer.

1.2.1.1 The Corynetoxin Producing Bacteria *Rathyibacter toxicus*

The toxin responsible for the ARGT is produced by the bacteria *Rathyibacter toxicus*, formally classified as *Clavibacter toxicus* (Riley and Ophel, 1992). *R. toxicus* is a rod shaped, gram positive, non-spore forming, non motile, capsulated bacteria that produces corynetoxins comprised of uracil, N-acetyl glucosamine, tunicamine and long fatty acid chains (Edgar *et al.*, 1982). The toxins act by inhibiting N-glycosylation of proteins (Jago *et al.,* 1983). This effects the vascular system and oxygen distribution within the infected animal. The resultant symptoms of toxicity in the infected animal are primarily neurological problems as the brain is the most severely effected organ of the reduced oxygen availability. *R. toxicus* has an obligate association with the nematode *Anguina funesta*, without which it cannot infect ryegrass (Price 1973, Bird and Stynes, 1977). Infested seed heads can be detected visually by the observation of a characteristic yellow slime on seed heads (Fisher, 1977).

1.2.1.2 Bacteriophage Associated with Toxin Producing *R. toxicus*

Hexagonal particles resembling bacteriophage were first observed by electron microscopy in toxic ryegrass galls after anthesis, the same time toxin concentration increased

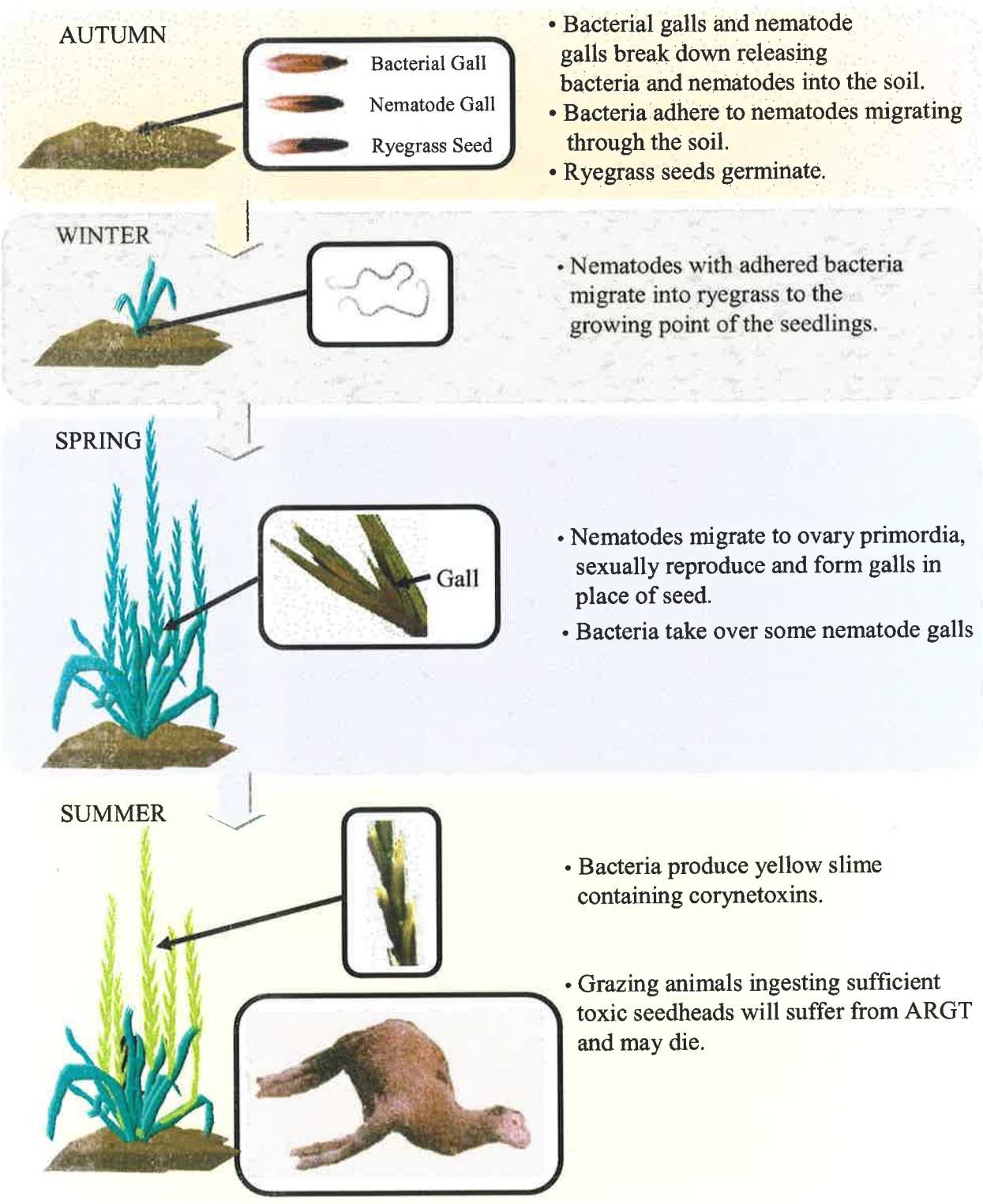


Figure 1.1: The roles of the nematode, *A. funesta* and the bacteria, *R. toxicus* in ARGT. Photographs used with permission of A. McKay.

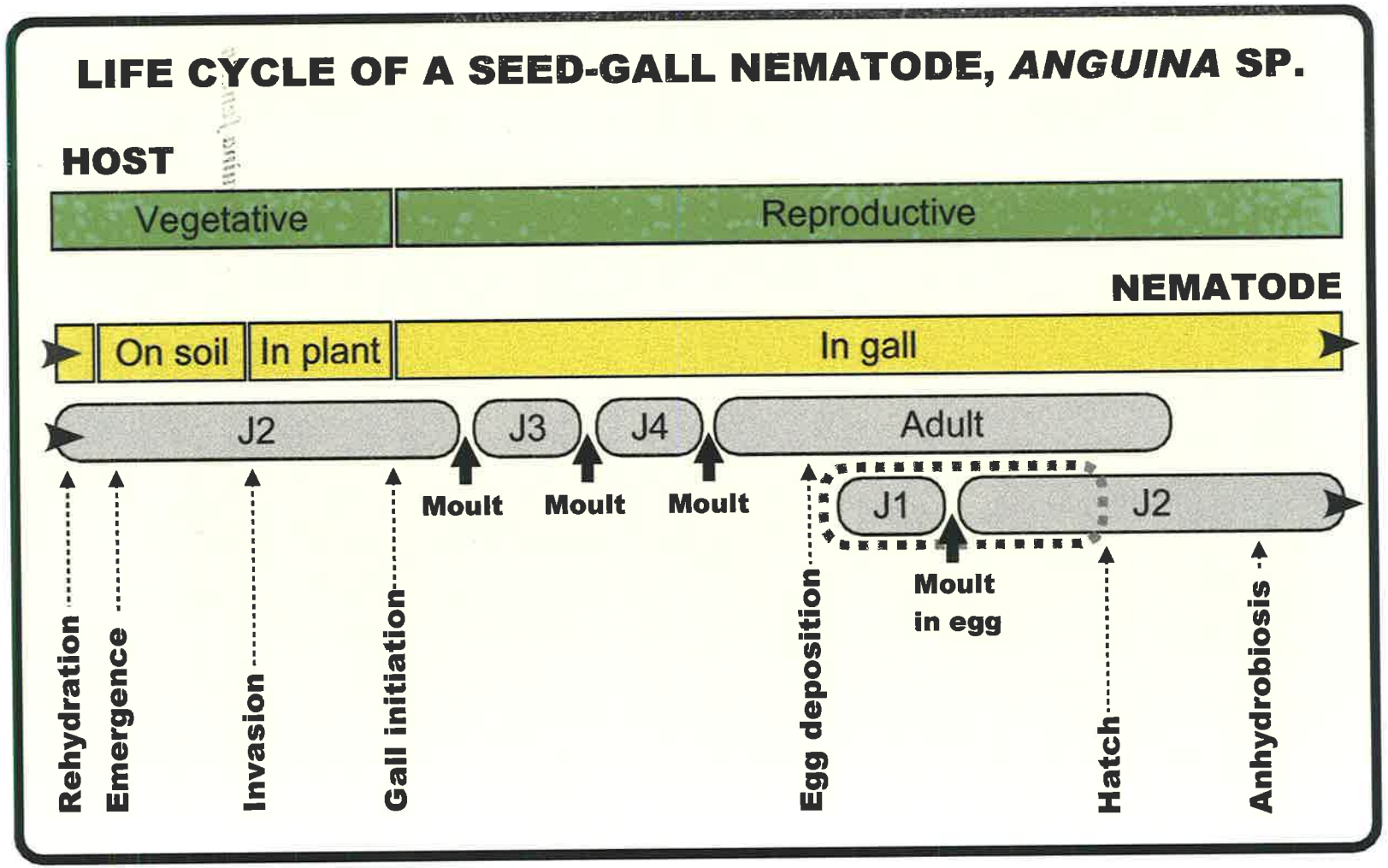
ten fold, but were absent in non-toxin producing bacteria in culture (Bird *et al.,* 1980; Stynes and Bird, 1983). Bacteriophage capable of specifically lysing strains of *R. toxicus* were isolated from toxic *L. rigidum* galls, as well as from galls containing corynetoxin producing *R. toxicus* on two alternative host plants; *Agrostis avenacea C.C. Gmelin* and *Polypogon monspeliensis (L.) Desf.* (Riley and Gooden, 1991; McKay *et al.*, 1993). Corynetoxin producing *R. toxicus* strains are described as phage carriers in a psuedolysogenic state as they contain multiple copies of bacteriophage DNA not integrated into the bacterial chromosome and express bacteriophage structural proteins (Ophel *et al.*, 1993). Toxin producing cells are not stable in culture, which suggests there are other factors that may have a role in stabilising the phage carrier state of the bacteria within the gall (Ophel *et al.*, 1993).

Although the association has been observed, the exact role of the bacteriophage on corynetoxin production by *R. toxicus* is not known. Possible contributions made by the bacteriophage include genetic input to switch on bacterial toxin production, encode the final step of the toxin biosynthetic pathway or to promote export of the toxin by making the bacterial capsule and membrane more permeable to the toxin (Ophel *et al.*, 1993).

1.2.1.3 Life Cycle of the Plant Pathogenic Nematode *Anguina funesta*

Anguina funesta survives dry summer conditions inside a seed gall on the soil surface as an anhydrobiotic second stage juvenile (Figure 1.2). The galls decay during winter, releasing the nematodes which allows them to be distributed in surface water (Price *et al.*, 1979). This event coincides with the tillering of the host plant. Nematodes invade the host plant and congregate near the near the apical meristem until initiation of ovary primordia (McKay, 1981). The nematodes stimulate the ovary primordia to develop into galls, usually

Figure 1.2: Life cycle of the seed-gall nematode *Anguina funesta*. Used with permission from Riley (2001).


LIFE CYCLE OF A SEED-GALL NEMATODE, ANGUINA SP.
HOST
Vegetative
Reproductive
NEMATODE
On soil
In plant
In gall
J2
J3
J4
Adult
J1
J2
Moult
Moult
Moult
Moult in egg
Rehydration
Emergence
Invasion
Gall initiation
Egg deposition
Hatch
Anhydrobiosis

also suppressing the development of stamen primordia (Price *et al.,* 1979). Most galls are initiated by two nematodes, but can be initiated by up to 9 nematodes (McKay, 1986). The invading nematodes moult three times to become adults that mate within the developing floret (Price *et al.*,1979). The resultant developing nematodes moult once in the egg, hatch as second stage juveniles and develop into a survival stage after several days, coinciding with anthesis (McKay *et al.*, 1981). Galls usually average between 1000 to 2000 juveniles, but may contain up to 3560 juveniles (Riley and McKay, 1991a). As the plant senesces the survival nematodes become anhydrobiotic (Bird and Stynes, 1981) and are dispersed by wind or running water (Price , 1973) or transported in infested seed.

1.2.1.4 Bacteria-Nematode Interaction

Observed bacterial infection of *L. rigidum* is only achieved when associated with nematode infection (Riley and McKay, 1991a). The interaction between the bacterium and nematode is strong, involving the fusion of the nematode glycocalyx with the bacterial capsule, displacement or breakdown of the epicuticular membrane in the nematode and the thickening of the remaining epicuticular membrane (Bird and Stynes, 1977; Bird, 1985). Bacterial adhesion is specific to the infective second stage nematode juveniles present in surface water during winter (Bird and Riddle, 1984). The ability of bacteria to adhere to *A. funesta* varies between nematode populations (Riley and McKay, 1991b). There are some populations of *A. funesta* to which bacteria are unable to adhere. The nature of the recognition or bacterial site on the surface of the nematode is not known.

1.2.1.5 The Annual Ryegrass *Lolium rigidum*

Lolium is a small genus of eight species found in meadows, pastures and weed infested habitats (Terril, 1968; Clayton and Renvoize, 1986). *Lolium rigidum* is an annual ryegrass suited to the short and variable growing season of the cereal and sheep belt of southern Australia. All members of the *Lolium* genus contain 2n=14 chromosomes, but may be classified as either inbreeding or outbreeding based on their ability to self pollinate (Terrell, 1968; Clayton and Renvoize, 1986). *L. rigidum, L. perenne, L. multiform* and *L. canariense* are all outbreeding, wind-pollinated species of ryegrass. The inability of *L. perenne* to self-pollinate is genetically controlled by two self incompatibility (SI) loci (Fearon *et al.,* 1994). Fertilisation is unable to occur between gametes sharing SI alleles. Such a mechanism prevents fertilisation occurring between highly related plants.

L. rigidum contains 4.33 picograms per 2C nuclear DNA which is the equivalent to approximately 4.2 x 10^9 base pairs (Hutchinson *et al.,* 1979; Rees *et al.*, 1982). To date few markers have been mapped in *L. rigidum*. However, the mapping of the *L. perenne* genome has been the focus of a genome project conducted by Dr. M Hayward and co-workers at the Institute of Grasslands and Environmental Research in Aberystwyth, Wales with the aim of being used in marker assisted selection programs for the improvement of forage grasses. The highest density molecular map has covered 930 cM, based on 463 AFLP markers, 3 isozyme and 5 expressed sequence tagged (EST) markers (Bert *et al.,* 1999). Although the average density of these markers was one per 2 cM, the distribution of markers was not uniform due to the repetitive nature of DNA in the heterochromatin of pericentric regions of the chromosome.

other lolium maps have also been published.

1.2.1.6 The Affected Grazing Animal

Early signs of toxicity, such as the loss of the co-ordination of hind legs, can develop within four days of initial ingestion of infected seed heads. The progression of the disease leads to tetanic convulsions with arched heads, extended limbs and muscle spasms involving the face, lips and shoulders and ultimately death (McIntosh *et al.,* 1967). The lethal number of ingested bacterial galls is 20 000, which is the equivalent to 3-5 mg of corynetoxin/kg of animal (Jago and Culvenor, 1987). Lethal doses can be the result of a short duration, high level consumption or long duration, low level consumption of bacterial galls (Jago and Culvenar, 1987). Less affected animals appear to recover and seem normal. The mortality rate for livestock feeding in infested pasture can exceed 90% if they are not removed soon after the first sign of toxicity (McKay and Ophel, 1993). However, symptoms of toxicity may persist for up to 10 days after their removal from an infested paddock (McIntosh *et al.*, 1967; Berry and Wise, 1975).

1.2.2 Approaches towards Disease Control

Multiple approaches have been used in an attempt to control ARGT. Each approach varies in its ease of implementation, cost and practicality and targets different stages of the disease cycle.

1.2.2.1 Monitoring Outbreaks

An ELISA based testing service has been developed that detects the levels of the bacteria in emerging seed heads and assesses the corresponding risk of toxicity to livestock

(McKay and Riley, 1993). Early detection enables stockowners to transfer livestock to an uninfested pasture before any signs of poisoning are evident.

1.2.2.2 Controlling *L. rigidum* Levels

As population densities of *A. funesta* are highly dependent on the density of *L. rigidum*, a reduction in *L. rigidum* density would also result in a decrease in *A. funesta* populations. *L. rigidum* levels have been controlled in pastures by using the herbicide paraquat to dessicate seed heads (Price, 1973; Stynes and Wise, 1980). Such an approach enabled large areas to be treated quickly and cheaply and reduced populations of *A. funesta* (McKay and Ophel, 1993). Other herbicides have been effectively used in the past to prevent ARGT outbreaks. However, *L. rigidum* has the capacity to rapidly become cross resistant to different herbicide groups (Powels and Matthews, 1993). The outcome of such an approach is the selection of *L. rigidum* populations highly herbicide resistant, rendering this control method ineffective in the long term (McKay and Ophel, 1993). Alternatively, the density of *L. rigidum* seed heads has been reduced by mowing or increasing grazing before toxin levels are significantly high to cause ARGT (Price, 1973; McKay *et al.,* 1981; McKay *et al.,* 1982; Synes and Bird, 1983).

1.2.2.3 Potential Biocontrol Agents

The plant parasitic twist fungus *Dilophospora alopecuri* has been found to occur naturally in WA and may be associated with a decline in ARGT in this region (Pink, 1989; McKay and Ophel, 1993). The spores of *D. alopecuri* are carried into *L. rigidum* by *A. funesta* and can colonise up to 80% of galls initiated by *A. funesta* (McKay *et al.,* 1981; Bird and

McKay, 1987). In trials, the fungal colonisation of galls results in a reduction in the *A. funesta* population, which decreases the number of bacterial galls the following season. Spores of *D. alopecuri*, as a biocontrol agent for ARGT, were first made available to farmers in 1998 (Yan and Riley, 1998). In a number of reported case studies, the application of the twist fungus onto *A. funesta* infested paddocks has resulted in significant reductions in the levels of bacterial galls and the risk of ARGT outbreak in subsequent grazing seasons. (Yan and Riley, 1999).

Another potential biocontrol approach is the use of a closely related, non-toxic bacteria that could displace *R. toxicus* from its association with *A. funesta*, thereby reducing or limiting corynetoxin production. Native strains of the non-toxigenic bacteria *Clavibacter tritici* have been examined but failed to adhere to *A. funesta* (Riley and Reardon, 1995).

1.2.2.4 Development of a Commercial Cultivar of *L. rigidum* Resistant to *A. funesta*

McKay (1994) has developed a commercial cultivar of *L. rigidum* 99% resistant to *A. funesta*. The cultivar, Guard, was the product of several rounds of selective crossing between resistant lines of *L. rigidum* and consists of three lines highly resistant to *A. funesta* based on testing involving the gall formation assay. A limitation of this cultivar is that it is unsuitable for regions with short growing seasons as it flowers relatively late and has a short growth habit during winter. A more recent commercial cultivar, Safeguard, is an early flowering *A.funesta* resisitant ryegrass, and was developed by Valley Seeds, the South Australian Research and Development Institute (SARDI), and the Meat Corporation (Allen and Bywater, 2002).

1.2.3 *L. rigidum* Selective Breeding Programs

A *L. rigidum* breeding program aimed at isolating lines highly resistant to *A. funesta* was initiated in 1985 (McKay, 1993). *L. rigidum* seed was collected from natural populations, plants screened for *A. funesta* reproduction and classified as either resistant or susceptible to *A. funesta*. Plants classified as resistant to *A. funesta* were intercrossed, as were plants classified as susceptible to *A. funesta*. The most resistant and susceptible progeny lines were retained for further intercrosses. This selection process was repeated each season until 1991 (McKay, 1994). Further intercrossing of the six most resistant lines selected in 1988 was carried out to isolate progeny that were both true breeding for resistance to *A. funesta* and had desirable field characteristics such as vigorous growth (McKay, 1994). Seeds from three selected lines were combined to form the commercial *A. funesta* resistant cultivar Guard.

1.2.4 Genetics of Resistance *A. funesta* in *L. rigidum*

At the end of 1989, R14.9 was selected as the line with the lowest level of susceptibility to *A. funesta* galling and hence was considered the most resistant line. S1150 was selected as the line with the highest level of *A. funesta* gall formation and was considered the most susceptible line (McKay, 1993). The progeny from a cross between R14.9 and S1150 were tested for resistance to *A. funesta*. The ratio of resistant:susceptible (R:S) progeny was about 1:3 (A. McKay, 1994). R14.9 was considered to be heterozygous at the locus/loci responsible for resistance to *A. funesta* as it did not breed true for resistance. In plant lines in which resistance is controlled by a single gene, equal numbers of resistant and susceptible progeny would be expected from a cross between a heterozygous, resistant carrier (*Aa*) and a susceptible plant (*aa*) homozygous for the recessive susceptible allele (Figure 1.3a). To

Parents	*Aa*	x	*aa*
Progeny	*Aa*		*aa*
Frequency	1	:	1
Resistance	R		S
R:S	1:1		

(a) Resistance controlled by a single gene

Parents	*AaBb*	x	*aabb*	
Progeny	*AaBb*	*Aabb*	*aaBb*	*aabb*
Frequency	1 :	1 :	1 :	1
Resistance	R	S	S	S
R:S	1:3			

(b) Resistance controlled by two, complementary genes

Figure 1.3: A Comparison of expected frequencies of resistant and susceptible progeny and their corresponding genotypes in genetic systems controlled by either one or two genes.
(a) A representative cross between a heterozygous resistant plant and a homozygous susceptible plant in which resistance is conferred by a single gene.
(b) A representative cross between a double heterozygous resistant plant and a double homozygous susceptible plant in a system in which resistance is conferred by two genes with complementary action.

account for the observed ratio of about 1:3 of R:S in the progeny, McKay (1994) proposed, without statistical testing, two dominant complementary genes were controlling resistance to *A. funesta* in *L. rigidum*. In this model R14.9 is double heterozygous (*AaBb*) for both *A. funesta* resistance genes and S1150 is double homozygous recessive (*aabb*) at the *A. funesta* resistance loci (Figure 1.3b). The model proposes that plants phenotypically resistant to *A. funesta* may be one of four genotypes; *AABB, AaBB, AaBb* or *AABb* and plants phenotypically susceptible may be one of five genotypes; *Aabb, AAbb, aaBB, aaBb* or *aabb* (Table 1.1).

Of the 96 progeny tested by the gall formation assay from a cross between S1150 and R3339, 23 were classified as resistant and 73 were classified as susceptible to *A. funesta* (A. McKay, pers. comm.). A number of the tested resistant progeny were not as resistant to *A. funesta* as R14.9 or R3339 as measured by the gall formation assay. These progeny were classified as moderately resistant as up to 10 of the 20 tested seed heads contained galls and were proposed to be a distinct class from fully resistant lines of *L. rigidum*.

1.2.5 Construction of Near Isogenic Lines (NILs) of *L. rigidum*

Near isogenic lines (NILs) share almost identical genetic backgrounds, but differ in one selected phenotype of interest (Martin *et al.,* 1991). NILs facilitate the identification and cloning of genes involved in the selected phenotype and can assist in both the structural and functional analysis of these genes. To produce NILs of *L. rigidum* resistant and susceptible to *A. funesta* McKay (1994) initially crossed R14.9 with S1150 (Figure 1.4). Selected progeny resistant to *A. funesta* were backcrossed with S1150. Recurrent selection and backcrossing occurred over four generations to produce the line R799, resistant to *A. funesta* with a genetic background theoretically 93.8% identical to S1150.

Table 1.1: A comparison of potential genotypes of resistant and susceptible lines in one and two gene models for inheritance of resistance

Resistance controlled by a single gene	
Genotype	**Phenotype**
AA	Resistant
Aa	Resistant
aa	Susceptible
Resistance controlled by two, complementary genes	
Genotype	**Phenotype**
AABB	Resistant
AABb	Resistant
AaBB	Resistant
AaBb	Resistant
AAbb	Susceptible
aaBB	Susceptible
Aabb	Susceptible
aaBb	Susceptible
aabb	Susceptible

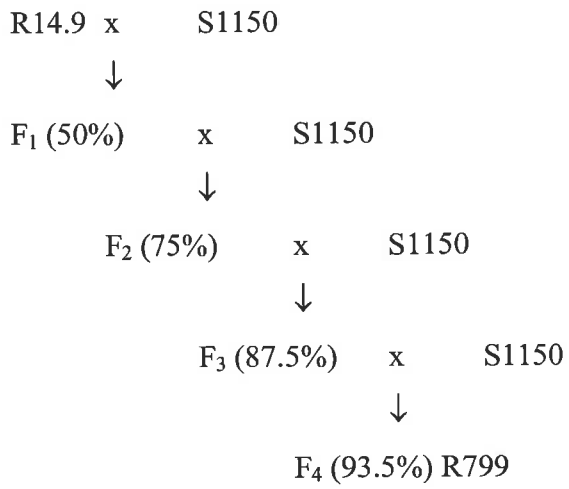


Figure 1.4: Breeding program to isolate NILs of *L. rigidum* resistant and susceptible to *A. funesta*

The level of relatedness of each generation with S1150 is indicated in parentheses.

1.2.6 Preliminary Analysis of RAPDs with Potential Association to Resistance to *A. funesta* in *L. rigidum*.

In an attempt to isolate molecular markers linked to resistance to *A. funesta* in *L. rigidum*, a range of primers were used in RAPD PCRs with DNA isolated from a limited set of resistant or susceptible *L. rigidum* plants (McKay, 1994). RAPD bands were observed in reactions with the primers OPD-15 and OPW-8 with template DNA from lines of *L. rigidum* resistant to *A. funesta* but not with DNA from lines of *L. rigidum* susceptible to *A. funesta*. Although the resistance status of the limited number of lines used in this study was recorded, the pedigrees of these lines were not. Further characterisation of the RAPD band profiles produced in reactions with the primers OPD-15 and OPW-8 and DNA from a more extended range of resistant and susceptible lines of *L. rigidum* would need to be conducted to determine if the observed RAPD bands are always associated with resistance to *A. funesta*.

1.3 OVERALL AIMS, RATIONAL AND APPROACHES

1.3.1 Chapter 3

Aim: To determine the probable genotypes of parental lines of *L. rigidum* in relation to *A. funesta* resistance through the analysis of the numbers of progeny resistant and susceptible to *A. funesta*.

Rational: Under the complementary two gene model of resistance to *A. funesta* in *L. rigidum*, a plant of a given resistance phenotype can only be one of a limited number of resistance genotypes. Therefore, a limited number of alternative genetic descriptions or models may represent any given phenotypic description of a cross. As the phenotypes of both parents in a cross may be assessed through the gall formation assay, the range of genetic models representing any given cross are known. Statistical testing of the observed numbers of progeny resistant or susceptible to *A. funesta* relative to the expected numbers predicted from each potential genetic model should provide support or reject each model to allow the attribution of genotypes to the parental lines.

Approaches:

- Re-analysis of crosses carried out by McKay, to assess if the observed numbers of progeny resistant and susceptible to *A. funesta* statistically support the deduced genotypes.
- Investigation of the use of small scale progeny testing of factorial crosses as an alternative approach to large scale progeny testing of a single cross to determine genotypes in relation to resistance.

- Analysis of the outcomes of crosses involving *L. rigidum* lines moderately resistant to *A. funesta.*

1.3.2 Chapter 4

Aim: To identify RAPD(s) markers associated with *A. funesta* resistance in *L. rigidum.*

Rational: Screening of new *L. rigidum* cultivars would be faster and easier with the use of a molecular marker(s) associated with resistance to *A. funesta* rather than the gall formation assay. Combining the RAPD approach with NILs of *L. rigidum* differing in resistance to *A. funesta* increases the opportunity of isolating RAPDs associated with resistance to *A. funesta.*

Approach: Initially, random sequence primers will be used in RAPD PCR using genomic DNA from R799 and S1150 as template and the RAPD profiles comparised. Any reactions that produce RAPDs using DNA from R799 but not S1150 will be repeated with a wider range of *L. rigidum* lines resistant or susceptible to *A. funesta* to examine if the association of the RAPD with resistance is maintained.

1.3.3 Chapter 5

Aim: To amplify and analyse RGA scquences from *L. rigidum.*

Rational: RGA sequences have been found in a wide range of plants and are often found to occur in clusters with R-genes. The isolation of RGA sequences is a more targeted approach than RAPDs towards cloning regions of the genome containing or linked to resistance genes.

Approach: Degenerate PCR primers targeted towards conserved regions of the NBS will be used to amplify NBS containing sequences within the *L. rigidum* genome. Characterised, amplified RGAs will be analysed for an association with resistance to *A. funesta* in a number of lines of *L. rigidum*.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals

Chemicals used for *in vitro* studies were of analytical grade and are listed in alphabetical order of supplier.

- lithium chloride (LiCl), xylene cyanol: Ajax Chemicals, Auburn, NSW, Australia.
- 2-mercaptoethanol, boric acid, bromophenol blue, chloroform, ethanol, ethylenediaminetetra acetic acid disodium salt (EDTA), glacial acetic acid, glucose, hydrochloric acid (HCl), iso-amyl alcohol, magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), phenol, potassium acetate (KOAc), potassium chloride (KCl), propan-2-ol, sodium acetate (NaOAc), sodium chloride (NaCl), sodium citrate, sodium dodecyl sulphate (SDS), sodium hydroxide (NaOH): BDH, Pool, UK.
- Agarose LE and bovine serum albumun: Boehringer Mannheim.
- Bacto-agar, bacto-trytone and yeast extract: Difco Laboratories, Detroit, MI, USA.
- Guanidine hydrochloride: International Biotechnologies Inc.
- Glycerol: Merck, Whitehouse Station, NJ, USA.
- Ficol 400: Pharmacia.
- Deoxyribonucleotide triphosphates (dATP, dTTP, dGTP and dCTP): Promega.
- 2[N-morpholino]ethanesulfonic acid (MES), 3-[N-morpholino]propanesulfonic acid (NaMOPS), Ampicillin, calcium chloride ($CaCl_2$), diethyl pyrocarbonate (DEPC), dithiothreitol (DTT), ethidium bromide, hexadecyltrimethyl ammonium bromide (CTAB), manganese chloride ($MnCl_2$), polyethylene glycol 8,000 (PEG-8000),

polyvinylpyrrolidine-360 (PVP), rubidium chloride (RbCl), sodium phosphate, Tris (hydroxymethyl) amino-methane (trizma base), Triton X-100: Sigma.

2.1.2 Radiochemicals

[α-^{32}P]dCTP (3,000 Ci/mmol): Geneworks, Adelaide, Australia

2.1.3 Enzymes

- RNasin, *Taq* DNA polymerase, T_4 DNA ligase: Promega.
- Pancreatic RNase A: Sigma Chemical Company, St. Louis, MO, USA.
- Restriction enzymes: Boehringer Mannheim, Mannheim, FRG.

2.1.4 Kits

- Jetquick Plasmid miniprep Spin Kit, Jetquick Gel Extraction Spin Kit: Genomed Inc., N.C. USA.
- Megaprime™ DNA labelling system: Amersham, UK.
- 5' RACE system for Rapid Amplification of cDNA ends: Gibco-BRL (Life Technologies), Gaithersburg, MD, USA.

2.1.5 Plasmids

- pBLUESCRIPT II SK +, Stratagene Cloning Systems, La Jolla, CA, USA.
- pGEM-T, Promega

2.1.6 Molecular Weight Standards

▪ pGEM DNA molecular weight marker, a combination of equimolar amounts of pGEM3 DNA digested separately with *Hin*fI, *Rsa*I and *Sin*I, (Promega) was used at a final concentration of 500 ng/μl in a 1x Type II loading solution. Approximate fragment sizes in bp: 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 36.

▪ λ DNA digested with *Hind*III: (Geneworks, Adelaide, Australia) Fragment sizes in bp: 23130, 9416, 6557, 4361, 2322, 2027, 564, 125

▪ 200 bp ladder: (Geneworks, Adelaide, Australia). Fragment sizes in 200 bp increments ranging from 200 bp to 6 Kb with brighter reference bands at 2 and 5 Kb.

2.1.7 Oligodeoxyribonucleotides

Oligonucleotides directed towards the P-loop, kinase-2 and hydrophobic membrane spanning (GLPL) domains of RGA sequences used in reactions to amplify RGA sequences from *L. rigidum* were based on oligonucleotides used to amplify RGA seqeunces from *Z. mays* (Collins *et al.,* 1998). Oligonucleotides 1f, 2f, 3f and 4f were designed to enable amplification of *L. rigidum* class specific probes and the oligonucleotide 1R designed for use in the production of a *L. rigidum* RGA 5' RACE product (Table 2.1). All oligonucleotides used in Chapter 5 were synthesized by Dr Neil Shirley of the Department of Plant Science, The University of Adelaide, using an Applied Biosystems Model 380B synthersizer (Perkin Elmer, Norwalk, CT, USA) and purified by ion exchange HPLC using a MonoQ column (Pharmacia, Uppsula, Sweden).

Table 2.1: Oligonucleotides directed towards RGA sequences

P-loop (GVGKTT)		Primer Sequence
PLOOPAA	(P1)	5'-AAG AAT TCG GNG TNG GNA AAA CAA C-3'
PLOOPAT	(P2)	5'-AAG AAT TCG GNG TNG GNA AAA CTA C-3'
PLOOPAC	(P3)	5'-AAG AAT TCG GNG TNG GNA AAA CCA C-3'
PLOOPAG	(P4)	5'-AAG AAT TCG GNG TNG GNA AAA CGA C-3'
PLOOPGA	(P5)	5'-AAG AAT TCG GNG TNG GNA AGA CAA C-3'
PLOOPGT	(P6)	5'-AAG AAT TCG GNG TNG GNA AGA CTA C-3'
PLOOPGC	(P7)	5'-AAG AAT TCG GNG TNG GNA AGA CCA C-3'
PLOOPGG	(P8)	5'-AAG AAT TCG GNG TNG GNA AGA CGA C-3'
Kinase2 (L V/L/I VLDDV)		
kinase2d	(K2d)	5'-CTA CTG NTN CTN GAC GAC GT-3'
kinase2e	(K2e)	5'-CTA CTG NTN CTN GAC GAT GT-3'
kinase2f	(K2f)	5'-CTA CTG NTN CTN GAT GAC GT-3'
kinase2g	(K2g)	5'-CTA CTG NTN CTN GAT GAT GT-3'
GLPL (GLPLAL)		
GLPL1		5'-AAC TCG AGA GNG CNA GNG GNA GGC C-3'
GLPL2		5'-AAC TCG AGA GNG CNA GNG GNA GAC C-3'
GLPL3		5'-AAC TCG AGA GNG CNA GNG GNA GTC C-3'
GLPL4		5'-AAC TCG AGA GNG CNA GNG GNA GCC C-3'
GLPL5		5'-AAC TCG AGA ANG CCA ANG GCA AAC C-3'
GLPL6		5'-AAC TCG AGA ANG CCA ANG GCA ATC C-3'
RGA class specific primers		
1f		5'-G(C/A)T GCT TTG GAA GAG TAT GAA-3'
1r (5' RACE)		5'-GTA TC(A/G) TG(T/C) CGA GTG GTA ACT A-3'
2f		5'-GTT GG(A/G) A(A/G)C TAT TT(T/C) GTA GCA A-3'
3f		5'-GTC AAA AGG CAT TCC GTA GAT T-3'
4f		5'-GCA TTT GGT GAA GAG CAT CC-3'

Random 10-mer oligonucleotides used for RAPD analysis were supplied by Operon Technologies Inc., Alameda, CA, USA. These included the 20 oligonucleotide series OPAM, OPE and OPJ and the oligonucleotides OPD-15 and OPW-08.

2.1.8 Strains of *E. coli*

- *Escherichia Coli* (*E. coli*) JM109 *rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ*(lac-proAB)*[F' *tra*D36 *pro*AB *lac*I^{q}ZΔM15]: Promega Corporation, Madison, WI, USA.
- Novablue Singles: Novagen, Madison, WI, USA

2.1.9 Plant Lines

All plant lines used in this study were obtained from either Dr Alan McKay's ARGT resistance breeding program (SARDI) or Valley Seeds, Australia as indicated in Table 2.2.

Table 2.2: *L. rigidum* Plant Lines and Cultivars

Plant Line/Cultivar	Susceptibility to *A. funesta*	Pedigree	
799	R	Recurrent backcross to 1150	c, d
Guard	R	(6 X 167 X 127)	a, d
Guard II	R	(6 X 167 X 127)	a, d
Guard C2	R	(6 X 167 X 127)	b, d
Guard C3	R	(167 X 30)	b, d
Guard WA	R	(Guard X 10)	a, d
WA Early Flowering	R	-	a, d
WAR	R	-	e
C19.1	R	(*L. rigidum*X *L.multiform* Westerwold (Progrow)),	b, d
C17	R	(*L. rigidum*X *L.multiform* Westerwold (Progrow) 23061.4	b, d

		X 23523.1 II generation)	
C18	R	(*L. rigidum*X *L.multiform* Westerwold (Progrow) 23062.4 X 23522.5 II generation),	b, d
793-A	R	S1150 backcross	e
799D	R	S1150 backcross	e
3094	R	Wimmera x Progrow	e
3297	R	Wimmera x Progrow	e
3100	R	Wimmera x Progrow	e
3339	R	-	e
1150	S	Wimmera susceptible control	c, d
Wimmera	S	-	c, d
Turretfield	S	Wimmera local population	c, d
WA Ecotype (WA656)	S	-	c, d
WA-S	S	-	e
Springfield	S	Local Waite poulation	e
Waite	S	Local Waite poulation	e
792E	S	S1150 backcross	e
793B	S	S1150 backcross	e
Italian R/S	Heterozygous resistance	-	e
796-D	S	S1150 backcross	e
10.19	Untested	(1150 X 799)	d, f
10.20	Untested	(1150 X 799)	d, f
R15789.5	MR	(R3330 X 1150)	c, d
R1386.4	MR	(R3330 X 1150)	c, d
S15796.2	S	(R3330 X 1150)	c, d
R15794.1	R	(R3330 X 1150)	c, d
S15782.3	S	(R3330 X 1150)	c, d

a, Plant grown of this line from seed supplied by A. McKay or **b,** Valley Seeds; **c,** plant material obtained from A. McKay; **d,** DNA isolated from plant material in this study; **e,** DNA obtained from A. McKay of this plant line; **f**, seed obtained from set up crosses in this study. Lines of unrecorded pedigree are represented with a "-".

2.1.10 Standard Solutions

Solutions were prepared with microfiltered (milliQ) water and either filter sterilised under sterile conditions in a laminar flow, or autoclaved at 121°C for 20 min.

10x DNase buffer	100 mM Tris pH 8, 100 mM $MgCl_2$ (DEPC), 10 mM DTT. Stored at -20°C.
100x Denhardt's solution	2% (w/v) Ficol 400, 2% (w/v) bovine serum albumen, 2% (w/v) PVP. Stored at -20°C.
20x SSC	3 M NaCl, 0.3 M sodium citrate pH 7.4.
20x SSPE	3.6 M NaCl, 0.2 M Sodium phosphate, 0.02 M EDTA, pH 7.7
10x T4 DNA Ligase Buffer	300 mM Tris-HCl (pH 7.8), 100mM $MgCl_2$, 100 mM DTT, 10mM dATP
10x *Taq* buffer	50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100
10x Type II loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficol 400
CTAB Buffer	2%(w/v) CTAB, 1% (w/v) PVP, 1.4 M NaCl, 0.2% (v/v) 2-mecaptoethanol, 0.2M EDTA, 0.1M Tris-Cl, pH 8.0
Denaturation solution	1.5 M NaCl, 0.5 M NaOH
Neutralisation buffer	1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA
Hybridisation solution	5x SSPE, 5x Denhardt's solution, 0.5% (w/v) SDS
RNA Lysis Buffer	8M Guanidine hydrochloride, 20mM MES, 20 mM EDTA
Solution I	50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA pH 8.0,

	autoclaved
Solution II	0.2 M NaOH, 1% SDS
Solution III	3.0 M KOAc, 11.5% glacial acetic acid
Stripping solution	0.1x SSC, 0.1% (w/v) SDS, 0.2 M Tris-HCl pH 7.5
1x TAE buffer	4 mM Tris, 2 mM glacial acetic acid, 0.09 mM EDTA
0.5x TBE buffer	50 mM Tris, 0.5 mM EDTA, 43 mM boric acid, pH 8.3,
TE	10 mM Tris-HCl pH 7.5, 0.1 mM EDTA

2.1.11 Bacterial Media

Luria broth (LB)	1.0% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.0% (w/v) bacto-tryptone. pH 7.0, autoclaved
LB-agar	LB, 1.5% (w/v) agar, autoclaved
SOC	2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 20 mM glucose, 0.25 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. Filter sterilised
TFB I	30 mM KOAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% (v/v) glycerol, adjusted to pH 5.8 with 0.2 M glacial acetic acid. Filter sterilised
TFB II	10 mM NaMOPS pH 7.0, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol. Filter sterilised

2.1.12 University of California (UC) Soil Mix

Soil mix used was prepared as follows by the plant growth facility at the Waite Campus of the University of Adelaide. The mix consisted of 400 litres sterilised, coarse, washed sand heated to 100°C for 30 min to which 300 litres (dry volume) Eurotorf peatmoss was added and mixed for 10 s. After 10 min of cooling time, the following fertilisers were added and mixed with the sand/peat mix for 20 sec; 700 g Calcium hydroxide, 480 g Calcium carbonate, 600 g Nitrophoska. Nitrophoska contained 15% total nitrogen, 3.9% total phosphorus, 12.4% Potassium sulphate, 1.25% Magnesium carbonate, 3.4 % dicalcium phosphate, 5.3% sulphates, 0.3% Iron oxide, 0.0002% Copper oxides, 0.007% Zinc oxide, 0.01% Calcium borate and 0.0003% Molybdenum oxide. The pH of the UC soil mix with fertilisers was 6.8.

2.2 METHODS

2.2.1 Plant Methods

2.2.1.1 Germination of Seed

Seeds were removed from long-term desiccation at 4°C, placed onto one filter paper per seed-set and subjected to ten days in a misting chamber to remove germination inhibitors from the seed coat. Ryegrass seeds were germinated on moist filter paper, incubated in the dark at 25°C and selected for planting out when the roots were sufficiently developed (approximately two weeks, post-germination).

2.2.1.2 Plant Growth Conditions

The University of California (UC) soil mix used in pot experiments was prepared by the plant growth facility at the Waite Campus of the University of Adelaide. Plants used in crossing experiments or for use in the preparation of DNA or RNA were grown in 130 or 250 mm pots in a glasshouse, at day and night temperatures of 25°C and 18°C, respectively. Plants used in the gall formation assay were grown outdoors in 130 mm pots and were watered each morning with an automatic fine-mist sprinkler system in order to maintain plant surface moisture.

2.2.1.3 Plant Crossing

Ryegrass crosses were carried out according to the method of McKay and co-workers (SARDI, Adelaide, SA, *pers. comm.*). Individual ryegrass plants were grown in 130 mm pots until the seed heads begin to emerge from the boots. The parents of a cross were placed together in a 250 mm pot and the surrounding space was filled with UC mix. Single crossing bags were made from 2 greaseproof paper sheets (300 mm x 500 mm) with top and sides sealed together by double sided tape. A viewing window was made by cutting a rectangular hole on one side, covered with a plastic sheet that was sealed to the bag with double-sided tape. The bag was slipped over the rim of the 250 mm pot containing the parents, and tightly tied with string under the rim of the pot. The parents in the sealed crossing bags were placed into 100 mm deep trays, that were filled with water each week. Each crossing bag was gently tapped for several minutes, once a day to facilitate pollen movement between the parents of the cross. Plants were left in the bags for 10 weeks, until the seedheads had finished flowering and started to dry out. The dry seedheads from both parents of each cross were cut off, placed

in labeled paper bags, and dried in a 37°C incubator for 1 week. The seeds from each cross were harvested by hand and stored in a dessicator with dessicant at 4°C.

2.2.1.4 Gall Formation Assay

The nematode resistant phenotype of ryegrass was determined by the gall formation assay (McKay, 1986). The seedling was planted into the soil and five nematode galls were embedded in the soil around the base of the seedling shoot. The first four seed heads to emerge from the boot of each plant were collected. Ten florets on each head were scored for gall formation by examination of the two spikelets closest to the stem. Ryegrass plants were classified as resistant if they produced less than four seed galls per seed head when infected with a standard inoculum of five galls. Susceptible plants were those that produce greater than four galls.

2.2.1.5 Preparation of Ryegrass Genomic DNA

Leaves from young ryegrass plants grown under glasshouse conditions were harvested, placed immediately into liquid nitrogen and stored at -80°C. For PCR applications, genomic DNA was extracted by grinding approximately 100 mg of leaves under liquid nitrogen with a mortar and pestle and using the method of Raeder and Broda (1985). For restriction digestion and Southern analysis, larger scale extractions using 1 g of leaves were required. The ground leaf tissue was added to 7.5 ml of preheated CTAB buffer in a 30 ml Corex tube at 60°C in a water bath and incubated for 30 min with occasional swirling of the tube. To this extract 5 ml of chloroform was added and mixed gently, followed by centrifugation at 1,600 *g* for 15 min.

The aqueous phase was removed, transferred to a clean tube and mixed with 5 ml of cold isopropanol and incubated for 1 h at 4°C to precipitate the nucleic acids. The precipitated DNA was wound onto a sterile glass pipette loop and washed in cold 70% ethanol. The DNA was pelleted by centrifugation at 1,600 g for 10 min and the ethanol was drained off. The pellet was dissolved in 0.7 ml of TE and transferred to an Eppendorf tube to which 250 µl of phenol and 250 µl of chloroform were added, mixed and centrifuged at 15,400 g for 2 min to separate the phases. The aqueous phase was removed and transferred to a clean Eppendorf tube and one tenth the volume of 2 M NaOAc (pH 5) was added followed by 2 volumes of 100% ethanol and mixed. The genomic DNA was pelleted by centrifugation at 15,400 g for 20 min. DNA pellets were washed with 1 ml ice-cold 70% ethanol, dried and resuspended in 0.5 ml of TE.

2.2.1.6 Preparation of Ryegrass Total RNA

All solutions used in RNA preparation were treated by the addition of DEPC to a final concentration of 0.05%, incubated overnight and autoclaved at 121°C for 30 min to remove ribonuclease activity. All glassware used for RNA extractions was baked for 16 h at 300°C and certified RNase-free filtered pipette tips and Eppendorf tubes were used.

About 3 g of frozen ryegrass plant material was ground to a fine powder with a mortar and pestle, under liquid nitrogen. The powder was transferred to a 50 ml Falcon tube, to which 8 ml of RNA lysis buffer and 34 µl of 2-mercaptoethanol (final concentration of 50 mM) was added. The tube was vortexed, 8 ml of phenol/chloroform was added, followed by vortexing for 1 min. The extract was split into 8 x 2 ml tubes and centrifuged in a Eppendorf 5415C bench centrifuge at 15,400 *g* for 10 min in a pre-chilled rotor. The aqueous phase was

transferred to a 15 ml Corex tube and 450 µl (1/20 vol.) 1 M acetic acid and 6.3 ml 100% ethanol (0.7 vol.) was added. The Corex tube was stored at -20°C overnight to precipitate nucleic acids before centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet was washed two times with ice-cold 70% ethanol. The ethanol was drained off and the pellet was resuspended in 0.5 ml distilled H_2O (dH_2O) and 0.5 ml 4 M LiCl and stored at -20°C overnight to allow the precipitation of RNA. The RNA was pelleted by centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 100 µl of dH_2O to which 10 µl of 3 M NaOAc and 200 µl of ethanol were added. The solution was mixed and incubated at -20°C for 2 h, followed by centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant was discarded, the pellet was washed twice with 70% ethanol, dried briefly and resuspended in 200 µl dH_2O. The sample was spun briefly to pellet undissolved material and the supernatant was transferred to a fresh tube. DNA was digested for 30 min at 37°C by the addition of 50 U DNase I, in the presence of 1x DNase I Buffer and 50 U RNasin. An equal volume of phenol and chloroform was added, mixed and centrifuged at 15 400 g for 2 min to separate the phases. The aqueous phase was removed and transferred to an Eppendorf tube containing an equal volume of chloroform and mixed. The two phases were separated by centrifugation at 15 400 g for 2 min. The aqueous phase was removed and transferred to a clean Eppendorf tube and 1/20 the volume of 3M NaOAc (pH 5) and 2 volumes of 100% ethanol were added, mixed and precipitated overnight at -20°C. The RNA was pelleted by centrifugation at 15,400 g for 20 min. RNA pellets were washed once with 70% ethanol, air dried breifly and resuspended in 200 µl dH_2O.

2.2.1.7 Approach to Genotype Allocation of Parental Ryegrass Plant Lines

The chi-squared (χ^2) test was used to determine whether deviations of observed from expected numbers of resistant and susceptible progeny were statistically significant. Expected ratios were generated from proposed genetic models that may represent the phenotypic cross undertaken (Table 2.3). The underlying model in each case was the complementary two gene model for resistance to *A. funesta* infection (McKay, 1994) that dictated, based on the observed parental phenotypes, the range of genotypes of a particular plant line. Both the expected and observed numbers of the distinct classes of progeny were used to calculate the χ^2 value for any given cross. Each χ^2 value corresponds to a probability (p) value. The p value represents the probability of achieving as great or greater deviation of observed from expected due to chance alone if the genetic model is correct. A p value of below 0.05, resulted in the rejection of the model and conversely a p value of above 0.05 provided support for the model. By statistically testing the full range of genetic models to account for a particular phenotypic cross, information on the genotypes of the plants involved in the cross may be inferred.

2.2.1.8 Calculation of Genetic Distance from RAPD Profiles

Generated PCR products between 200 bp and 2.6 kb were scored for their presence (score of one) or absence (score of zero) in the RAPD profile of each plant line. The RAPDistance software package version 1.04 (Armstrong *et al.*, 1994) was used to record and analyse profile data. The software package contained several algorithms to calculate the genetic similarity of pairs of samples, each of which is characterised by the presence (1) or absence (0) of bands.

From the vectors of "1's" and "0's" a pairwise genetic distance matrix was calculated using the formula, $1 - F = [1 - (2n_{xy})/(n_x + n_y)]$, where $2n_{xy}$ equals the number of shared bands, and n_x and n_y represents the number of bands observed in individual x and individual y, respectively (Nei and Li, 1979). Resultant genetic similarities derived from the distances calculated with the formula of Nei and Li (1979) were expressed as F_p (genetic similarity between parental lines), F_s (genetic similarity between siblings) and F_{sp} (genetic similarity between sibling and parental lines).

An alternative formula, $1 - M = [1 - (n_{xy} + n00)/(n)]$ was also used, where n_{xy} represents the number of shared bands, n00 represents the number of shared null alleles and n is the total number of band positions (Apostol *et al.*, 1993). Genetic similarities calculated with the simple matching formula of Apostol *et al.*, (1993) were expressed as M_p (genetic similarity between parental lines), M_s (genetic similarity between siblings) and M_{sp} (genetic similarity between sibling and parental lines).

RAPDistance calculated pairwise distances between the DNA samples using the band data from a RAPDistance datafile, and provided the results as triangular matrices. The distance data (D) was converted into a percentage similarity (S) using the formula, S = (1 - D) x 100. The similarity matrix described the genetic similarity between DNA samples.

2.2.1.9 Minimum Distance Calculation

The expected minimum distance (MD) from the target locus to the closest RAPD marker was calculated using the formula of Martin *et al.*, (1991); $MD = c/2(nx + 1)$, where c = genome size in cM, n = number of primers, x = average number of products per RAPD primer.

Table 2.3: Expected segregation ratios amongst progeny from all possible parental genotype combinations in a complementary two gene system. Shaded boxes designate crosses producing all resistant progeny.

	AABB	*AABb*	*AaBB*	*AaBb*	*AAbb*	*aaBB*	*Aabb*	*aaBb*	*aabb*
AABB	*AABB*	*AABB* *AABb*	*AABB* *AaBb*	*AABB* *AABb* *AaBB* *AaBb*	*AABb*	*AaBB*	*AABb* *AaBb*	*AaBB* *AaBb*	*AaBb*
AABb	*AABB* *AABb*	3:1 *AABB* 2AABb *AAbb*	*AABB* *AaBB* *AABb* *AaBb*	3:1 *AABB* 2AABb *AaBB* 2AaBb *AAbb* *Aabb*	1:1 *AABb* *AAbb*	*AaBB* *AaBb*	1:1 *AABb* *AaBb* *AAbb* *Aabb*	3:1 *AaBB* 2AaBb *Aabb*	1:1 *AaBb* *Aabb*
AaBB	*AABB* *AaBb*	*AABB* *AABb* *AaBB* *AaBb*	3:1 *AABB* *AaBb* *AaBB* *aaBB*	3:1 *AABB* *AABb* 2AaBB 2AaBb *aaBB* *aaBb*	*AABb* *AaBb*	1:1 *AaBB* *aaBB*	3:1 *AABb* 2AaBb *aaBb*	1:1 *AaBB* *AaBb* *aaBB* *aaBb*	1:1 *AaBb* *aaBb*
AaBb	*AABB* *AABb* *AaBB* *AaBb*	3:1 *AABB* 2AABb *AaBB* 2AaBb *AAbb* *Aabb*	3:1 *AABB* *AABb* 2AaBB 2AaBb *aaBB* *aaBb*	9:7 *AABB* 2AABb 2AaBB 4AaBb *AAbb* *aaBB* 2Aabb 2aaBb *aabb*	1:1 *AABb* *AaBb* *AAbb* *Aabb*	1:1 *AaBB* *AaBb* *aaBB* *aaBb*	3:5 *AABb* 2AaBb 2Aabb *AAbb* *aaBb* *aabb*	3:5 *AaBB* 2AaBb *Aabb* *aaBB* 2aaBb *aabb*	1:3 *AaBb* *Aabb* *aaBb* *aabb*
AAbb	*AABb*	1:1 *AABb* *AAbb*	*AABb* *AaBb*	1:1 *AABb* *AaBb* *AAbb* *Aabb*	All S *AAbb*	*AaBb*	All S *AAbb* *Aabb*	1:1 *AaBb* *Aabb*	All S *Aabb*
aaBB	*AaBB*	*AaBB* *AaBb*	1:1 *AaBB* *aaBB*	1:1 *AaBB* *AaBb* *aaBB* *aaBb*	*AaBb*	All S *aaBB*	1:1 *AaBb* *aaBb*	All S *aaBB* *aaBb*	All S *aaBb*
Aabb	*AABb* *AaBb*	1:1 *AABb* *AaBb* *AAbb* *Aabb*	3:1 *AABb* 2AaBb *aaBb*	3:5 *AABb* 2AaBb 2Aabb *AAbb* *aaBb* *aabb*	All S *AAbb* *Aabb*	1:1 *AaBb* *aaBb*	All S *AAbb* 2Aabb *aabb*	1:3 *AaBb* *Aabb* *aaBb* *aabb*	All S *Aabb* *aabb*
aaBb	*AaBB* *AaBb*	3:1 *AaBB* 2AaBb *Aabb*	1:1 *AaBB* *AaBb* *aaBB* *aaBb*	3:5 *AaBB* 2AaBb *Aabb* *aaBB* 2aaBb *aabb*	1:1 *AaBb* *Aabb*	All S *aaBB* *aaBb*	1:3 *AaBb* *Aabb* *aaBb* *aabb*	All S 1aaBB 2aaBb 1aabb	All S *aaBb* *aabb*
aabb	*AaBb*	1:1 *AaBb* *Aabb*	1:1 *AaBb* *aaBb*	1:3 *AaBb* *Aabb* *aaBb* *aabb*	All S *Aabb*	All S *aaBb*	All S *Aabb* *aabb*	All S *aaBb* *aabb*	All S *aabb*

2.2.2 Bacterial Methods

2.2.2.1 Growth of Bacteria

Cultures of *E. coli* JM109 were grown overnight at 37°C, using solid LB or shaking in LB broth. Liquid cultures of JM109 were established by inoculating 10 ml of LB broth in a 25 ml bottle, with a single bacterial colony. Where appropriate, the antibiotic selective agent ampicillin was added to a concentration of 50 μg ml^{-1} or 100 μg ml^{-1}.

2.2.2.2 Preparation of Competent Cells

Competent cells were prepared by a modification of the methods of Kushner (1978) and Hanrahan (1983). An overnight culture of JM109 *E. coli* was prepared in 10 ml LB broth and incubated shaking at 37°C. One ml of the culture was used to inoculate 50 ml LB broth and incubated shaking at 37°C until the culture had reached an OD_{600} = 0.3. Five ml of this culture was placed into 100 ml of 37°C LB and grown to an OD_{600} = 0.4-0.5. The culture was placed on ice for 5 min and centrifuged at 4,620 *g* for 10 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 30 ml of TFB I. The cells were incubated for 2 min on ice, then centrifuged at 4,620 *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 4 ml of TFB II. The cell suspension was dispensed into 50 μl aliquots in 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80°C.

2.2.2.3 Transformation of *E. coli*

About 50-80 ng of DNA from ligation reactions was added to 50 µl aliquots of competent cells thawed on ice. The cells were incubated with the DNA for 5 min on ice, heat shocked in a 42°C water bath for 30 s and then placed immediately on ice for a further 2 min. The cells were mixed with 100 µl of SOC medium and grown at 37°C for 1 h on a shaker. The cell suspension was centrifuged in an Eppendorf 5415C bench centrifuge at 15,400 *g* for 30 s. The supernatant was discarded and the pellet was resuspended in 100 µl of LB, then transferred to LB-agar plates containing 50 µg ml^{-1}ampicillin and pre-spread with a solution containing 1 mg of X-Gal and 2.5 mg of IPTG, and incubated at 37°C overnight.

2.2.3 General DNA Based Methods

Standard molecular protocols were carried out according to Sambrook *et al.,* (1989) or using procedures specified by the manufacturer, except where indicated.

2.2.3.1 Mini-preparation of Plasmid DNA from Bacterial Cells

Plasmid DNA was isolated using a procedure based on that of Sambrook *et al.,* (1989), for the small-scale isolation of plasmid DNA by alkaline lysis. A 2 ml liquid culture of plasmid-containing bacteria was added to an Eppendorf tube and centrifuged at 15,400 *g* for 1 min. The supernatant was discarded, and the cells resuspended in 100 µl ice-cold Solution I by vortexing for 1 min. To lyse the cells, 200 µl of freshly made, ice cold Solution II was added and mixed by gentle inversion. To the lysate, 150 µl Solution III was added and the tube mixed gently before incubating on ice for 5 min. The tube was centrifuged for 10 min and 350 µl of the supernatant was transferred to a 1.5 ml Eppendorf tube. An equal volume of

phenol/chloroform mixture (1:1) was added to the supernatant, vortexed and centrifuged at 15,400 *g* for 2 min to separate the phases. The aqueous phase was removed and transferred to a clean Eppendorf tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added. The tube was vortexed for 1 min and centrifuged for 2 min. The aqueous phase was transferred to a new Eppendorf tube and the plasmid DNA was precipitated by the addition of 35 µl of 3M NaOAc, pH 5.2, and 1 ml of ice cold 100% ethanol, followed by an incubation at -20°C for 30 min. The nucleic acids were pelleted by centrifugation at 15,400 *g* for 15 min. DNA pellets were washed with 1 ml ice-cold 70% ethanol, dried and resuspended in 20 µl of TE containing 40 mg ml^{-1} pancreatic RNase A.

2.2.3.2 Restriction Endonuclease Digestions

DNA was enzymatically cleaved with restriction endonucleases under the conditions specified by the manufacturer, Boehringer Mannheim. One unit of enzyme was used for each microgram of DNA.

2.2.3.3 Ligation of DNA

Ligation reactions were carried out in a volume of 10 µl using approximately equimolar amounts of prepared vector and insert DNA in 1x T_4 DNA ligase buffer and 1.5-3 U T_4 DNA ligase. The reaction was incubated at 22°C for 3 h or overnight at 4°C.

2.2.3.4 Separation of DNA Fragments by Agarose Gel Electrophoresis

Agarose minigels were prepared using 30-50 ml of 0.8-2.0% (w/v) molten agarose in 0.5x TBE, or 1x TAE buffer, a 10 x 7 cm gel casting tray and an appropriate well-forming

comb. One tenth volume loading buffer type II was added to DNA samples before being loaded into the wells. Gels were run in their corresponding 0.5x TBE or 1x TAE buffer at 80-120 mA, until the bromophenol blue dye migrated down 2/3 the length of the gel. DNA was stained with 0.5 mg L^{-1} ethidium bromide for 15 min, destained in water for 15 min and observed with UV light (260 nm) on a transilluminator. Gels were photographed using Polaroid 667 photographic system.

2.2.3.5 DNA and RNA Quantitation

Genomic and plasmid DNA was quantified by the minigel method of Sambrook *et al* (1989) using molecular weight markers of known concentration and/or with a Beckman DU-68® spectrophotometer using the Nucleic Acid Soft-Pac™ Module Program 10, Warburg/Christian Concentrations (Warburg and Christian, 1942). The amount of nucleic acid was calculated by the following program: [Nucleic Acid] = (-36.0 x A280) + (62.9 x A260), and the absorbances at 260 and 280 nm were corrected for by the background at 320 nm. The concentration of RNA was calculated from the absorbance at 260 nm, given at OD_{260}=1 the RNA concentration was known to be approximately 40 µg/ml.

2.2.3.6 DNA Sequencing of Plasmid Clones

Plasmid DNA was prepared for sequencing using the JETQUICK Plasmid miniprep Spin Kit (Genomed) and diluted to a concentration of 250 ng/µl. The plasmid DNA was sequenced by Dr Neil Shirley of the Nucleic Acid and Protein Chemistry Unit, University of Adelaide using the oligos M13f and M13r (NEB). Sequencing reactions were analysed on an Applied Biosystems Model 373A automated sequencer.

2.2.3.7 Polymerase Chain Reaction (PCR)

All PCRs were preformed in 0.2 µl tubes in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Waltham, MA, USA) with the exception of RAPD PCRs which were preformed in 25 µl capillary tubes in a FTS-1C Capillary Thermocycler, (Corbett Research, Mortlake, NSW, Australia). All reaction components used in PCR were dispensed with filtered micro pipette tips (AxyGen Scientific Inc., Fremont, CA, USA) to reduce aerosol cross-contamination.

2.2.3.8 Preparation of Clone Inserts/ DNA Isolation from Agarose

DNA was extracted from excised agarose gel plugs using the JETQUICK gel extraction spin kit (Genomed).

2.2.3.9 Preparation of ^{32}P-labelled DNA fragments

For labelling reactions, 25 ng of template consisting of gel-purified plasmid-derived PCR products were labelled with 30 µCi α-^{32}P-dCTP using the Megaprime™ DNA labelling system (Amersham). Labelled DNA was separated from unincorporated nucleotides in a ProbeQuant G-50 Micro column (Amersham).

2.2.3.10 Slotblot of Plasmid DNA

Plasmid DNA was transferred and fixed onto nylon membrane using a Bio-Rad slotblotter (Hercules, CA, USA). Three sheets of pre-wet 3MM filter paper, (Whatman International Ltd. Maidstone, UK) were loaded into the slotblotter, followed by the Zeta probe (Bio-Rad) nitrocellulose membrane. The apparatus was assembled according to manufacturers instructions. Plasmid DNA samples were diluted in 0.5 ml of 0.4 M NaOH, 0.01 M EDTA to a final concentration of approximately 5 pg/μl, incubated at 100°C for 10 min before being loaded into the slotblotter. Once all wells had been loaded, vacuum was applied until the wells became dry. Wells were rinsed under vacuum with 0.5 ml per well of 0.4 M NaOH. The slotblotter was disassembled and the Zeta probe membrane washed in 2x SSC and UV crosslinked in a UV Genelinker (Bio-Rad).

2.2.3.11 Southern Blot of Genomic DNA

For RFLP analysis, 8 μg of *L. rigidum* genomic DNA was digested with one of a selection of restriction enzymes overnight at 37°C. The fragments were fractionated by electrophoresis in 0.8 % agarose gels (6 mm in thickness and 200 mm in length) in 1x TAE buffer at 50 volts for 11 h. Gels were stained with ethidium bromide, photographed, and soaked in denaturation solution for 30 min while shaking. The gels were rinsed in dH_20 and placed in neutralisation buffer for 15 min while shaking. The last step was repeated before the DNA within the gel was transferred in 20x SSC according the capillary blot method of Southern (1975) to Hybond^{TM}-N+ positively charged nylon membrane (Amersham). The membranes were rinsed briefly in 2x SSC and placed on 3 sheets of filter paper soaked in 0.4 M NaOH for 20 min to fix the DNA to the membrane.

2.2.3.12 Hybridisation and Autoradiography

The membranes were separated by nylon mesh inside a 30 cm bottle containing 10 ml of hybridisation solution. A 1 mg/ml solution of sonicated salmon sperm DNA was heated to 100°C for 5 min and placed on ice, before 200 µl was added to the hybridisation solution. All membranes were pre-hybridised for a minimum of 12 h at 65°C. Radiolabelled probes were denatured by heating at 100°C for 5 min before addition to the hybridisation solution. Probes were hybridised for 20 h at 65°C in a rolling bottle hybridisation oven. Following hybridisation, the membrane was washed twice at 65°C in 2x SSPE, 0.1 % SDS for 10 min and once at 65°C in 1x SSPE, 0.1 % SDS for 15 min. If additional washes were required, a further high stringency wash of 0.1x SSPE, 0.1 % SDS at 65°C for 10 min was preformed. Membranes were exposed to X-Ray film, X-Omat (Kodak, Australia) at -70°C, inside cassettes containing intensifier screens. Membranes were stripped for re-probing by washing in 0.4 M NaOH at 45°C for 30 min and then transferred into stripping solution for a further 15 min at 45°C.

2.2.4 DNA Based Methods Used in the Isolation of RAPD Markers

2.2.4.1 RAPD PCR Reaction Conditions

Polymorphic regions of ryegrass genomic DNA were amplified by a modification of the method of Williams *et al.,* (1990). Modifications were required for ryegrass DNA and the reaction kinetics of the capillary-tube thermocycler used.

The 10 µl reaction volume consisted of 1x *Taq* Buffer, 3 mM $MgCl_2$, 0.2 mM each of the deoxyribonucleotide triphosphates dATP, dCTP, dGTP and dTTP, 13.5 pM of a single

operon primer, 0.5 units of *Taq* polymerase (Promega) and 20 ng of ryegrass genomic DNA. Individual reaction mixtures were prepared on ice and withdrawn into 25 µl positive displacement capillary tubes, leaving an air-space at the end of the tube. The capillary tubes were sealed in a heat sealer supplied with the Corbett Research Capillary Thermocycler FTS-1C and stored on ice until all tubes had been sealed.

The reactions were performed in a FTS-1C thermocycler under the following conditions: 3 cycles at 94°C for a 60 s denaturation, annealing at 36°C for 30 s and extension at 72°C for 90 s. This was followed by 45 cycles at 95°C for 15 s, 36°C for 1 s and 72°C for 90 s followed by 1 cycle at 72°C for 5 min to ensure that reaction products were completely extended. Ramp rate was set at 4, for slow annealing.

The completed PCR reactions were analysed by electrophoresis on 2% agarose gels cast and run in 0.5x TBE buffer (pH 8.3). Gels were stained with ethidium bromide and photographed under UV light. In the event of unresolved band profiles generated in PCR, the reactions were repeated at the higher annealing temperature of 37°C to increase the specificity of primer binding.

2.2.4.2 Reproduceability of RAPD profiles

To examine reproducibility, the 15 primers used in reactions that generated products potentially associated with resistance were re-screened against the same panel of DNA from plant lines. To be considered reproducible, resistance associated RAPD-PCR products must consistently have been generated across the same resistant lines as they had in the previous round. Products must also have been of the same apparent electrophoretic mobility when resolved on 2% agarose gels.

2.2.4.3 Selection Criteria for a RAPD Marker Linked to Resistance in a 2 Gene System

The criteria applied to putative resistance associated PCR products were:

· found consistently in all resistant plants and not in the majority of susceptible plants,

· amplification yield was consistent across resistant plant samples,

· resolved from other PCR products of a similar molecular weight,

· produced consistently in repeated PCRs, using the appropriate primer/template combination

· did not co-migrate with control primer generated artifacts.

2.2.4.4 Use of NILs in the Isolation of RAPD Markers Linked to Resistance to Nematode Infection

The experimental approach consisted of two stages and was modified from the original NIL approach (Martin *et al.*, 1991). A range of RAPD primers were initially used in reactions with DNA from a limited number of resistant and susceptible plants primarily susceptible S1150 and its resistant recurrent F_4 backcross, R799 (regarded as NILs) to identify primers used in reactions which generated bands specific to resistant plants. The resistance associated primers identified in the first stage were used in reactions with DNA from a larger population of plant lines to identify primers used in reactions which consistently produced a PCR product(s) specific to DNA derived from resistant plants.

2.2.5 DNA Based Methods Used in the Isolation of Ryegrass RGAs

2.2.5.1 Two Round PCR Conditions for RGA Amplification

A two round PCR, depicted in Figure 2.1, was undertaken to amplify RGA sequences in *L. rigidum*. The first round PCR reactions of 20 μl consisted of 1x Taq Buffer, 2.0 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 0.25 μM of each primer, 1U *Taq* polymerase and 500 ng of *L. rigidum* genomic DNA. The reactions were performed in a PTC-100™ Programmable Thermal Controller (MJ Research) under the following conditions: 95°C for 2 min followed by 40 cycles at 95°C for a 30 s denaturation, annealing at 40°C for 30 s and extension at 72°C for 2 min. This was followed by a final extension at 72°C for 10 min.

The second round PCR reactions of 10 μl consisted of 1x Taq Buffer, 2.0 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 0.25 μM of each primer, 0.5 U *Taq* polymerase and 0.5 μl of a 1/20 dilution of bulked first round PCR reaction as template DNA. The reactions were performed in a PTC-100™ Programmable Thermal Controller (MJ Research) under the following conditions: 95°C for 2 min followed by 10 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 50 s; 25 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 50 s. This was followed by a final extension at 72°C for 10 min. Gel purified, second round products were cloned into the vector pGEM-T.

2.2.5.2 PCR Conditions for the Amplification of Class Specific Probes

PCR reactions of 20 μl consisted of 1x Taq Buffer, 2.0 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 0.25 μM of each primer (GLPL2 and either 1f, 2f,

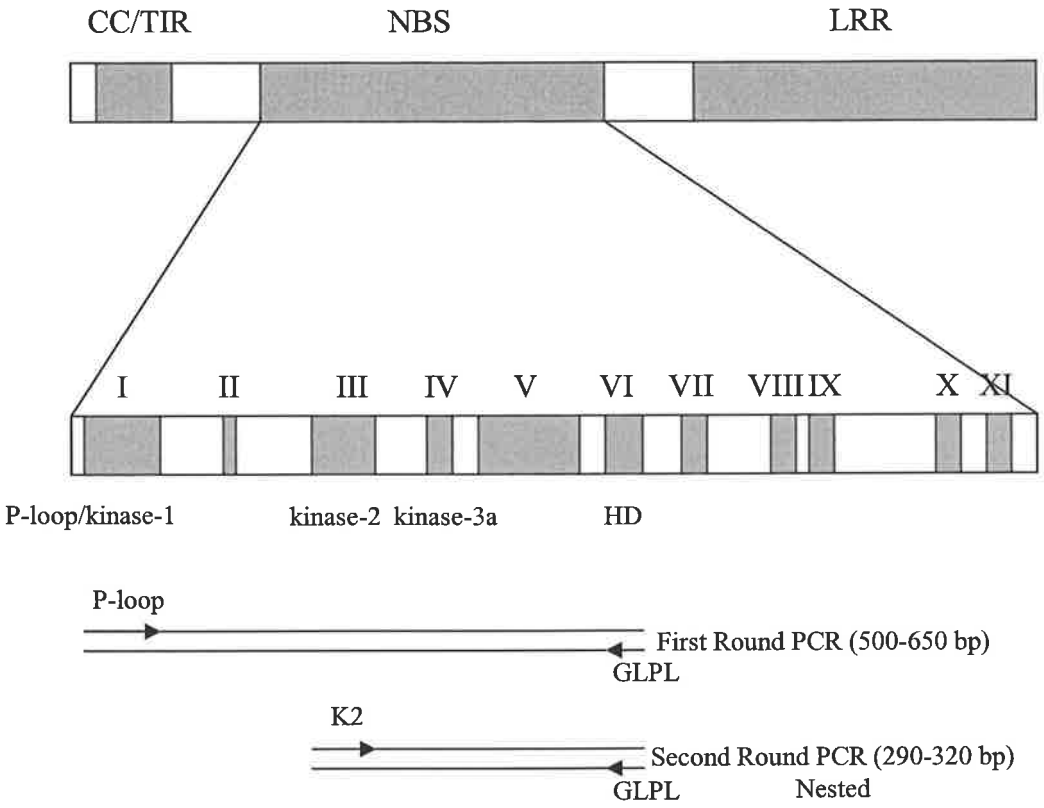


Figure 2.1: Two stage PCR strategy for the amplification of RGA sequences
The schematic diagram depicts the generalised structure of the deduced amino acid sequence of the NBS-LRR R-genes. The sequence contains NBS and LRR with either a Coiled-coil motif (CC) or a region with homology to the *Drosophila Toll* or human interleukin receptor (TIR) at the N-terminal of the protein. PCR amplification of RGA sequences focused on the NBS region known to contain 11 conserved motifs (Pan *et al.,* 2000), which are shaded and numbered I to XI. The first round of amplification utilises degenerate primers directed towards sequences encoding the P-loop (I) and the hydrophobic membrane spanning domain (HD, VI) to amplify a product of 500-650 bp from genomic DNA. The second round of amplification used the PCR products of the first round of amplification as a template with the nested primer directed towards sequence encoding the kinase-2 domain in combination with the primer directed towards the HD domain to amplify a product of between 290 and 320 bp.

3f, or 4f), 1U *Taq* polymerase and 10 ng of the corresponding representative class plasmid DNA (atm59, atm68, atm1 and atm8, respectively). The reactions were performed in a PTC-100™ Programmable Thermal Controller (MJ Research) under the following conditions: 25 cycles of 95°C for a 1 min denaturation, annealing at 56°C for 30 s and extension at 72°C for 30 s. This was followed by a final cycle of 95°C for 30 s, 56°C for 30 s and extension at 72°C for 3 min.

2.2.5.3 RGA 5' RACE Reaction

The 5' RACE System for Rapid Amplification of cDNA Ends (GIBCO-BRL) was used to obtain 5' RACE products. Material used but not supplied with the kit included RNA isolated from *L. rigidum* and the gene specific primers, GLPL2 used as a first strand primer and 1R used as a nested primer. RACE products were directly cloned into pGEM-T.

2.2.5.4 Analysis of RGA Sequences

All sequence data was analysed using the analysis software package, Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group (GCG), 575 Science Drive, Madison Wisconsin, USA.

Chapter 3

Genetics of Disease Resistance

3.1 INTRODUCTION

Resistance to *A. funesta* in *L. rigidum* appears to be controlled by two dominant, independently assorting genes (McKay, 1994). Genotypes of the crossed plants that formed the basis of the evidence of the complementary two gene model were inferred from the resultant numbers of resistant and susceptible progeny. The initial aims of research conducted in this chapter were to re-examine the data generated by McKay (1993, 1994) for statistical significance as a potential genetic model to account for the observed numbers of resistant and susceptible progeny and its ability to definitively genotype resistant and susceptible plants.

Progeny representing a potential third phenotypic class, moderate resistant (MR), resulted from a cross between R3339 and S1150 (McKay, pers. comm.). It was of interest to examine the resistance status of progeny arising from crosses involving lines of moderate resistance. Factorial crosses between MR plants and plants previously classified as susceptible or resistant in phenotype were undertaken. The aims of the factorial crosses were to examine both the potential use of such crosses and subsequent progeny testing as a method of allocation of genotype to parental lines and to analyse the phenotypes of progeny arising from crosses involving MR plants. In all analyses conducted on crosses, the complementary two gene model of inheritance of resistance to *A. funesta* was assumed to be in operation.

3.2 RESULTS

3.2.1 Reanalysis of Crosses for Statistical Significance

There were three alternative genetic models supported by the testing of the goodness of fit of the observed with expected numbers in the χ^2 test to explain the outcomes of a cross between R14.9 and S1150 (Table 3.1). Each of the three models supported the genotype of R14.9 as heterozygous at each resistance gene locus (*AaBb*). The supported genetic models indicated S1150 was either homozygous susceptible (*aabb*) at each locus (p = 0.10) or heterozygous at either one of the resistance loci, while homozygous susceptible (*Aabb* or *aaBb*) at the other loci (p = 0.46). No genetic model supported the genotype for R14.9 of either *AABb* or *AaBB* as the observed ratio of R:S progeny had p values below 0.05.

The cross between R3339 and S1150 produced 23 resistant and 73 susceptible progeny (McKay, pers. comm.). It was noted that of the resistant progeny many were not as resistant as the previously characterised R14.9 and were classified as moderately resistant. The only model supported by statistical testing supported a genotype of *AaBb* for R3339 and *aabb* for S1150 (p = 0.81) (Table 3.2). Models with the resistant plant of the genotype *AABB* were excluded, as they did not allow for the occurrence of susceptible progeny. Models allocating the genotypes of R3339 and S1150 as homozygous resistant at alternative resistance loci (*AABb* x *aaBB* or *AaBB* x *AAbb*) were also excluded on this basis. The sole model supported by statistical analysis predicted any resistant progeny arising from such a cross could only be of the genotype *AaBb* (Table 2.3). This had implications for the subsequent factorial crosses conducted which involved the progeny from this cross, as it indicated all resistant progeny arising from the cross between R3339 and S1150 were genetically identical with respect to their resistance genotype.

Table 3.1: Statistical analysis of numbers of resistant and susceptible progeny from the cross R14.9 x S1150

Genetic Model			Model	Expected	χ^2_1	*P*	Accept/ Reject Model
R14.9		S1150	R:S	R:S			
AABb	x	*AAbb*	1:1	36:36	8	0.0047	✗
AABb	x	*aaBB*	1:0	72:0	-	-	✗
AABb	x	*Aabb*	1:1	36:36	8	0.0047	✗
AABb	x	*aaBb*	3:1	54:18	67	<0.0001	✗
AABb	x	*aabb*	1:1	36:36	8	0.0047	✗
AaBB	x	*AAbb*	1:0	72:0	-	-	✗
AaBB	x	*aaBB*	1:1	36:36	8	0.0047	✗
AaBB	x	*Aabb*	3:1	54:18	67	<0.0001	✗
AaBB	x	*aaBb*	1:1	36:36	8	0.0047	✗
AaBB	x	*aabb*	1:1	36:36	8	0.0047	✗
AaBb	x	*AAbb*	1:1	36:36	8	0.0047	✗
AaBb	x	*aaBB*	1:1	36:36	8	0.0047	✗
AaBb	x	*Aabb*	3:5	37:45	0.53	0.47	✓
AaBb	x	*aaBb*	3:5	37:45	0.53	0.47	✓
AaBb	x	*aabb*	1:3	18:54	2.7	0.10	✓

Expected ratios of R:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 24 resistant and 48 susceptible plants to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✗).

Table 3.2: Statistical analysis of numbers of resistant and susceptible progeny from the cross R3339 x S1150

Genetic Model			Model	Expected	χ^2_1	*P*	Accept/ Reject Model
R3339		S1150	R:S	R:S			
AABb	x	*AAbb*	1:1	48:48	26	<0.0001	✗
AABb	x	*aaBB*	1:0	95:0	-	-	✗
AABb	x	*Aabb*	1:1	48:48	26	<0.0001	✗
AABb	x	*aaBb*	3:1	72:24	133	<0.0001	✗
AABb	x	*aabb*	1:1	48:48	26	<0.0001	✗
AaBB	x	*AAbb*	1:0	95:0	-	-	✗
AaBB	x	*aaBB*	1:1	48:48	26	<0.0001	✗
AaBB	x	*Aabb*	3:1	72:24	133	<0.0001	✗
AaBB	x	*aaBb*	1:1	48:48	26	<0.0001	✗
AaBB	x	*aabb*	1:1	48:48	26	<0.0001	✗
AaBb	x	*AAbb*	1:1	48:48	26	<0.0001	✗
AaBb	x	*aaBB*	1:1	48:48	26	<0.0001	✗
AaBb	x	*Aabb*	3:5	36:60	7.5	0.0061	✗
AaBb	x	*aaBb*	3:5	36:60	7.5	0.0061	✗
AaBb	x	*aabb*	1:3	24:72	0.056	0.81	✓

Expected ratios of R:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 23 resistant and 73 susceptible plants to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✗).

3.2.2 Parental Phenotype Testing

The control plants S1150 and R799 demonstrated the extremes of resistance and susceptibility phenotypes that were possible in the gall formation assay in extensive testing of all parental lines (Table 3.3). The resistant line R799 was totally resistant, as no galls were detected in the seedheads of this plant. The susceptible line, S1150, demonstrated the upper range of susceptibility as the maximum numbers of galls (20 per seedhead) were always found in each infected seedhead.

Table 3.3: Comparative Results of Gall Formation Assay Performed on Lines in Two Consecutive Years

Plant line	First year limited score	Status	Second year extensive score	Status
R799	0,0	R	0,0,0,0,0,0,0,0	R
S1150	20,20	S	20,20,20,20,20,20,20,20	S
R15789.5	3,6,14	MR	0,1,0,5,3,0,1,0,3,3,7,1,0,0,2,0	MR-R
R1386.4	8	MR	1,1,0,0,0,7,0,1,2,2,0,0,3,0,1,3	MR-R
S15796.2	15	S	15,20,20,20,8,0,0,20,4,20,20,20,20,9,1,1	S
R15794.1	1,3,2,1	R	0,1,4,1,0,8,0,3,0,4,3,1,0,7,5,6,9,2	MR-R
S15782.3	15,18	S	0,1,0,0,1,0,0,0,0	R

Limited first year scores were obtained from McKay (pers. comm.). Individual counts represent the number of nematode galls per seedhead (0 to 20). The corresponding resistance status was derived from the overall level of infection.

The scoring of a number of seedheads of S15796.2 that contained more than 10 galls (9 of the total 16 tested) resulted in its classification as a susceptible plant. However, a number of seedheads were uninfected (5 of the 16 tested), demonstrating that not all seedheads of susceptible plants contain galls in this assay. Based on the counts obtained in the second year

of testing, R15789.5 was classified as moderately resistant as, although less than four galls per seedhead in 13 of the 15 seedheads, two seedheads contained five and seven galls, respectively. R1386.4 was classified as moderately resistant, as one of the 16 seedheads tested contained seven galls, while the remaining 15 heads contained under four galls per seedhead. The phenotypically resistant line R15794.1 was classified as moderately resistant as five of the total 18 seedheads scored contained more than four but less than 10 galls. The remaining heads contained less than four galls. No more than one gall per seedhead in the nine seedheads produced by S15782.3 was detected. Consistent with its resistance phenotype, S15782.3 was subsequently referred to as R15782.3.

3.2.3 Factorial Crossing

The lack of seed set from any of the self crosses was consistent with *L. rigidum* being outbreeding (Table 3.4). In addition to the self crosses, eight of the 22 factorial crosses also failed to set seed. All outcrosses involving line R15782.3 produced seed. This indicated that, of the plants tested, R15782.3 may be the most genetically divergent. At the other extreme, all but one cross involving line R15794.1 did not produce seed.

A surprising result was the success of the cross between the control lines S1150 and R799. As these lines were expected to share 93.5% of their genetic material, it was highly likely that these lines would also share self incompatibility alleles and hence be incompatible.

Of the crosses that did produce seed, the number of seeds collected was less than for outbreeding commercial cultivars of ryegrass. The number of seeds collected from successful crosses ranged from 50 to 200 seeds compared to 500 or more seeds obtained for crosses conducted with three parents by A. McKay (pers. comm.).

Table 3.4: Seed Set from Controlled Crosses of Various Lines

plant line	R15782.3	S15796.2	R15789.5	R15794.1	R1386.4	S1150	R799
R799	+	+	+	-	+	+	-
S1150	+	++	-	-	+	-	
R1386.4	+	-	+	-	-		
R15794.1	+	-	-	-			
R15789.5	+	-	-				
S15796.2	+	-					
R15782.3	-						

Crosses with a positive sign (+) resulted in seed set. Crosses with a double positive sign (++) were conducted in duplicate with both resulting in seed set. Crosses with a negative sign (-) failed to result in seed set.

3.2.4 Progeny Phenotype Testing

Up to 10 progeny from each of the factorial crosses were tested with respect to their susceptibility to *A. funesta* (Table 3.5). The lack of information on the resistance status of the progeny from all crosses severely impeded the ability of this analysis to determine the genotypes of the parents, as the full complement of factorial crosses was not achieved. However, the allocation of putative genotypes was still possible in a number of situations due to the informative nature of a number of the crosses.

Table 3.5: Numbers of Resistant (R), Moderate Resistant (MR) and Susceptible (S) progeny generated from controlled crosses of various lines, as tested by gall formation assay

plant line	S15782.3	S15796.2	R15789.5	R15794.1	R1386.4	S1150	R799
R799	10R, 0MR, 0S	9R,1MR, 0S	8R,1MR, 0S	-	6R, 0MR, 1S	8R, 1MR, 0S	-
S1150	0R, 1MR, 9S	5R,2MR, 12S	-	-	5R, 2MR, 2S	-	
R1386.4	6R, 2MR, 2S	-	5R, 2MR, 3S	-	-		
R15794.1	5R, 3MR, 2S	-	-	-			
R15789.5	3R, 0MR 4S	-	-				
S15796.2	1R, 2MR, 7S	-					
S15782.3	-						

Crosses with a negative sign (-) failed to result in seed set.

3.2.4.1 Allocation of Potential Genotypes to Resistant and Susceptible Lines

One of the aims of the analysis of the factorial crosses was to investigate their potential use in determining the resistance genotypes to previously uncharacterised lines. Analyses of key crosses are represented in detail. The potential genotypes deduced by analysis of the remainder of the crosses are presented in the summary Table 3.10 and Appendix A. With the exception of the cross between S15796.2 and S1150, 10 or less progeny from each cross were tested for susceptibility. Due to the small sample size, less emphasis was placed on the significance testing of various genetic models to examine the observations. Testing of the

various models served as a rough guide to the most likely genotypes in an attempt to assist in the planning of future experiments.

3.2.4.2 Analysis of the Cross Between S15796.2 and S1150

Duplicate crosses of S15796.2 and the control plant S1150 were made and hence represented the cross with the most progeny tested. Both plants were phenotypically susceptible and therefore, under the complementary two gene model, could theoretically be one of five genotypes. However, the generation of resistant plants from crosses involving two susceptible parents is only possible if the parents carry resistance alleles at alternative resistance loci. Of the progeny that were tested from this cross, 7 were classified resistant and 12 susceptible. The generation of the seven resistant progeny could only have occurred if the control plant S1150 had a genotype *aaBb* or *Aabb* rather than the genotype *aabb* previously assigned by McKay (1994). The restricted set of combinations of genetic crosses was tested for the significance to which they could account for the observed generation of resistant and susceptible progeny (Table 3.6).

Two genetic models were supported ($p = 0.23$) as the observed numbers of resistant to susceptible progeny did not deviate significantly from the expected ratio of 1:1. Both models assigned S15796.2 or S1150 as homozygous resistant at one locus and the other parent as heterozygous resistant at the alternative resistance locus. The model assigning both S15796.2 and S1150 as heterozygous resistant at single but alternative resistance loci was also statistically supported ($p = 0.25$). If the control plant S1150 was assumed to be *aaBb* then the line S15796.2 may be either *Aabb* or *AAbb* (Table 3.10).

Table 3.6: Statistical analysis of numbers of resistant and susceptible progeny from the cross S1150 x S15796.2

Genetic Model			Model	Expected	χ^2_1	*P*	Accept/ Reject Model
S1150		S15796.2	R:S	R:S			
aaBb	x	*AAbb*	1:1	9.5:9.5	1.32	0.25	✓
aaBb	x	*Aabb*	1:3	4.75:14.25	1.42	0.23	✓
Aabb	x	*aaBb*	1:3	4.75:14.25	1.42	0.23	✓
Aabb	x	*aaBB*	1:1	9.5:9.5	1.32	0.25	✓
aabb	x	*AAbb*	0:1	0:19	-	-	✗
aabb	x	*Aabb*	0:1	0:19	-	-	✗
aabb	x	*aaBB*	0:1	0:19	-	-	✗
aabb	x	*aaBb*	0:1	0:19	-	-	✗
aabb	x	*aabb*	0:1	0:19	-	-	✗

Expected ratios of R:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 7 resistant and 12 susceptible plants to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✗).

3.2.4.3 Analysis of the Cross Between S15796.2 and R799

The genotype of R799 was not known and could potentially be one of four resistant genotypes; *AABB*, *AaBB*, *AaBb*, or *AABb*. Although only resistant progeny were produced in this cross, the genotype *AABB* for R799 was able to be excluded from any subsequent analysis as susceptible progeny were produced in crosses with S15796.2, R15789.5, R1386.4 and S1150 (Table 3.5). Statistical testing of the six alternative genetic models with the observed result of 10 resistant and no susceptible plants led to the support of three of these models (Table 3.7). The potential genotypes of the control line R799 were restricted to either *AaBB* ($p = 1$) or *AaBb* ($p = 0.068$) in models with S15796.2 of the genotype *AAbb*. Alternatively if the genotype of S15796.2 was *Aabb*, the only genetic model supported by testing was R799 with the genotype *AaBB* ($p = 0.068$). Three models were rejected due to the inability of chance to account for significant deviations of observed from expected number of the two classes of plants ($p = <0.0001$, $p = 0.0016$). However, with a small sample size, a large deviation may occur due to chance and result in an erroneous rejection of the genetic model.

3.2.4.4 Analysis of the Cross Between S15796.2 and R15782.3

Phenotypically the cross between S15796.2 and R15782.3 was identical to the cross of S15796.2 and R799 and as such the same genetic models were tested for their ability to explain the observed results (Table 3.10). Evidence that R15782.3 was genotypically distinct from R799 was observed by the large percentage of susceptible progeny produced in its cross with S15796.2 compared to the large percentage of resistant progeny in the cross between R799 with S15796.2 (Table 3.5). This was supported by statistical testing leading to the rejection of all three genetic models ($p = 0.001$, $p = 0.001$, $p = 0$) that were supported in the

Table 3.7: Statistical analysis of numbers of resistant and susceptible progeny from the cross R799 x S15796.2

Genetic Model			Model	Expected	χ^2_1	*P*	Accept/ Reject Model
R799		S15796.2	R:S	R:S			
AaBb	x	*Aabb*	3:5	3.75:6.25	17	<0.0001	✖
AaBB	x	*Aabb*	3:1	7.5:2.5	3.3	0.068	✓
AABb	x	*Aabb*	1:1	5:10	10	0.0016	✖
AaBb	x	*AAbb*	3:1	7.5:2.5	3.3	0.068	✓
AaBB	x	*AAbb*	1:0	10:0	0	1	✓
AABb	x	*AAbb*	1:1	5:5	10	0.0016	✖

Expected ratios of R:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 10 resistant plants to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✖).

analysis of the cross between R799 and S15796.2 (Table 3.8). Genetic models that were supported narrowed the potential genotype of R15782.3 to either *AaBb* ($p = 0.62$) or *AABb* ($p = 0.20$), if the genotype of S15796.2 was *Aabb*. Alternatively, if the genotype of S15796.2 was *AAbb*, the model that assigned R15782.3 as *AABb* was supported ($p = 0.20$).

3.2.4.5 Analysis of the Cross Between R15789.5 and R799

Analysis of the genetic models accounting for a cross between two resistant plants and their goodness of fit with the observed numbers of nine resistant and no susceptible progeny generated from the cross between R15789.5 and R799 is presented in Table 3.9. The only rejected model failed to support the genotype of both lines as double heterozygous (*AaBb*) for resistance ($p = 0.0081$). One supported model allocated the genotype of the parental lines as homozygous resistant at alternative loci to produce solely resistant progeny ($p = 1$). Alternative supported models assigned the genotypes of one parent as homozygous resistant at one loci and a heterozygous at the other loci (*AABb* or *AaBB*) and the second parent as double heterozygous (*AaBb*) ($p = 0.083$).

3.2.5 Factorial Analysis of Data Generated from Individual Crosses

One of the proposed advantages of using factorial crosses in the assignment of genotypes was the ability to examine the outcomes of crosses cooperatively. The inability to generate progeny from each cross severely limited the power of such an approach. Restricting the genetic models of each cross to include only those with R15782.3 and R15798.5 of the genotype *AaBb* and S1150 of the genotype *aaBb*, assisted in determining the genotypes of one of the remaining lines (Table 3.11). Re-examination of the cross between R15782.3 and

Table 3.8: Statistical analysis of numbers of resistant and susceptible progeny from the cross R15782.3 x S15796.2

Genetic Model			Model	Expected	χ^2_1	*P*	Accept/ Reject Model
R15782.3		S15796.2	R:S	R:S			
AaBb	x	*Aabb*	3:5	3.75:6.25	0.24	0.62	✓
AaBB	x	*Aabb*	3:1	7.5:2.5	11	0.001	✗
AABb	x	*Aabb*	1:1	5:5	2	0.21	✓
AaBb	x	*AAbb*	3:1	7.5:2.5	11	0.001	✗
AaBB	x	*AAbb*	1:0	10:0	-	-	✗
AABb	x	*AAbb*	1:1	5:5	1.6	0.21	✓

Expected ratios of R:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 3 resistant and 7 susceptible plants to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✗).

Table 3.9: Statistical analysis of numbers of resistant and susceptible progeny from the cross R15789.5 x R799

Genetic Model			Model	Expected	χ^2_1	*P*	Accept/ Reject Model
R15789.5		R799	R:S	R:S			
AABb	x	*AABb*	3:1	6.75: 2.25	3	0.083	✓
AABb	x	*AaBb*	3:1	6.75: 2.25	3	0.083	✓
AABb	x	*AaBB*	1:0	9:0	0	1	✓
AaBB	x	*AABb*	1:0	9:0	0	1	✓
AaBB	x	*AaBb*	3:1	6.75: 2.25	3	0.083	✓
AaBB	x	*AaBB*	3:1	6.75: 2.25	3	0.083	✓
AaBb	x	*AABb*	3:1	6.75: 2.25	3	0.083	✓
AaBb	x	*AaBb*	9:7	5.06:3.94	7	0.0082	✗
AaBb	x	*AaBB*	3:1	6.75:2.25	3	0.082	✓

Expected ratios of R:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 9 resistant plants to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✗).

Table 3.10: Summary of deduced genotypes derived from statistical analysis of individual crosses

	Deduced Genotypes of Plant Lines						
Cross	**R799**	**S1150**	**S15796.2**	**R15782.3**	**R1386.4**	**R15794.1**	**R15789.5**
R799 x S1150	*AABb*	*aaBb*					
R799 x S15796.2	*AaBB*, *AaBb*		*Aabb*, *AAbb*				
R799 x R15782.3	*AaBb*, *AABb*, *AaBB*			*AaBb*, *AABb*, *AaBB*			
R799 x R1386.4	*AaBB*, *AABb*, *AaBb*				*AaBb*, *AABb*, *AaBB*		
R799 x R15789.5	*AaBB*, *AABb*, *AaBb*						*AaBb*, *AABb*, *AaBB*
S1150 x S15796.2		*aaBb*	*Aabb*, *AAbb*				
S1150 x R15782.3		*aaBb*, *aabb*		*AaBb*			
S15796.2 x R15782.3			*Aabb*, *AAbb*	*AaBb*, *AABb*			
R15782.3 x R1386.4				*AaBb*, *AABb*, *AaBB*	*AaBb*, *AABb*, *AaBB*		
R15782.3 x R1574.1.				*AaBb*, *AABb*, *AaBB*		*AaBb*, *AABb*, *AaBB*	
R15782.3 x R15789.5				*AaBb*			*AaBb*
R1386.4 x R15789.5					*AaBb*, *AABb*, *AaBB*		*AaBb*, *AABb*, *AaBB*
Overall	*AaBB*, *AABb*	*aaBb*	*Aabb*, *AAbb*	*AaBb*	*AaBb*, *AABb*, *AaBB*	*AaBb*, *AABb*, *AaBB*	*AaBb*

S15796.2 restricted potential genetic models to two in which R15782.3 was of the genotype *AaBb* (Table 3.8). Statistical analysis rejected the model with R15782.3 of the genotype *AAbb* ($p = 0.001$), while supporting the model with S15796.2 of the genotype *Aabb* ($p = 0.62$).

In the cross between R15789.5 and R799, potential genetic models were limited to three with R15789.5 of the genotype *AaBb* (Table 3.9). The model with R799 as a double heterozygous carrier was rejected ($p = 0.008$). This was consistent with two previous individual analyses of crosses between R799 with R15782.3 or S1150 in which models with R799 of the genotype *AaBb* were rejected. Therefore, while the factorial analysis was able to provide a result consistent with those found previously, it did not provide a novel or definitive result.

Table 3.11: Summary of deduced genotypes of plant lines derived from statistical analysis of individual crosses and factorial analysis

Plant line	Genotype	Phenotype
R799	*AaBB,AABb*	R
S1150	*aaBb*	S
S15796.2	*Aabb*	S
R15782.3	*AaBb*	R
R1386.4	*AaBb,AABb,AaBB*	MR
R15794.1	*AaBb,AABb,AaBB*	MR
R15789.5	*AaBb*	MR

3.2.6 Analysis of Progeny from Factorial Crosses of Moderately Resistant Plants

Analysis of the cross that generated the moderately resistant plants used in this study revealed that resistant progeny could only have been of the genotype *AaBb* (3.2.1.2). It was

proposed that lines were required to be homozygous resistant at at least one resistance locus, to be fully resistant and plants lines double heterozygous for resistance may only be of moderate resistance (McKay, 1994). Factorial intercrosses between moderately resistant lines were undertaken to examine the inheritance of resistance amongst the progeny of moderately resistant plants. Unfortunately, with a small sample size in conjunction with three potential classes of resistance, results of the statistical analysis were interpreted with caution.

It was expected that, if the proposed basis of moderate resistance was correct, a ratio of 5:4:7 of fully resistant: moderate resistant: susceptible would be expected in a cross between two moderately resistant plants. Unfortunately, only one of the four factorial crosses between moderately resistant plants resulted in seed set, severely limiting the analysis. As can be seen in Table 3.12, in the cross between R1386.4 and R15789.5, the observed numbers of five resistant, two moderate resistant and two susceptible plants were consistent with the expected ratio of 5:4:7 ($p = 0.26$).

The model for the basis of moderate resistance predicted a ratio of 4:2:1 of R:MR:S amongst progeny of a cross between plants lines of genotypes *AaBb* and *AaBB* or *AABb*. Two of the three attempted crosses of this type successfully set seed (R15789.5 x R799 and R1386.4 x R799). The observed numbers were consistent with the expected numbers of the three classes of progeny ($p = 0.15$, $p = 0.29$, respectively), despite the small sample numbers (Table 3.12).

A ratio of 1:2:5 of R:MR:S would be expected in the progeny of a cross between a moderately resistant and a susceptible plant of the genotype *aaBb*. Alternatively, if S1150 was of the genotype *aabb*, a ratio of 0:1:3 of R:MR:S would be expected amongst progeny in a cross with a plant of the genotype *AaBb*. Of the four crosses performed of this type, only two set seed (R15796.2 x S1150 and R1386.4 x S1150). Results obtained for the cross between

Table 3.12: Statistical analysis of numbers of resistant, moderate resistant and susceptible progeny in crosses involving moderately resistant plant lines

Genetic Model			Model R:MR:S	Expected R:MR:S	χ^2_2	*P*	Accept/ Reject Model
MR ***AaBb***	**x**	**MR** ***AaBb***					
R1386.4	x	R15789.5	5:4:7	2.8:2.25:3.95	2.7	0.26	✓
MR ***AaBb***	**x**	**R** ***AABb,AaBB***					
R15789.5	x	R799	4:2:1	5.1:2.6:1.3	3.8	0.15	✓
R1386.4	x	R799	4:2:1	4:2:1	2.5	0.28	✓
MR ***AaBb***	**x**	**S** ***aaBb***					
R15796.2	x	S1150	1:2:5	2.4:4.8:11.9	4.5	0.11	✓
R1386.4	x	S1150	1:2:5	1.1:2.3:5.6	16	0.0004	✗
AaBb		***aabb***					
R15796.2	x	S1150	0:1:3	0:4.75:14.25	-	-	✗
R1386.4	x	S1150	0:1:3	0:2.25:6.75	-	-	✗

Expected ratios of R:MR:S were derived from genetic models that may explain the genotypes of parental lines which in turn were responsible for expected numbers of resistant (R), moderate resistant (MR) and susceptible (S) progeny plants. These values were used in conjunction with observed numbers of 5 R, 2 MR, 2 S (R1386.4 x R15789.5), 8 R, 1 MR, 0 S (R15789.5 x R799), 6 R, 1 MR, 0 S (R1386.4 x R799), 5 R, 2 MR, 12 S (R15796.2 x S1150) and 5 R, 2 MR, 2 S (R1386.4 x S1150) to calculate the chi-squared (χ^2_2) and corresponding probability (p) value. On the basis of the value of p, the model was either accepted (✓) or rejected (✗).

R15796.2 and S1150 (*aaBb*), displayed in Table 3.12, were statistically consistent with the expected numbers of the three categories of resistant progeny (p = 0.11). However, if the S1150 is assumed to be of the genotype *aabb*, the observed numbers of progeny deviated significantly from the expected numbers leading to the rejection of the model (p = 0). Analysis of the progeny from the cross between R1386.4 and S1150 (*aaBb*) revealed the results were not consistent with expected numbers (p = 0.0004). In particular, the numbers of resistant progeny, seven resistant out of total nine scored (78%), were in excess of expected numbers for this class (37.5%). Observed results were also inconsistent with the expected numbers for the cross between S1150 (*aabb*) and R1386.4 (p = 0).

3.3 DISCUSSION

3.3.1 Reanalysis of Data Forming the Basis of the McKay Model

Statistical analysis of the cross conducted by McKay (1993) between R14.9 and S1150 supported three alternative genetic models to explain the observed frequencies of resistant and susceptible progeny. The genotype for R14.9 was consistent with the double heterozygous genotype deduced by McKay (1993) in all three supported models. However, the models supported the genotype of S1150 as either double homozygous susceptible (*aabb*), consistent with the genotype deduced by McKay (1993), or as a carrier of a single resistance allele (*aaBb* or *Aabb*). Therefore, while testing supported the inferred genotype, alternative genotypes may also explain the observed outcomes.

As statistical testing allowed the rejection of all but one of the genetic models in the analysis of the cross between R3339 and S1150, allocation of parental genotypes was possible. The supported genotype of S1150 as *aabb* was consistent with both the deduced genotype by

McKay (1993) and one of the three potential genotypes deduced from statistical analysis of the cross between R14.9 and S1150.

The conclusions that can be made from these two crosses reflect the resolving power of such analysis to allow genotype allocation. Observed numbers of resistant to susceptible progeny deviated significantly from an expected 3:5 ratio in the cross between R3339 and S1150, allowing rejection of genetic models with this expected outcome. In contrast, in the cross between R14.9 and S1150 observed numbers did not deviate significantly from those expected from a 3:5 ratio and hence models with these expected numbers could not be rejected as potential models. This approach was therefore limited by its inability to discriminate between models with the same expected ratios of resistant to susceptible progeny and its inability to generate definitive genotype allocations in all analyses.

3.3.2 Comparison Between Parental Phenotypes Obtained in the Initial and Subsequent Years of Testing

Extensive scoring of the control lines R799 and S1150 demonstrated the full range of infection was possible when testing mature plants. Overall, most lines tested were of the same phenotype between the consecutive years. However, two lines were found to differ in phenotype between the two years. Line R15794.1, classified initially as resistant, was reclassified as only moderately resistant following more extensive scoring in the subsequent year. Possible reasons for this are variation in field conditions between the two years, subtly affecting nematode invasion and/or host susceptibility or simply that, by chance, the small sample initially scored did not include any seedheads with more than four galls.

Plant R15782.3 demonstrated a dramatic change from a susceptible phenotype to that of a resistant phenotype. It is theoretically possible that the susceptible plant escaped infection in the second year. This possibility was discarded as a low level of gall formation was observed. If the line was too advanced, nematodes may have had a reduced opportunity of gall initiation resulting in an apparent phenotype of resistance. This also appeared unlikely as all lines tested in the second year used more advanced plants and susceptible phenotypes were observed in both lines S1150 and S15796.2. Mislabeling of line S15782.3 can not be discounted.

3.3.3 Factorial Crossing

L. rigidum is an outbreeding species of ryegrass (Jauhar, 1993). SI alleles prevent both self crossing and crossing with other lines with identical SI alleles. This was of particular relevance for the crosses undertaken, as the lines were known to be related and consequently lack diversity at SI loci. The failure of self crosses to set seed indicated that the lines were self incompatible and the crossing bags were an effective barrier capable of isolating plants from external contaminating pollen.

Set seed in the cross between S1150 and R799, was unexpected because they were assumed to share 93.5% of their genetic material. Although it was still theoretically possible that these lines were diverse at SI loci in addition to resistance loci, this observation cast some doubt over their true level of genetic relatedness.

Line R15794.1 was incompatible with all but one plant used in the factorial crosses. While sharing of SI alleles may explain this incompatibility, other factors such as flowering time and general vigour could also be responsible. As an example, a flowering date of

R15794.1 more than two weeks later than most plants involved in the crosses would result in no seed set. Lack of seed set may also have been a consequence of the poor general fitness of the clones of R15794.1 that were the least vigorous of the lines in this study. This slow growth may have also contributed to delayed flowering.

3.3.4 Allocation of Genotypes to Parental Lines

One of the primary aims of the factorial crosses was to examine the suitability, in conjunction with statistical analysis, of this method for allocation of genotypes to lines of known phenotype. Through the reanalysis of previous crosses it was possible to deduce the genotypes of a number of lines. All resistant progeny from the cross between R3339 and S1150 were deduced to be of the genotype *AaBb* (Table 3.2). This genotype was correctly allocated to two of the lines (R15782.3, R15789.5) by the analysis of the crosses involving these lines. The remaining two resistant lines (R1386.4, R15794.1) could not be limited to one genotype. However, the genotype *AaBb* remained a possibility.

The susceptible control line, S1150, was determined to be *aaBb*. This conflicted with the analysis of the cross between R3339 and S1150 which supported the allocation of the genotype *aabb* to S1150. The basis for the inconsistency was three resistant progeny from the cross between S1150 and the susceptible S15796.2, which indicated both lines contained at least one resistance allele. It is possible that these resistant progeny were susceptible plants that had escaped gall initiation. If this were the case, alternative, overlooked genetic models predicting all susceptible progeny may have been considered including those in which S1150 was of the genotype *aabb*.

3.3.5 Problems Encountered in the Use of Factorial Crosses in Genotype Allocation

The lack of progeny from all crosses severely limited the resolving power of this process to allocate only one genotype to each line. The underlying problem of SI was presumed to be the cause of the lack of seed set from a number of crosses. It was not possible to predict which crosses would be incompatible and not produce progeny. Rather than eliminate the possibility of informative analysis of progeny arising from crosses between lines believed to be related, all potential crosses were conducted and analysed.

The compromise of decreased numbers of progeny tested for the ability to score more crosses did not appear to be beneficial for the analysis of crosses between two resistant lines. The only definintive conclusion to be made from such crosses was the exclusion of possible double homozygous resistance genotype (*AABB*) from either of the lines in the crosses with susceptible plants amongst the progeny. Even though most lines were able to produce progeny when crossed to the resistant control line R799, the analysis on the small number of progeny seldom led to an allocation of genotypes. This was due to the inability of the statistical testing to exclude any of the potential genetic models and discriminate between a 9:7 and a 3:1 expected ratio in crosses in which only small numbers of progeny were phenotype tested. If larger numbers of progeny were tested, the observed ratios may still support several genetic models.

3.3.6 Classification of Moderately Resistant Plants

Moderately resistant plants used in this study originated from a cross between R3339 and S1150. Genotypically this represented a cross between a double heterozygous resistant line

(*AaBb*) and a double homozygous susceptible line (*aabb*), and would be expected to produce resistant to susceptible progeny in the ratio of 1:3. All resistant progeny resulting from such a cross would be of the genotype *AaBb*. Any variation in the level of resistance must be due to factors other than the primary resistance loci. Such factors may include seasonal variation in pathogen infection, general vigour of the line and presence or absence of other genes that may act to modify the action of the resistance genes. The difference between a fully resistant and moderately resistant plant may not represent distinct classes of resistance but may reflect a broad spectrum of resistance phenotypes measured by the gall formation assay. Therefore, any cutoff score distinguishing the two classes of resistance would be arbitrary.

3.3.7 The Complementary Two Gene Model of Inheritance of Resistance to *A. funesta* in *L. rigidum*

The complementary two gene model remained the most likely to explain the mode of resistance gene inheritance in *L. rigidum*. Although conflicting genotypes for S1150 and R799 were deduced from different crosses, it appeared this was more likely due to experimental limitations and efficiencies of the factorial crosses rather than due to an alternative genetic model. The small sample size of progeny tested in conjunction with the possibility of incorrect phenotype allocation combine to prevent speculation on an alternative genetic basis to explain observed outcomes.

Biochemical models of mechanisms for plant disease resistance usually involve pathogen recognition of a receptor (reviewed by Dangl and Jones, 2001). Upon binding of the pathogen or a pathogen derived product to the specific plant receptor, a signal is conveyed to a signal protein, which in turn conveys the signal to the nucleus to activate the transcription of

defence related genes. The *A* and *B* resistance genes in *L. rigidum* may represent two components of such a system, such that the product of the *A* gene is the nematode specific receptor and the *B* gene product is the communicator of the signal to the plant cell nucleus.

3.3.8 Future Directions

3.3.8.1 Crosses with Susceptible Lines Only

The most informative crosses conducted in this study were those involving susceptible lines. On a number of occasions the genotypes of both lines involved in a single cross could be deduced by statistical testing on the small numbers of resistant and susceptible progeny scored. If all lines could be successfully crossed with susceptible lines this could remove the need for factorial crosses to be conducted. A better approach in the future may be to construct a panel of unrelated double homozygous susceptible plants and single resistance gene carriers. Any line of unknown genotype may be crossed to the panel of susceptible plants, maximising the chances of compatible crosses and the analysis of the progeny leading to an informative allocation of genotype.

3.3.8.2 Limited Support for Small Scale Testing

Resistance genotypes for a number of the plants tested in the factorial crosses had been determined previously through analysis that had been conducted on up to 100 phenotype tested progeny (McKay, 1993). Statistical analysis conducted on up to 10 progeny also supported the same genotype allocation in some cases. This supported the determination of phenotypes for smaller numbers of progeny, which may be an advantage when screening large

numbers of crosses. The confidence with which a genotype allocation is made may further be improved by increasing the number of progeny tested in several types of crosses, such as those occurring between two resistant lines.

3.3.8.3 Isolation of Double Homozygous Resistant Lines

The only information generated by the analysis of crosses between resistant lines was the exclusion of the possibility that either line was *AABB*. This approach may therefore be useful in screening for double homozygous resistant lines. Such a line would always produce resistant progeny regardless of the genotype of the other parental line, reducing the need for excessive numbers of progeny plants to be phenotype tested.

3.3.9 Conclusions

Factorial crosses between related lines provided limited information on the resistance genotype of parental lines due to self-incompatibility. Allocation of parental genotypes was possible in a number of crosses that successfully set seed. This highlighted the validity of this approach, but only if conducted between non-highly related lines or in lines where prior compatibility has been established. On the basis of the analysis of the experimental data, the complementary two gene model of McKay (1994) was retained as the most plausible model to explain the behavior of genes involved in *A. funesta* resistance in *L. rigidum*.

Chapter 4

RAPD Analysis of *L. rigidum* Lines

4.1 INTRODUCTION

The unreliability and lengthy nature of the biological assay for plant nematode resistance was an impediment in the development of the first cultivar of *L. rigidum* resistant to *A. funesta* (McKay *et al.,* 1993). A DNA based diagnostic test able to discriminate between resistant and susceptible lines could be used for breeding new resistant cultivars, as well as to monitor resistant plant populations in the field. Random amplified polymorphic DNA (RAPD) markers can be used to detect DNA polymorphisms between lines (Williams *et al.,* 1990). When used in conjunction with Near-Isogenic Lines (NILs), RAPD markers can be used to identify genomic regions derived from the donor parent (Martin *et al.,* 1991). In comparisons between two NILs differing in resistance status, polymorphic markers have a high probability of linkage to the target resistance gene.

RAPD primers OPD-15 and OPW-8 were used to identify potential resistance markers and tested on various lines of *L. rigidum* either resistant or susceptible to *A. funesta* (McKay, 1994). Although the resistance status of these lines was reported the pedigrees were not. The mode of inheritance of resistance to *A. funesta* in *L. rigidum* appears to be consistent with a complementary two-gene model (McKay, 1994, Chapter 3). Unlike resistance conferred by a single gene, susceptible *L. rigidum* lines may carry a single resistance gene. It is therefore critical in RAPD analysis that susceptible control lines are not carriers of resistance gene alleles.

The approach described in this chapter used the resistant R799 and the susceptible S1150 lines, created as NILs of *L. rigidum*, as the initial control lines, in combination with an increased number of RAPD primers from previous attempts to screen for resistance associated

RAPD markers. The pedigree of line S1150 was known and it is not a carrier of a single resistance gene. The study was extended to include a wider range of resistant and susceptible *L. rigidum* lines in a second round of screening to further characterise potential resistance associated RAPD markers in R799.

4.2 RESULTS

4.2.1 Initial Screen of RAPD Primers

From the RAPD reactions that gave clear reproducible band profiles, 18 bands potentially associated with resistance were identified (Round 1a, Table 4.1). In reactions with primers OPAM-10, OPAM-11, OPAM-12, OPE-08, OPE-12, OPE-13, OPE-20, OPJ-03, OPJ-06, OPJ-08, OPD-15 and OPW-08 single bands potentially associated with resistance were identified. Reactions with primers OPAM-01, OPAM-03 and OPAM-08 each gave two bands potentially associated with resistance.

The number of visible bands observed in reactions using alternative RAPD primers varied considerably. No amplification products were detected in the reaction with the primer OPJ-02. Of the remaining reactions, the number of bands ranged from as few as three easily resolved bands per sample to some producing too many bands to be readily resolved. Reactions with primers OPAM-09, OPAM-20, OPE-03, OPE-04, OPE-05 and OPE-07 produced a large number of unresolvable products. These primers were subsequently excluded as potentially useful RAPD primers.

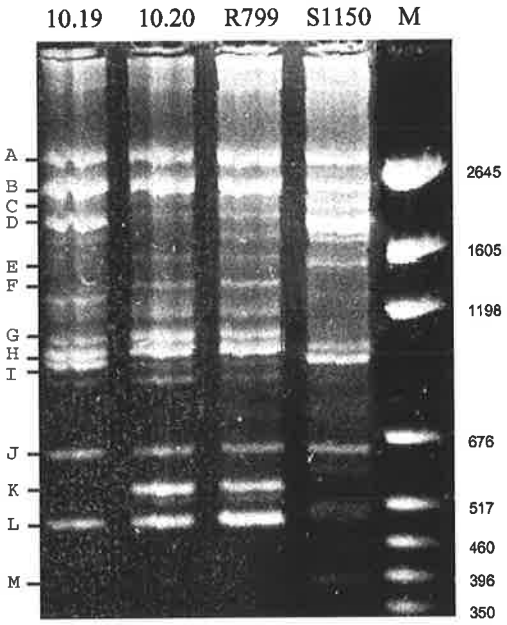
Most RAPD reactions produced polymorphic band profiles across the limited range of lines tested. Some polymorphisms were clear and easy to score, such as the band profile generated in the reaction using the primer OPE-12 (Figure 4.1). Other polymorphisms, such as those produced in reactions with the primers OPAM-9, OPAM-20 and OPE-03, appeared

Table 4.1: Summary of results of RAPD primers screened against *L. rigidum* lines

Primer	Amplification	Resolved Bands	Number of Resolved Band(s)		Control Artefacts
			Round 1a	Round 1b	
OPD-15	++	+	1	0	-
OPW-08	++	+	1	0	+
OPAM-01	++	+	2	1	+
OPAM-02	+	+	0	n/a	+
OPAM-03	++	+	2	0	+
OPAM-04	++	+	0	n/a	+
OPAM-05	+-	+	0	n/a	-
OPAM-06	+-	+	0	n/a	-
OPAM-07	+	+	0	n/a	+
OPAM-08	++	+	2	2	-
OPAM-09	+	-	0	n/a	+
OPAM-10	++	+	1	0	+
OPAM-11	++	+	1	0	+
OPAM-12	++	+	1	0	-
OPAM-13	++	+	0	n/a	+
OPAM-14	++	+	0	n/a	+
OPAM-15	+	+	0	n/a	+
OPAM-16	+	+	0	n/a	+
OPAM-17	+	+	0	n/a	+
OPAM-18	+	+	0	n/a	+
OPAM-19	++	+	0	n/a	+
OPAM-20	+++	-	0	n/a	+
OPE-01	+	+	0	n/a	+
OPE-02	+	+	0	n/a	+
OPE-03	++	-	0	n/a	+
OPE-04	++	-	0	n/a	+
OPE-05	+	-	0	n/a	+
OPE-06	+	+	0	n/a	+
OPE-07	++	-	0	n/a	+
OPE-08	++	+	1	0	+
OPE-09	+	+	0	n/a	-
OPE-10	+	+	0	n/a	+
OPE-11	+	+	0	n/a	-
OPE-12	++++	+	1	0	+
OPE-13	++	+	1	0	+
OPE-14	+	+	0	n/a	+

OPE-15	+	+	0	n/a	+
OPE-16	+	+	0	n/a	+
OPE-17	+	+	0	n/a	+
OPE-18	+	+	0	n/a	+
OPE-19	++	+	0	n/a	-
OPE-20	++	+	1	0	+
OPJ-01	+++	+	0	n/a	+
OPJ-02	--	-	0	n/a	-
OPJ-03	++	+	1	0	+
OPJ-04	+	+	0	n/a	+
OPJ-05	++	+	0	n/a	+
OPJ-06	++	+	1	0	+
OPJ-07	++	+	0	n/a	+
OPJ-08	++	+	0	n/a	-
OPJ-09	+	+	0	n/a	+
OPJ-10	+	+	0	n/a	+
OPJ-11	+	+	0	n/a	+
OPJ-12	+	+	0	n/a	+
OPJ-13	+	+	0	n/a	+
OPJ-14	+	+	0	n/a	+
OPJ-15	++	+	0	n/a	+
OPJ-16	++	+	0	n/a	+
OPJ-17	+	+	0	n/a	+
OPJ-18	+	+	0	n/a	+
OPJ-19	+	+	0	n/a	+
OPJ-20	+	+	0	n/a	+

The band profile, its resolution and resistance associated polymorphisms, was scored for each primer. Each attribute was scored as (+) present, (-) absent. Multiple scores (++) or (+-) respectively represent numbers of replicates in which consistent or inconsistent amplification was observed. Scoring for the presence or absence of artifact bands in the control reaction (no DNA template) occurred in Round 1a. n/a indicates non-assessed primers.



(a)

Band	10.19	10.20	R799	S1150
A	1	1	1	1
B	1	1	1	1
C	0	0	0	1
D	1	0	0	1
E	0	0	0	1
F	0	1	1	0
G	0	1	1	0
H	1	1	0	1
I	1	0	0	1
J	1	1	1	1
K	0	1	1	0
L	1	1	1	0
M	1	0	0	1

(b)

Figure 4.1: Example of RAPD band and corresponding vector profile generated with RAPD primer OPE-12

(a) RAPD band profiles generated with primer OPE-12. Lane M, pGEM molecular weight standards, sizes indicated in bp. Bands used for scoring to detemine genetic relatedness are indicated by letters A-M.

(b) Corresponding vector matrix used as input data for the RAPDistance software program.

ambiguous and were not useful as genetic markers (data not shown). Artifacts were detected in control reactions using 52 of the 62 RAPD primers screened. It was also observed that the band profiles generated in reactions with the RAPD primers on the lines R799 and S1150 did not display a high number of shared bands that would be expected of highly genetically related lines.

RAPD reactions that produced bands potentially associated with resistance were repeated and resolved on higher resolution agarose gels (Round 1b, Table 4.1). Of the 15 reactions with alternative primers that initially gave bands potentially associated with resistance, only 2 were repeatable. RAPD reactions with the primers OPAM-01 and OPAM-08 gave one and two bands potentially associated with resistance, respectively.

4.2.2 Secondary Screen of RAPD Primers

Reactions using primers OPAM-01 or OPAM-08 generated two bands potentially associated with resistance (Figure 4.2). However, the bands were not produced across all resistant lines and some were produced in a number of susceptible lines, thus excluding them from being tightly linked to resistance. The product OPAM-01a (Figure 4.2a) was generated from reactions with DNA extracted from resistant lines R799, Guard, R3094, 3339 and R3297 and also susceptible lines WAS, Springfield and WA 656. The product OPAM-01b (Figure 4.2a) was produced in reactions with only the resistant lines R799, R3084, R3297 and R3100. The band OPAM-08a (Figure 4.2b) was generated in RAPD PCRs from DNA of both resistant lines R799, Guard, S3094, S3100 and IR/S and susceptible line Turretfield. The RAPD product OPAM-08b (Figure 4.2b) was detected in PCR reactions with the resistant lines R799, WAR, R3094, R3100, IR/S and the susceptible lines Springfield and Waite.

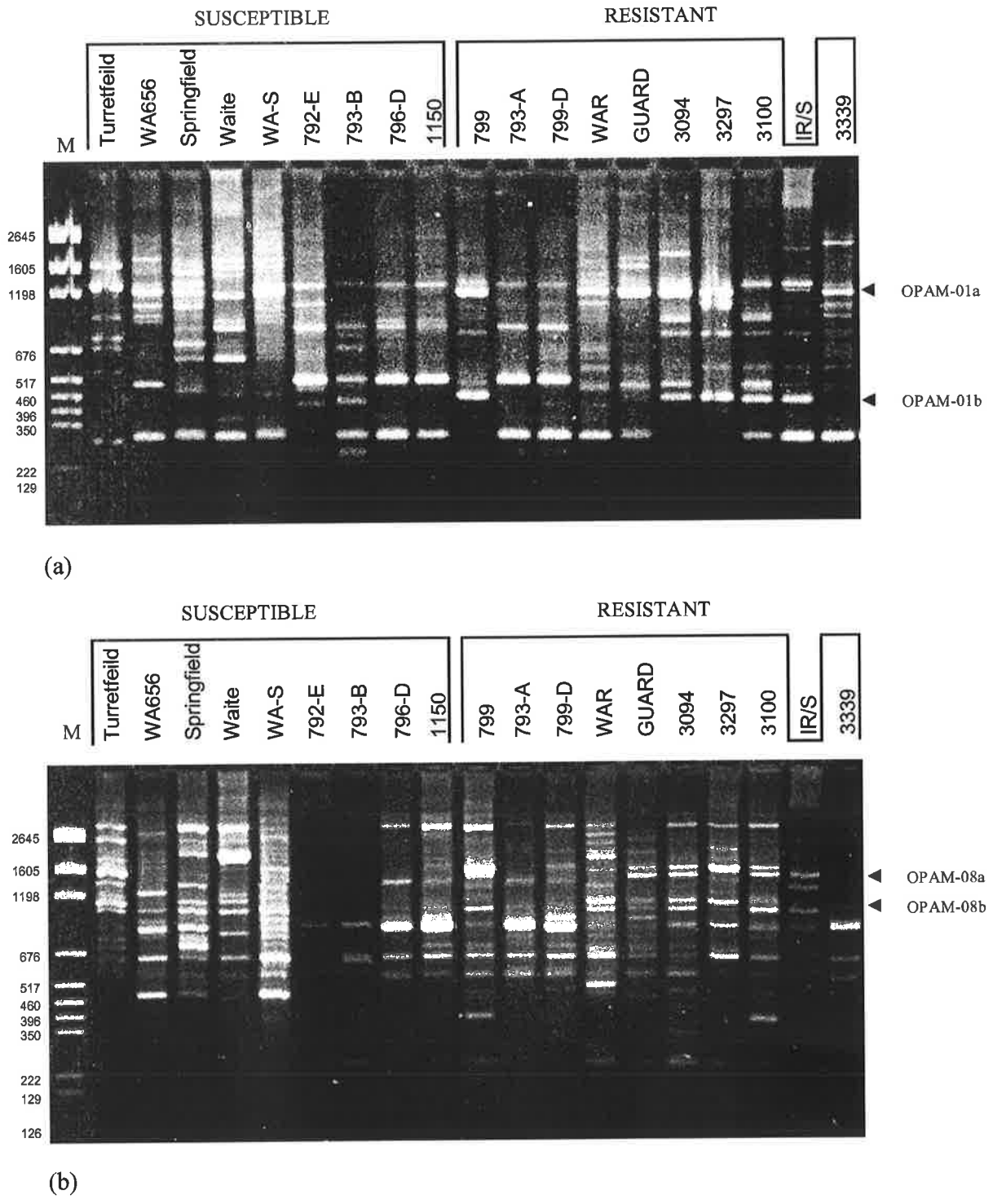


Figure 4.2: Secondary screen of RAPD band profiles generated from genomic DNA of resistant and susceptible ryegrass lines

(a) RAPD band profiles generated using primer OPAM-01. Arrows OPAM-01a and OPAM-01b indicate putative resistance associated bands identified in the primary screen. (b) RAPD band profiles generated using primer OPAM-08. Arrow heads OPAM-08a and OPAM-08b indicate putative resistance associated bands identified in the primary screen. Lane M in each gel corresponds to pGEM molecular weight standards, sizes indicated in bp. Genomic DNA isolated from *L rigidum* either resistant or susceptible to *A. funesta* as indicated or of intermediate resistance to *A. funesta* as was the case for Italian R/S (IR/S).

4.2.3 Measures of Genetic Relatedness

The selected primers OPAM-01, OPAM-03, OPAM-07, OPAM-12, OPE-06, OPE-12, OPE-13, OPD-15 and OPW-08 generated reproducible band profiles across all tested lines. An example of the banding profile generated by the primer OPE-12 and the corresponding binary matrix is presented in Figure 4.1. Based on the binary matrices, the numbers of polymorphic and monomorphic markers were calculated for each primer (Table 4.2 and Appendix B). The number of polymorphic markers ranged from 6 for OPAM-07 to 19 for OPD-15, with an average number of 11.4 polymorphic loci detected per primer. The genetic distance data were converted to an estimate of percentage similarity (Table 4.3).

Genetic similarity found between the parents R799 and S1150 calculated with the simple matching formula of Apostol *et al.* (1993) was $M_p = 23\%$ compared to $F_p = 38\%$ calculated using the formula of Nei and Li (1979). The genetic similarity between siblings 10.19 and 10.20 varied from $M_s = 51\%$ to $F_s = 58\%$ using the formulae of Apostol *et al.*(1993) and Nei and Li (1979), respectively. Siblings 10.19 and 10.20 varied in their genetic similarity to their parents R799 and S1150. The calculated genetic similarity ranged from $M_{sp} = 52\%$ between 10.19 and S1150 to $F_{sp}=70\%$ between 10.19 and R799.

4.2.4 Measurement of Minimum Distance

Based on the detection of a total of 128 products generated in reactions using 9 alternate RAPD primers (Table 4.2), the average number of products generated in each reaction in the secondary screen was 14.2. The expected minimum distance from the target locus to the closest RAPD marker for the 9 primers examined in the secondary screen varied from 82 cM for a genome size of 10500 cM to 330 cM for a genome size of 42000 cM. If the

Table 4.2: Attributes of primers used in RAPD reactions for generating RAPD markers from four individual lines; 10.19, 10.20, R799, S1150

Primer	Nucleotide sequence 5' to 3'	Number of polymorphic markers	Number of monomorphic markers
OPAM-01	TCACGTACGG	13	1
OPAM-03	CTTCCCTGTG	12	1
OPAM-07	AACCGCGGCA	6	3
OPAM-12	TCTCACCGTC	12	3
OPE-06	AAGACCCCTC	11	4
OPE-12	TTATCGCCCC	11	3
OPE-13	CCCGATTCGG	10	3
OPD-15	CATCGGTGCT	19	3
OPW-08	GACTGCCTCT	9	4
TOTAL		103	25

Table 4.3: Pairwise genetic similarity matrix for R799, S1150 and 10.19, 10.20 using 128 RAPD markers

	10.19	10.20	R799	S1150
10.19	-	58	70	61
10.20	51	-	68	61
R799	63	63	-	38
S1150	52	55	23	-

Genetic similarity (F) expressed as a percentage was derived from distances calculated with the formula of Nei and Li (1979) above the diagonal and those calculated with the simple matching formula (M) of Apostol *et al.*, (1993) below the diagonal. The distance matrix for the total data set is simply the sum of the nine single primer matrices.

assumption is made that reactions with the 62 alternate primers used in the initial screen also generated an average of 14.2 products, the MD would be reduced to 12 cM and 48 cM for 10500 cM and 42000 cM genomes, respectively.

4.3 DISCUSSION

4.3.1 Discussion Overview

Despite screening 62 primers, no markers were identified which matched the desired criteria. Possible reasons for this are discussed below. The future use of RAPD and other molecular markers as a means of identifying resistant ryegrass lines are also discussed.

4.3.2 RAPD-PCR Products Potentially Associated with Resistance Identified with Primers OPD-15 and OPW-08.

In the initial screen, single bands potentially associated with resistance were detected with the primers OPD-15 and OPW-08. Although these primers also generated products potentially associated with resistance in the previous study of McKay (1994), the generated band profiles and the sizes of the products potentially associated with resistance differed. Alterations in the RAPD amplification conditions have been shown to effect reproducibility of band profiles (Micheli *et al.,* 1994 and Skroch and Nienhus, 1995). Major alterations in conditions from those used by McKay (1994) were the use of independently isolated genomic DNA preparations as template DNA, a different concentration of RAPD primers and an alternative supplier of *Taq* polymerase. The quality of the DNA used as a RAPD-PCR template has been shown to greatly affect RAPD profile reproducibility (Micheli *et al.,* 1994). Both the presence of shorter, partially degraded genomic template and contaminants co-

precipitated with genomic DNA during ethanol precipitation may contribute to irreproducible results (Micheli *et al.,* 1994). Therefore, it may be possible that differences in the integrity of the genomic DNA from that extracted by McKay (1994) may account for differences in generated band profiles.

The potential resistance associated markers isolated in the initial screen were discounted as useful markers in the secondary screen. OPD-15 and OPW-08 failed to amplify the same resistance associated bands when screened against previously untested resistant lines of *L. rigidum*. The genetic variation detected by the presence or absence of these RAPD-PCR products was not closely linked to loci responsible for resistance to nematode infection.

4.3.3 Insufficient Primers Screened to Generate Resistance Associated Marker

The expected minimum distance from the target locus to the closest RAPD marker for the 62 primers screened was calculated to be between 12 cM and 48 cM. These calculations assume an average of 14.2 bands per primer, a genome size of between 10500 and 42000 cM and a random distribution of RAPD-derived sequences throughout the genome. The calculations do not include any variation in recombination along the length of any chromosome that may alter the exact genetic distance. Therefore, it may be possible an insufficient number of loci were screened to obtain the desired resistance marker. Due to the unpredictable nature of the number of markers generated by any given primer in any given plant species, it is difficult to establish the exact number of primers required for screening to obtain a tightly linked marker.

A number of RAPD markers mapping closely to known resistance genes in other plants have shown complete linkage to the resistance loci (Mohan *et al.,* 1994; Schachermayr *et al*, 1994; Yoshimura *et al.,* 1995 and Creusot *et al.*, 1999). While this is an ideal outcome, a number of useful markers considered to be closely linked have mapped 3.5 cM, 8 ± 2.4 cM, 1.4 cM and 5.3 cM from the resistance gene of interest (Maisonneuve *et al.* 1994; Schachermayr *et al.,* 1994; Yoshimura *et al.,* 1995 and Zhang *et al.* 1996). Using the inferred genome size range, reducing the minimum distance to 5 cM would require 86 additional primers for a genome size of 10500 cM or an additional 529 primers for a genome size of 42000 cM.

It is ~~possible~~ probable the RAPD markers may not be evenly distributed throughout the *L. rigidum* genome. Almost 60% of RAPD markers isolated in tomato by Saliba-Colombani *et al.* (2000) mapped around the centromeric regions of the tomato genome. The genetic distance between RAPD markers mapped in the blueberry varied from 3 to 30 cM (Rowland and Levi, 1994). In contrast, others such as Byrne *et al.* (1995) have reported the mapping of RAPD markers not deviating significantly from random distribution. In the case of non-random distribution of RAPD markers, it may be necessary to screen greater than the calculated additional numbers of primers to achieve a MD of 5 cM.

4.3.4 Level of Genetic Relatedness Between *L. rigidum* Lines R799 and S1150

The most striking result obtained concerned the calculated degree of genetic similarity between R799 and S1150. Theoretically, these two lines were expected to share 93.4% of their genetic material, as R799 was an F_4 backcross to S1150. Therefore, the probability of

detecting any polymorphic bands was expected to be low. In other studies involving NILs to identify RAPD markers linked to resistance, 2 of 280 primers screened on NILs of the rice *Xa-1* yielded polymorphic bands (Yoshimura *et al.*, 1995) and 3 of 395 primers screened uncovered polymophic markers between wheat *Lrq* NILs (Schachermayr *et al.*, 1994). However, the opposite was observed in *L. rigidum* putative NILs; the two lines did not share many monomorphic bands. This indicated R799 and S1150 were not as closely related as had been assumed. This had severe implications on the approach taken towards identification of resistance associated RAPD markers. The lack of true NILs as the basis for detecting resistance markers essentially invalidated this approach.

In comparison, siblings 10.19 and 10.20, assumed to share 50% of their genetic material, were found to be between 51% and 58% genetically similar. While the value for the percentage of genetic similarity was lower than the expected 85% similarity for RAPD markers, it was substantially greater than the percentage genetic similarity between R799 and S1150. Apostol *et al.*, (1993) found that in their experimental data for *Aedes aegypti* sibling similarity was 78.9%, 6% lower than the predicted 85% assuming Mendelian inheritance. In both *L. rigidum* and *A. aegypti* the calculated level of genetic similarity between siblings based on the number of shared RAPD markers was lower than the theoretically derived expected levels. The degrees of genetic similarity between the siblings and their parents (F_{sp} = 70%, 68%, 61%, 61% and M_{sp} = 63%, 63%, 52%, 55%) were also lower than the expected value of 85%.

4.3.5 Possible Underlying Causes for Lower Than Expected Levels of Similarity

It is probable that the values of the degree of genetic similarity calculated do not accurately reflect the true degree of genetic similarity. A range of factors may be contributing to this variation. The random nature of the primers used, and consequently the markers generated, may not be a true reflection of the percentage genetic similarity between the two plants. A limited set of primers was used in the analysis and this may have been non-representative of the overall levels of relatedness. Ambiguous polymorphisms may result from poor discrimination between alternative priming sites of slightly different nucleotide sequence. Scoring error, band homoplasy and the potential of bands to be inherited in a non-Mendelian manner such as chloroplast or mitochondrial sequences may also contribute to variation in similarity.

Concerns have been raised over the use of RAPD data to evaluate genetic relatedness (Perez *et al.,* 1998). As observed fragments did not relate to expected fragments in control RAPD experiments involving phage lambda DNA, the exact genetic variation uncovered by RAPD-PCR is questionable (Perez *et al.,* 1998).

4.3.6 The Future of RAPD Markers in *L. rigidum*

NILs were an essential component in the attempt to obtain resistance associated RAPD markers. In light of results indicating R799 and S1150 should not be considered to be NILs, it would not be worthwhile to pursue the current line of investigation with additional primers. Self-incompatibility in ryegrass would severely impede any attempts to obtain true NILs due to the difficulty in obtaining progeny from recurrent backcrosses. This is due to the

increasingly high level of relatedness between the progeny and the parent, in sequential backcrosses. This, in turn, increases the probability of shared self incompatibility alleles and hence decreases the chance of progeny arising.

The RAPD profiles generated in reactions with OPAM-01 or OPAM-08 for resistant lines were sufficiently unique to be used to monitor the durability of the original resistance cultivar planted in *A. funesta* infested fields. For these profiles to be used for this purpose, further investigation is required to determine the within-cultivar genetic variability of the bands generated in the OPAM-01 or OPAM-08 DNA profiles. Once established, sampling procedures that could provide useful estimates of continuing ARGT risk could be developed.

4.3.7 Alternatives to RAPD Technologies in the Search for Resistance Markers

There would be no expected advantage to employing an alternative DNA marker technology to isolate resistance-associated markers. The use of alternative DNA marker technology such as AFLPs, microsatellites and RFLPs would all encounter the same limitations as the RAPDs. This is due to their reliance on NILs as an essential component in the process of screening for markers found only in resistant lines. An alternative approach not reliant on NILs is bulk segregant analysis (Michelmore *et al.,* 1991). However, this approach is at its most powerful in plant disease systems in which resistance is controlled by a single locus. As demonstrated in Chapter 3, the mode of inheritance of resistance to nematode infection in ryegrass is likely to involve two independent complementary genes. Bulk segregant analysis also relies on homozygous F_2 populations. As ryegrass lines are incapable of producing progeny from a self-cross, homozygous F_2 populations would not be possible without the aid of double haploid technology. This technique has been attempted

unsuccessfully in *L. rigidum* with some lines used in this study (P. Davies, SARDI, pers. com.).

A more feasible line of investigation would be the candidate gene approach. This involves the cloning of resistance genes (R-genes) by the initial cloning of structurally conserved resistance gene analogues (RGAs, Leister *et al.,* 1996). A number of RGAs are linked to or are R-genes in other plant species. This targeted approach should increase the likelihood of obtaining R-genes or RGAs that are genetically linked to the actual R-gene. The results of applying this approach are described in Chapter 5.

4.5 CONCLUSIONS

Using 62 different primers on resistant and susceptible plants, about 900 RAPD loci were inspected for polymorphism. No RAPD loci were identified which were likely to be closely linked to the resistance genes. This was most likely due to an insufficient number of primers screened. Lines R799 and S1150 were found not to be NILs and hence not suitable for this application of RAPD analysis. This provides the most likely explanation for the lack of identification of appropriate resistance associated markers. The targeted resistance gene approach is the most feasible alternative approach and is investigated in Chapter 5.

Chapter 5

Resistance Gene Analogues of *L. rigidum*

5.1 INTRODUCTION

A comparison of cloned resistance genes (R-genes) from a variety of plants revealed common features shared through the deduced proteins. The majority of plant resistance genes encode a nucleotide binding site (NBS) domain and a leucine rich repeat (LRR) domain (Pan *et al.,* 2000). The conservation of particular motifs within the conserved domains allowed for the development of a PCR based strategy towards the isolation of resistance gene analogues (RGA) from other plant species (Leister *et al.,* 1996). This technique has been applied successfully in a number of monocot and dicot plant species (Kanazin *et al,* 1996, Leister *et al.,* 1996, 1998, Yu *et al.,* 1996, Aarts *et al.,* 1998, Collins *et al.,* 1998, Speulman *et al.,* 1998). Isolated RGA sequences were often found to exist in clusters, with some isolated RGAs showing linkage to known R-genes. Therefore, RGA sequences represent potential markers for resistance and potential candidate R-genes.

The aim of this study was to examine if RGA sequences were present in the *L. rigidum* genome and if so, examine their suitability as potential markers of resistance to *A. funesta*. Such an approach was more directly targeted towards the isolation of sequences known to be responsible for plant resistance compared to the more general RAPD approach described in Chapter 4.

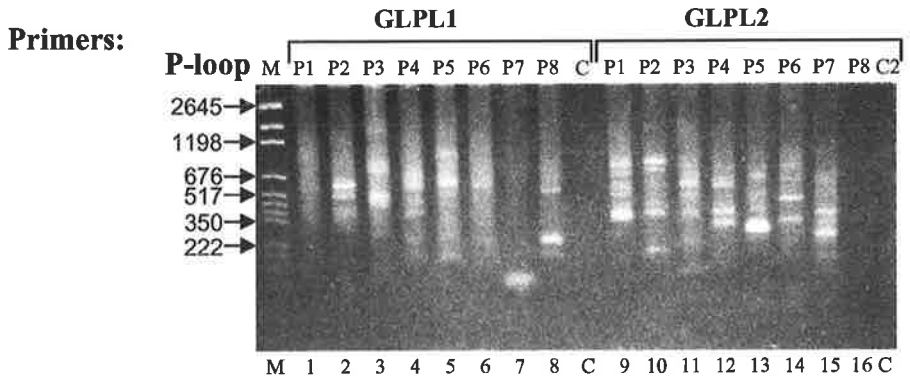
5.2 RESULTS

5.2.1 A Two Round PCR Approach Towards Amplification of 300 bp RGA PCR Products From *L. rigidum* Genomic DNA Using RGA Directed Primers

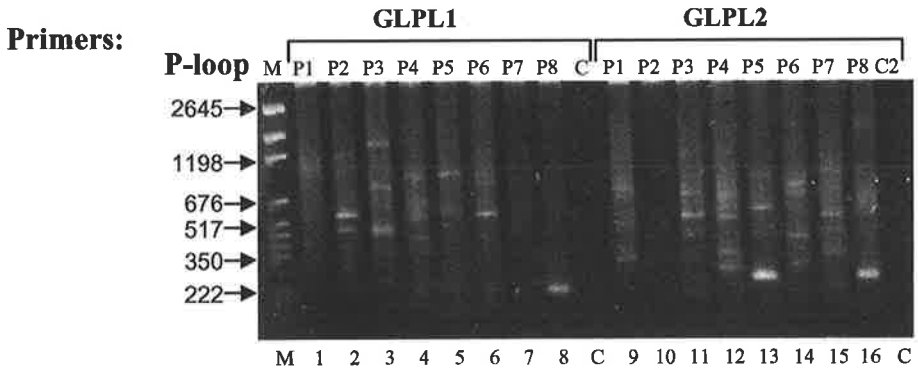
Examples of the resultant products of the first round of reactions are presented in Figure 5.1 for primers GLPL1 and GLPL2 in combination with all P-loop primers using R799 (A) and S1150 (B) genomic DNA as template. The results are reflective of the range of products generated with all 48 combinations of primers. Distinct PCR products were observed in the majority of reactions, but only 23 of the 48 combinations contained products within the predicted range of 500-650 bp when amplified from both R799 and S1150 genomic DNA (Table 5.1).

Table 5.1: Scoring of amplification of fragments of expected size for RGAs by PCR in first round PCR using combinations of P-loop and GLPLAL primers.
Each reaction was scored on the presence (+) or absence (-) of a PCR product within the size range of 500-650 bp. The scoring is presented in pairs for each primer combination and corresponds to alternative templates, R799 (left) and S1150 (right).

	P-loop Primer							
GLPLAL Primer	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
GLPL1	-/+	+/+	-/-	+/+	+/+	+/+	-/-	+/-
GLPL2	+/+	-/-	+/+	+/+	-/+	+/-	-/+	-/-
GLPL3	+/-	+/-	+/+	+/+	+/+	-/-	-/-	-/-
GLPL4	+/-	+/-	+/+	+/+	-/-	+/-	-/-	-/-
GLPL5	-/+	-/+	-/-	+/+	-/+	-/-	-/-	-/-
GLPL6	+/+	-/+	-/+	+/+	-/-	+/-	-/-	-/-



(A) Template: genomic DNA isolated from R799



(B) Template: genomic DNA isolated from S1150

Figure 5.1: First round PCR amplification products from genomic DNA of plant lines R799 and S1150. Unique combinations of primers directed towards sequence encoding P-loop and hydrophobic membrane spanning regions were used for amplification from genomic DNA template of R799 (A) and S1150 (B). Primer combinations for lanes in both agarose gels, are: 1 (GLPL1 + P1), 2 (GLPL1 + P2), 3 (GLPL1 + P3), 4 (GLPL1 + P4), 5 (GLPL1 + P5), 6 (GLPL1 + P6), 7 (GLPL1 + P7), 8 (GLPL1 + P8), 9 (GLPL2 + P1), 10 (GLPL2 + P2), 11 (GLPL2 + P3), 12 (GLPL2 + P4), 13 (GLPL2 + P5), 14 (GLPL2 + P6), 15 (GLPL2 + P7), 16 (GLPL2 + P8). The lane marked M in each gel corresponds to pGEM molecular weight standards with sizes indicated in base pairs (bp). The lanes marked C in each gel represent template-negative control PCR reactions using the primers GLPL1 and P8 (C) and GLPL2 and P8 (C2)

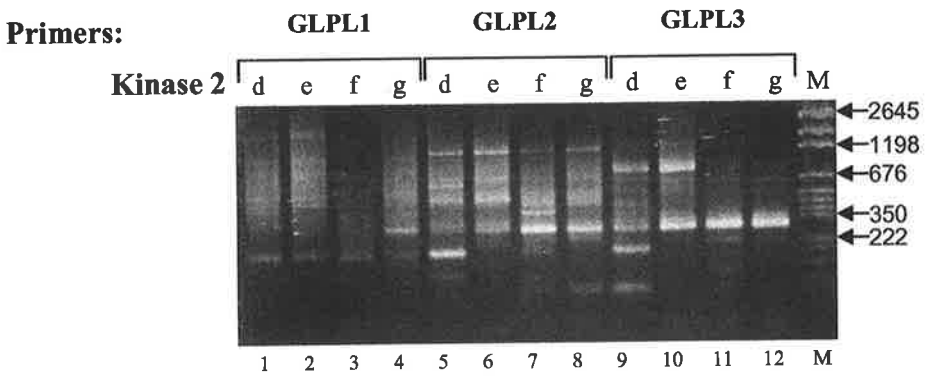
Amplification of an anticipated 300 bp product in reactions using the primer combinations of GLPL3 and K2e, f or g was observed for both plant lines (Table 5.2). This was in contrast to the absence of distinct PCR products in reactions using particular combinations of primers (e.g. GLPL1 and K2d/e with both R799 and S1150 genomic DNA) (Figure 5.2). Of the 24 alternative reactions using unique primer combinations, 17 with R799 DNA and 16 with S1150 DNA, amplified PCR products within the range predicted for RGA containing sequences. Second round PCRs using the primer GLPL3 in combination with K2e, f or g, produced the highest yield of the predicted size for a RGA PCR product.

Table 5.2: Scoring of amplification of fragments of expected size for RGAs by PCR in second round PCR using combinations of Kinase 2 and GLPLAL primers on pooled first round PCR sets. Samples of each reaction were scored on the presence (+) or absence (-) of a PCR product within the range of 290-320 bp. The scoring is presented in pairs for each primer combination, corresponding to alternative templates, R799 (left) and S1150 (right).

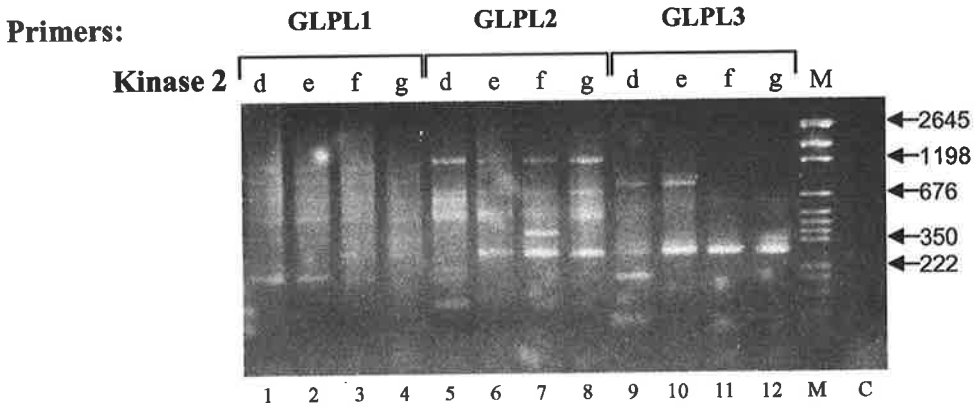
GLPLAL Primer	Kinase 2 Primer d	e	f	g
GLPL1	-/-	-/-	-/+	+/+
GLPL2	+/-	+/+	+/+	+/+
GLPL3	+/+	+/+	+/+	+/+
GLPL4	+/+	+/+	+/-	+/+
GLPL5	-/-	-/-	-/-	-/-
GLPL6	+/+	+/+	+/+	+/+

5.2.2 Cloning and Preliminary Analysis of Putative RGA PCR Products

Based on the results of previous PCR-based attempts of cloning RGAs in other plant species, it was unlikely that the observed 300 bp product in *L. rigidum* consisted of a single



(A) Template: Pooled round 1 PCR products derived from R799 genomic DNA



(B) Template: Pooled round 1 PCR products derived from S1150 genomic DNA

Figure 5.2: Second round PCR amplification products from first round templates. Unique combinations of primers directed towards sequence encoding the kinase-2 and hydrophobic membrane spanning regions were used for amplification from pooled first round PCRs derived from R799 (A) and S1150 (B). Primer combinations for lanes in both agarose gels, are: 1 (GLPL1 + K2d), 2 (GLPL1 + K2e), 3 (GLPL1 + K2f), 4 (GLPL1 + K2g), 5 (GLPL2 + K2d), 6 (GLPL2 + K2e), 7 (GLPL2 + K2f), 8 (GLPL2 + K2g), 9 (GLPL3 + K2d), 10 (GLPL3 + K2e), 11 (GLPL3 + K2f), 12 (GLPL3 + K2g). The lane marked M in each gel corresponds to pGEM molecular weight standards with sizes indicated in base pairs (bp). The lane marked C represents a template-negative control PCR reaction using primers GLPL1 and K2d.

homogenous sequence (Kanazin *et al.*, 1996; Leister *et al.*, 1996; Yu *et al.*, 1996; Collins *et al.*, 1998). The PCR product obtained by amplification of R799 derived DNA with GLPL3 and K2f primers was chosen for cloning as it yielded a distinct band of the correct size and in sufficient quantity to allow purification and subsequent cloning into the vector pGEM-T. A total of 91 clones, labelled as clones atm1 to atm91, were isolated.

Initially, clones atm1-16 were chosen for preliminary examination to confirm or reject their status as *L. rigidum* RGA clones. With the exception of atm4, all selected clones contained inserts of about 300 bp (Figure 5.3). Most clones contained inserts of the same size as atm1 (atm2, 3, 5-14) (Figure 5.3). Clone atm15 appeared to contain an insert smaller than atm1, while atm16 appeared to contain an insert relatively larger in size than atm1. Clones atm1, 15 and 16 were selected for sequencing analysis as they represented the full range of size variation observed amongst the subset of isolated clones. Further analysis of atm4 was not conducted, as it appeared unlikely to contain RGA sequences.

5.2.3 Sequence Analysis of atm1, atm15 and atm16

Sequencing of the clones atm1, atm15 and atm16 revealed inserts of 300 bp, 294 bp and 318 bp, respectively (Figure 5.4). The relative sizes were consistent with the PCR amplified inserts from the clones shown in Figure 5.3. The three clones shared between 46 to 56% sequence identity over the entire insert length. However, removal of the identical primer sequences reduced the shared identity of the genomically derived sequence to between 31% (atm15 compared to atm16) and 42% (atm1 compared to atm16).

One translation frame in each clone was found to contain a continuous open reading frame, while other frames frequently contained stop codons. The open reading frame in each

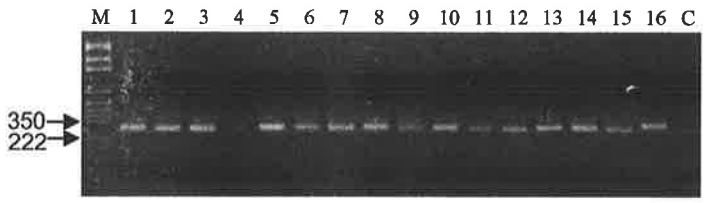
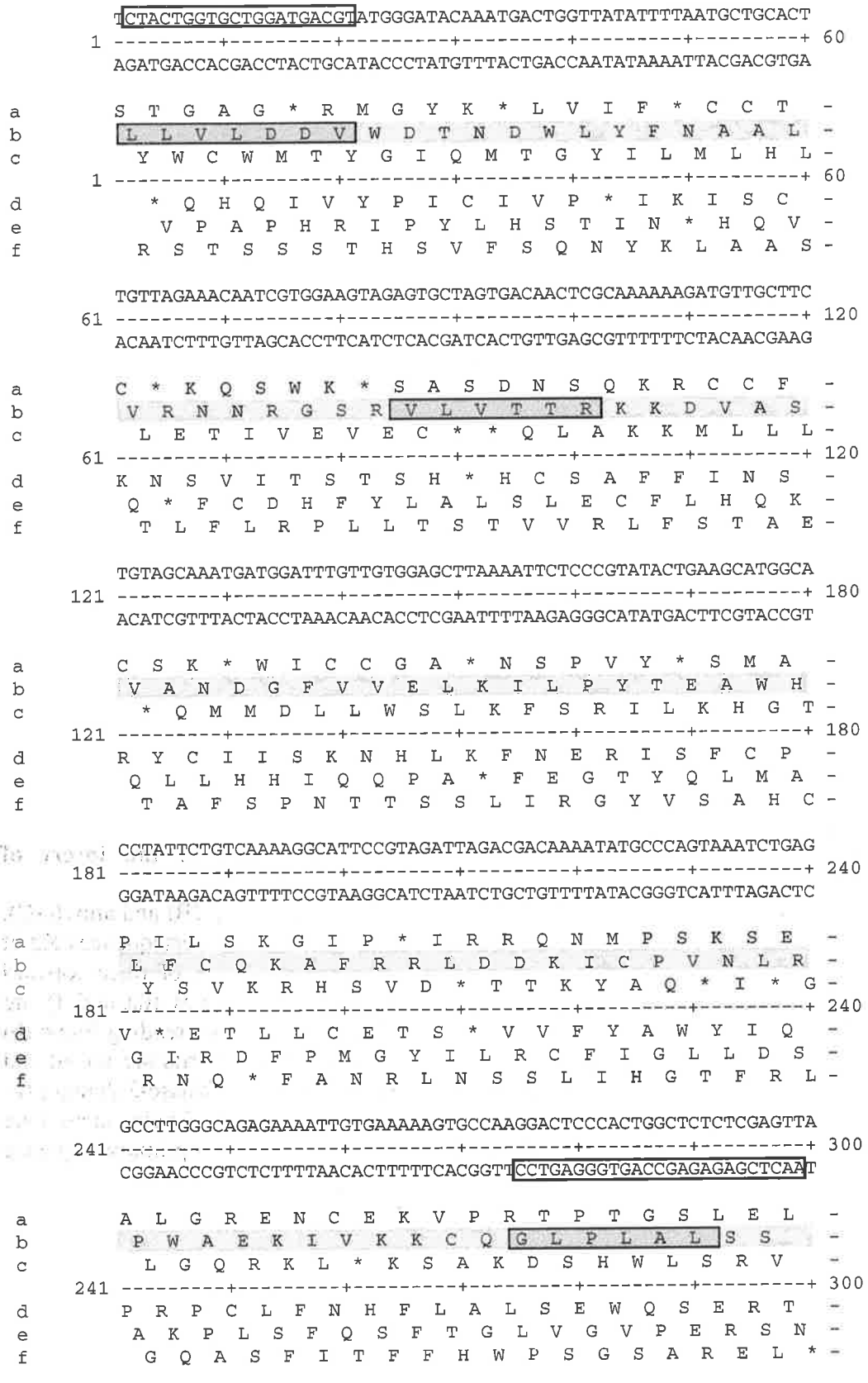


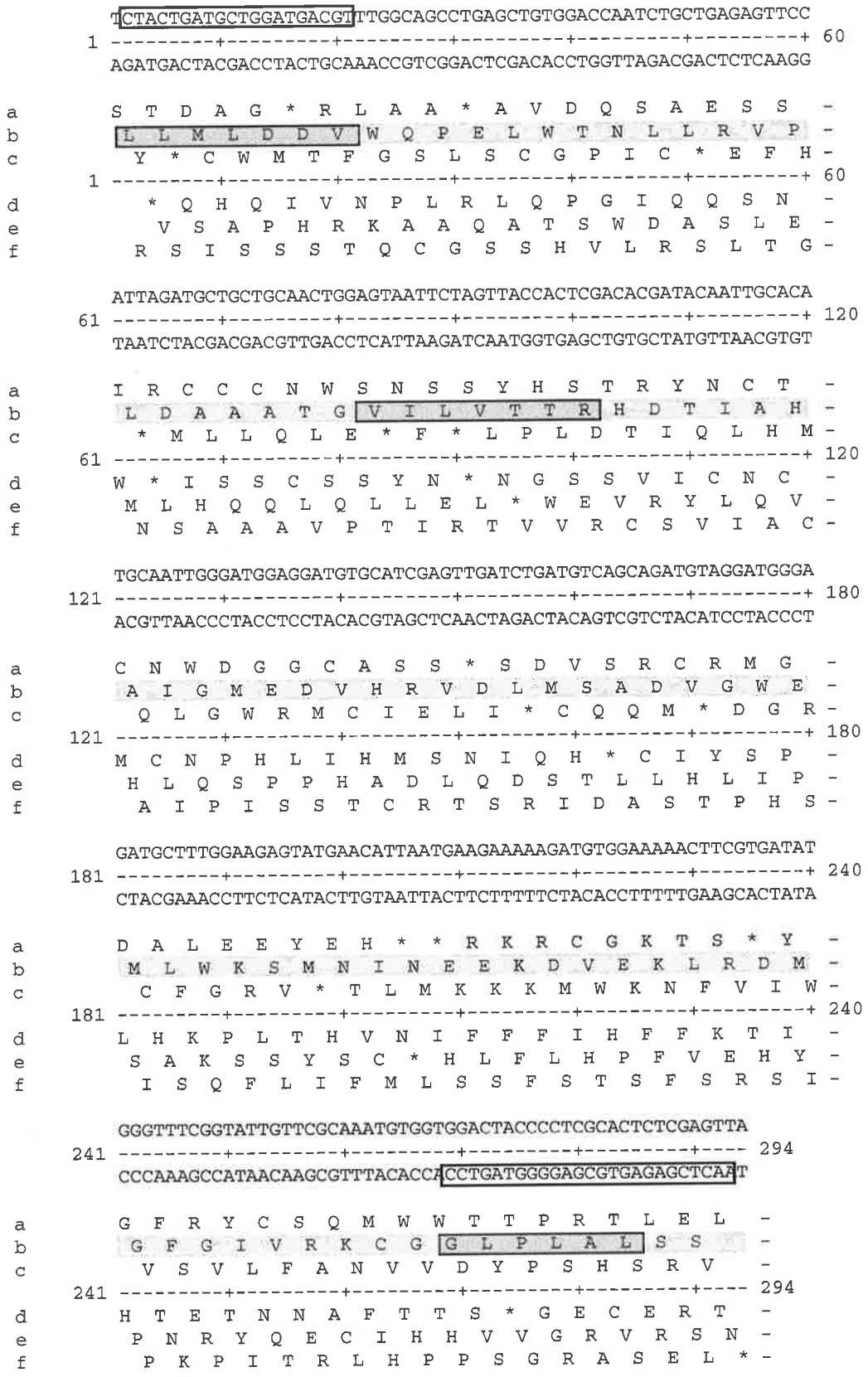
Figure 5.3: PCR amplification products from atm clones 1-16 using GLPL3 and K2f primers. Inserts contained within the clones atm1-16 were amplified using the primers GLPL3 and K2f. Lane 1 (atm1), 2 (atm2), 3 (atm3), 4 (atm4), 5 (atm5), 6 (atm6), 7 (atm7), 8 (atm 8), 9 (atm9), 10 (atm10), 11 (atm11), 12 (atm12), 13 (atm13), 14 (atm14), 15 (atm15), 16 (atm16). The lane marked M corresponds to pGEM molecular weight standards, sizes indicated in base pairs. The lane marked C represents a template-negative control PCR reaction using the primers GLPL3 and K2f.

Figure 5.4: DNA sequence and deduced translation of the insert of preliminary sequenced clones; atm1, atm15 and atm16

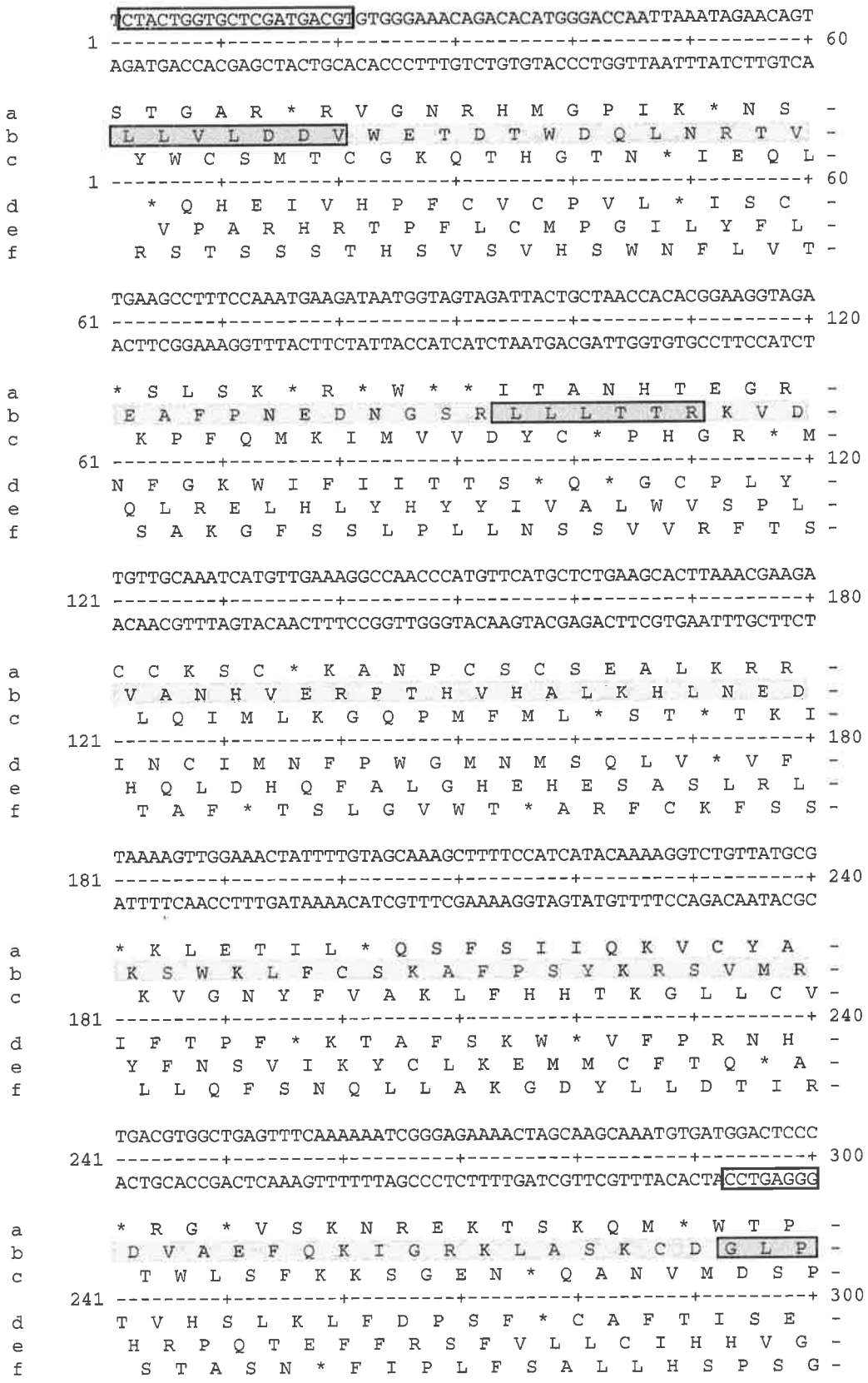
DNA sequence is presented for the inserts of atm1 (A), atm15 (B) and atm16 (C). Boxed regions within DNA sequences corresponds to the primersequences K2 at the 5' end and GLPL3 at the 3' end. Deduced translation of three forward reading frames (a, b and c) and three reverse reading frames (d,e,and f) are presented directly below the DNA sequence. A continuous open reading frame (b) is shaded gray. Within the open reading frame, conserved motifs are boxed and shaded dark gray. These correspond to the core motifs of the kinase-2 domain (L/V/L/I VLDDV) kinase-3 domain (XXXTTR) and hydrophobic membrane spanning domain (GLPLAL). The figure was constructed from primary sequence with the aid of the GCG program, 'map'.



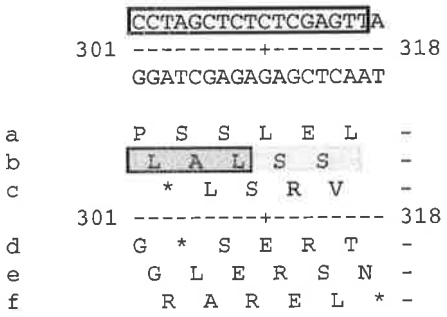
(A) DNA and deduced amino acid sequence of the insert of atm1



(B) DNA and deduced amino acid sequence of the insert of atm15



(Ci) DNA and deduced amino acid sequence of the insert of atm16



(Cii) DNA and deduced amino acid sequence of the insert of atm16 (cont.)

clone contained the primer encoded core motifs of the amino acids located in the kinase-2 domain and the hydrophobic domain. Analysis of the deduced amino acid sequence contained within the open reading frame of each clone revealed a conserved motif (6L6TTR), was located 20-23 residues downstream of the kinase-2 domain (Figure 5.5). This was consistent with both the sequence and the location of the kinase-3 domain contained in the NBS region of RGA sequences, supporting the status of the cloned sequences as RGAs.

5.2.4 Further Sequencing Analysis of atm Clones

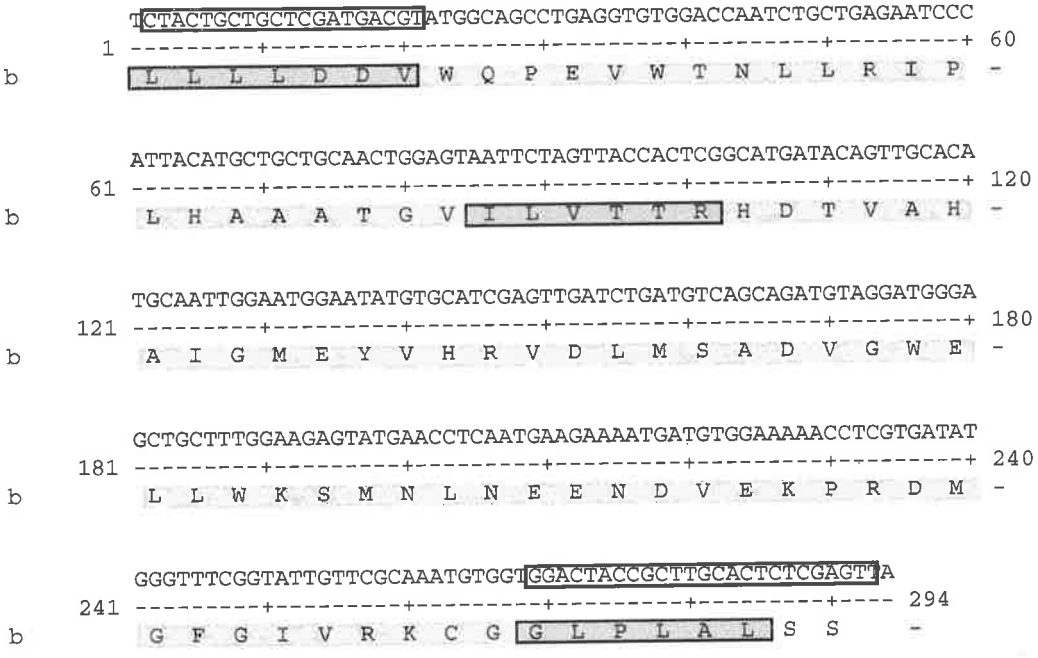
A further 19 atm clones were randomly selected for sequencing analysis (Figure 5.6). The majority of all sequenced clones (20 of 22) were at least 80% identical at the DNA level to at least one other clone (Table 5.3). The remaining two clones, atm8 and atm62, did not appear to be highly similar to any other sequenced clone. Analysis of the highly related sequences indicated all contained open reading frames of 95, 97 or 103 amino acids. Each open reading frame contained motifs corresponding to the primer encoded kinase-2 and hydrophobic domains and the genomically encoded kinase-3 domain.

The clone, atm8, did not show greater than 44% sequence identity with any of the other sequenced atm clones (Table 5.3). An open reading frame coding for 100 amino acids contained motifs of the primer encoded kinase-2, hydrophobic domains and core motifs of the genomically encoded kinase-3 domain, 22 residues downstream from the kinase-2 domain (Figure 5.6), supporting its status as an RGA sequence.

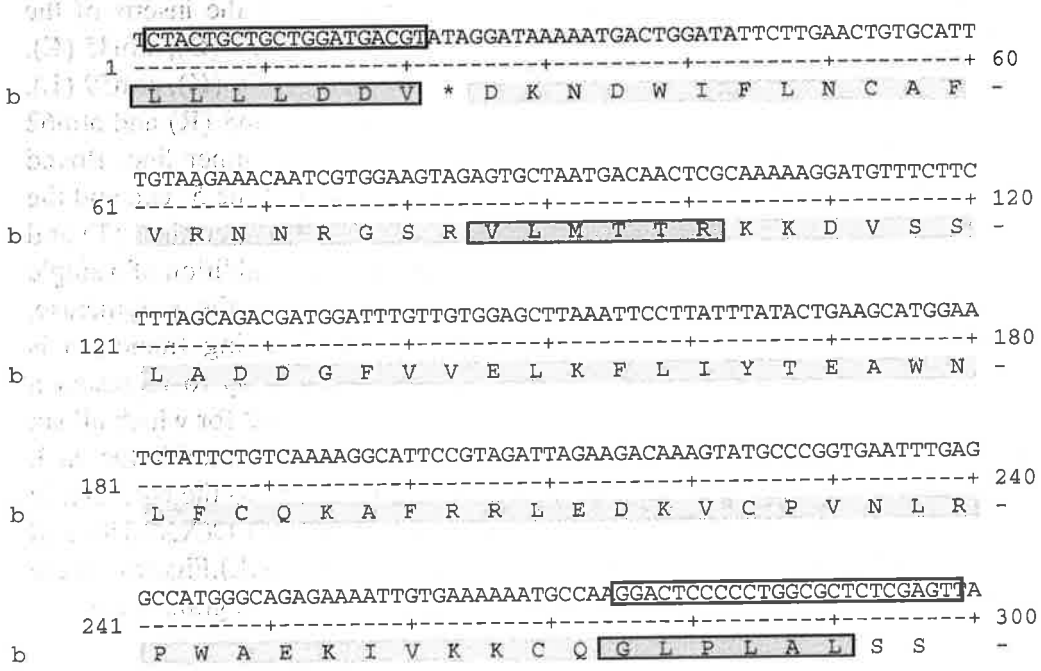
Like atm8, atm62 did not share a high degree of sequence identity with other atm clones (Table 5.3). Deduced translation in all six frames failed to identify any open reading frame larger than 43 amino acids (Figure 5.6). Analysis of all open reading frames revealed

Figure 5.5: Multiple alignment of deduced amino acid sequences of preliminary sequenced atm clones. Deduced amino acid sequences from the atm clones atm1, atm15 and atm16 were aligned using the 'PILEUP' program with the default settings of gap creation penalty at 3.0 and gap extension penalty at 0.1. Gaps represented by dashes (-) were introduced to maximise homology. The regions of homology are shown in black (100% conservation), gray (greater than 60% conservation). The conserved residues are indicated at the bottom of each alignment as a capital letter for 100% conservation, lower case for greater than 60% conservation. Numerals represent sequence similarity: 1= D/N, 2= E/Q, 3= S/T, 4= K/R, 5= F/Y/W, 6= L/I/V/M. Motifs conserved in RGAs are boxed and correspond to kinase-2, kinase-3 of the NBS and the hydrophobic domain.

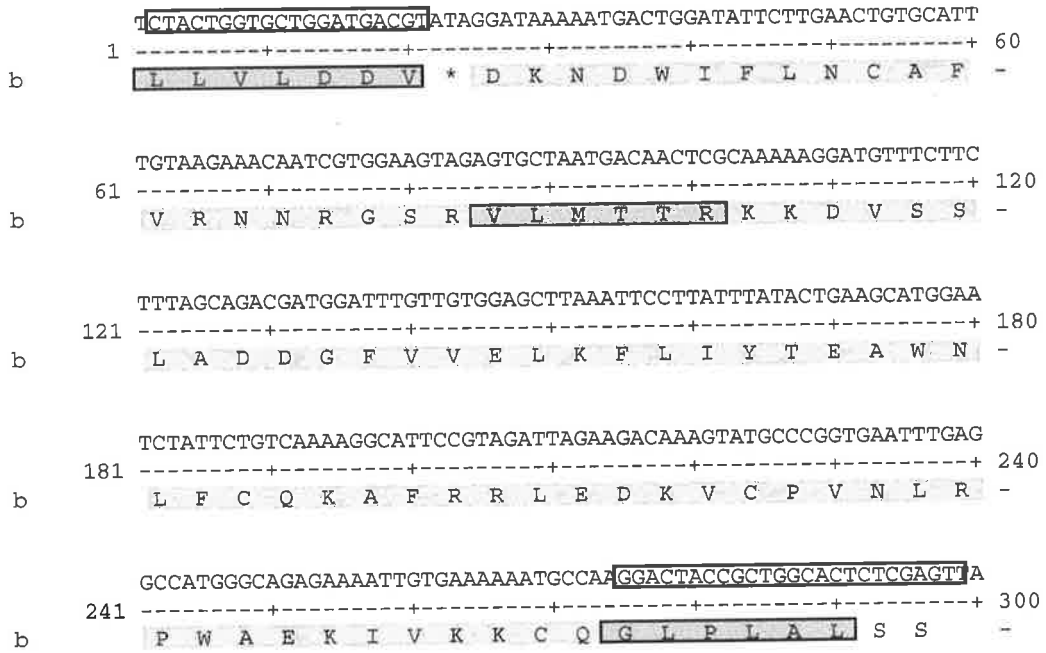
Figure 5.6: DNA and deduced amino acid sequences of selected second round
sequenced inserts of atm clones
DNA and deduced amino acid sequences are presented for the inserts of the following atm clones; atm22 (**A**), atm25 (**B**), atm28 (**C**), atm32 (**D**), atm35 (**E**), atm36 (**F**), atm39 (**G**), atm44 (**H**), atm53 (**I**), atm55 (**J**), atm56 (**K**), atm59 (**L**), atm67 (**M**), atm68 (**N**), atm79 (**O**), atm7 (**P**), atm84 (**Q**), atm8 (**R**) and atm62 (**S**) The DNA sequence of each insert is presented on the upper line. Boxed regions correspond to primer sequences for the K2 primer at the 5' end and the complementary sequence to the GLPL primer at the 3' end. Terminal (T) and (A) sequences are an artifact of the non-template dependent addition of a single deoxyadenosine (A) to the 3' ends of PCR products by the polymerase. Deduced amino acid sequence of the second forward reading frame (b) is presented directly below the DNA sequence and shaded gray to represent a continuous open reading frame for all sequences except atm62 for which all six possible reading frames (a-f) are shown. Three conserved motifs for each sequence are boxed and shaded dark gray. These correspond to the core motifs of the kinase-2 domain (L/ V/L/I VLDDV) kinase-3 domain (XXXTTR) and the hydrophobic membrane spanning domain (GLPLAL).Figures were constructed from primary sequence with the aid of the GCG program,'map'.



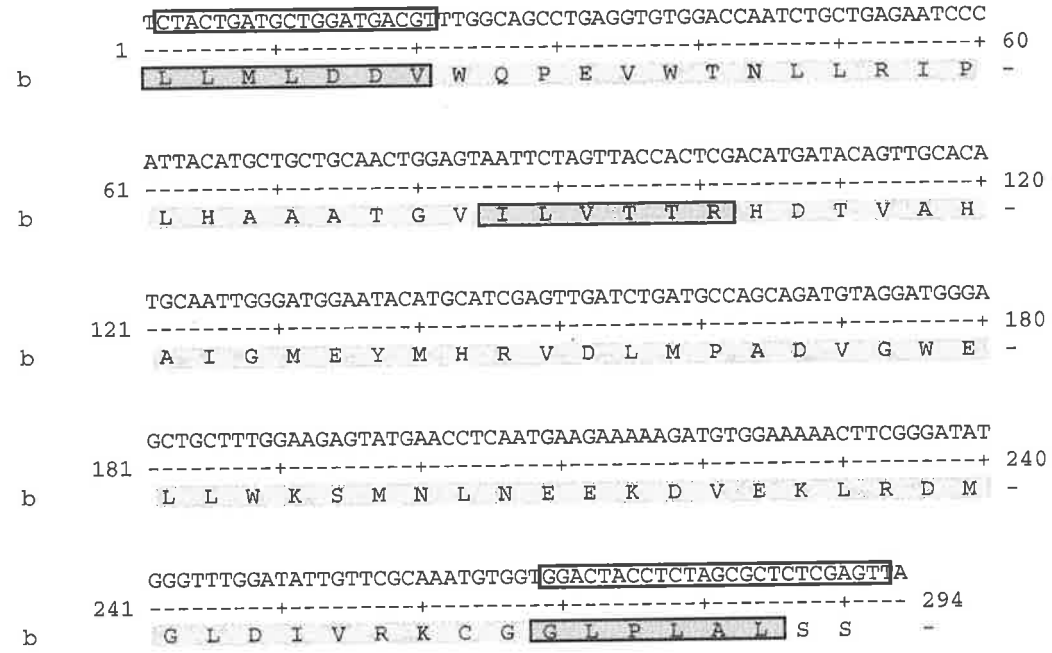
(A) DNA and deduced amino acid sequence of the insert of atm22



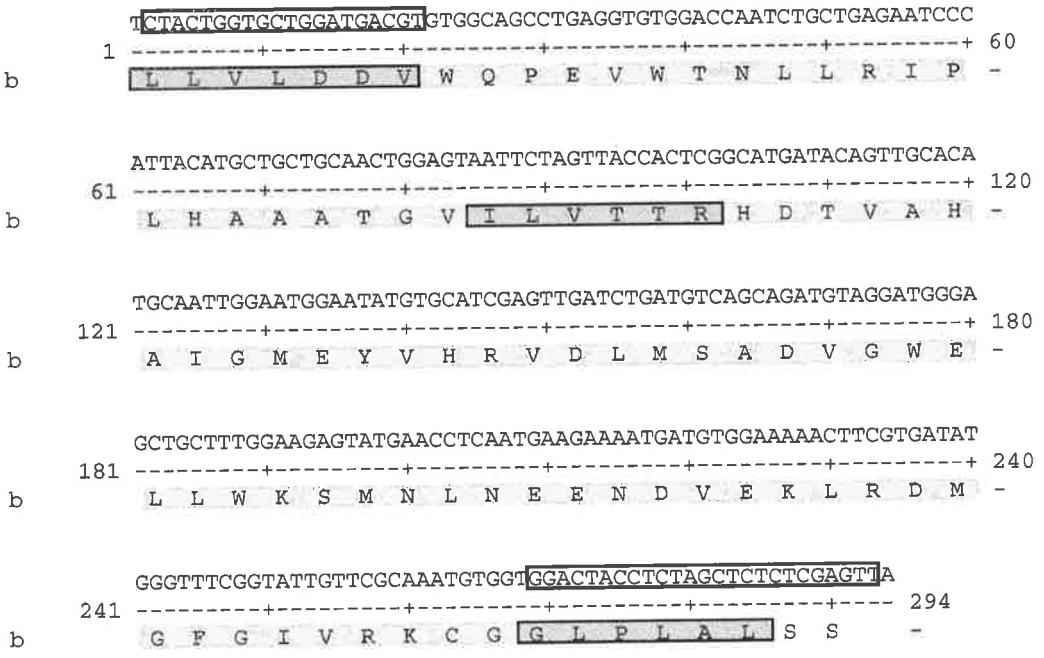
(B) DNA and deduced amino acid sequence of the insert of atm25



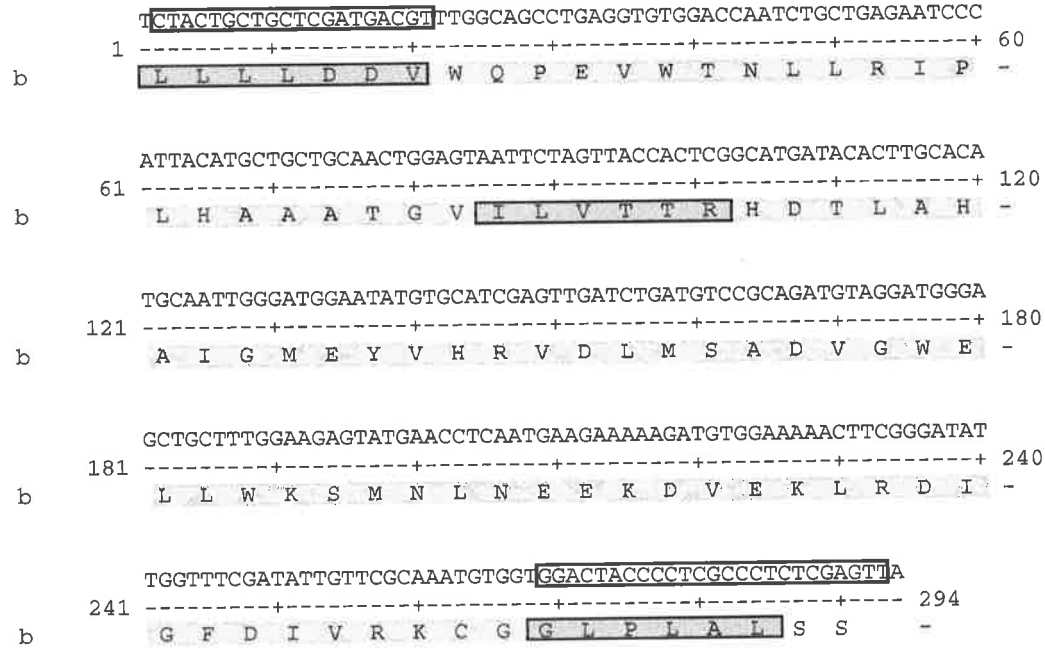
(C) DNA and deduced amino acid sequence of the insert of atm28



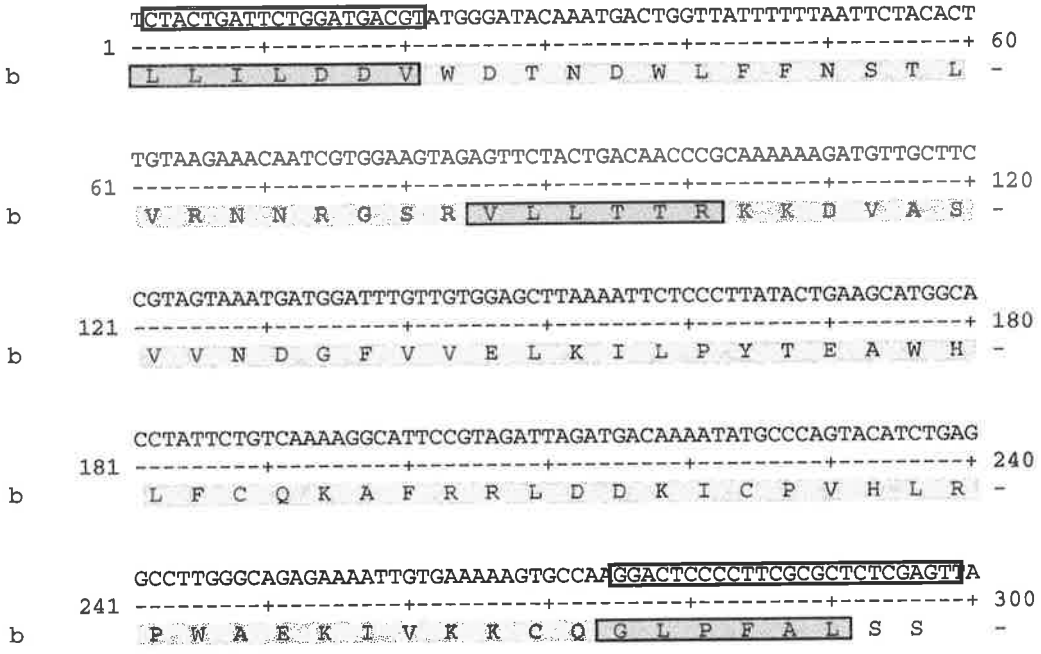
(D) DNA and deduced amino acid sequence of the insert of atm32



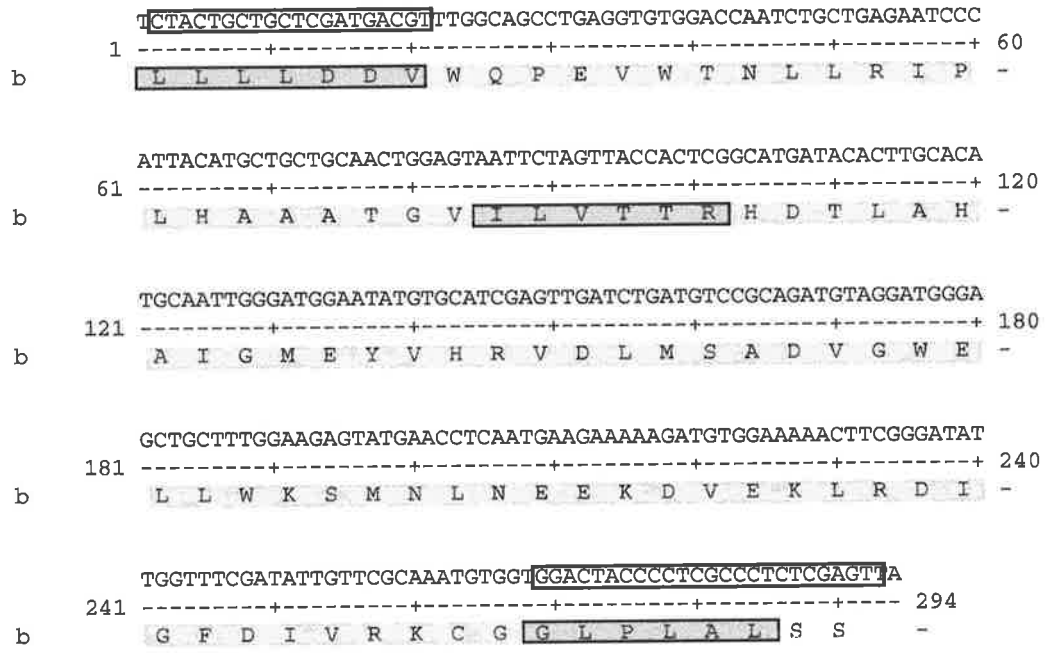
(E) DNA and deduced amino acid sequence of the insert of atm35



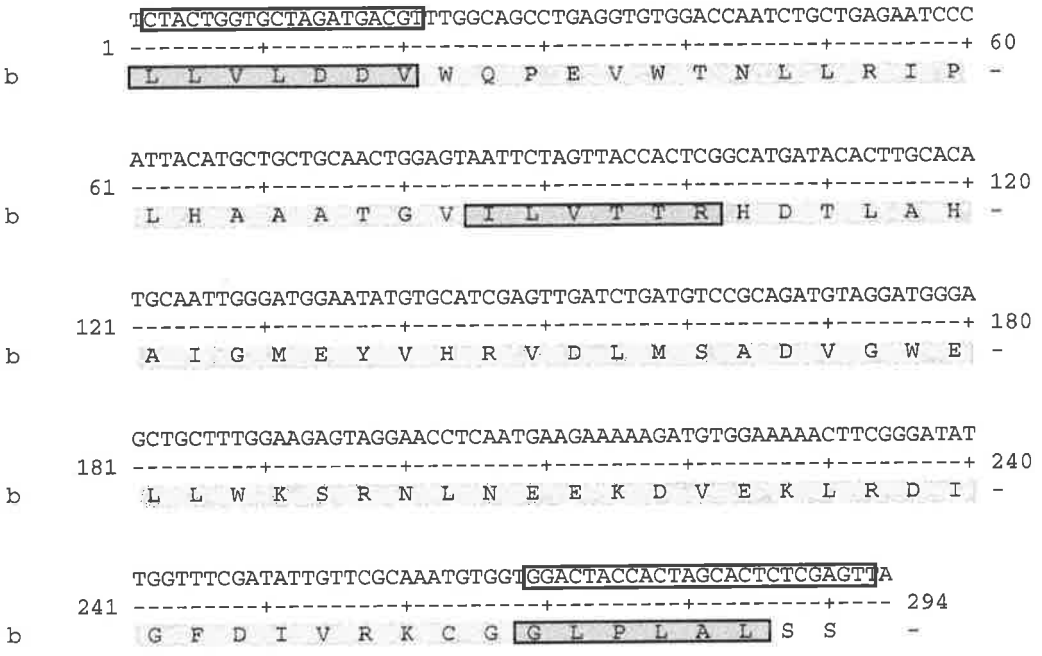
(F) DNA and deduced amino acid sequence of the insert of atm36



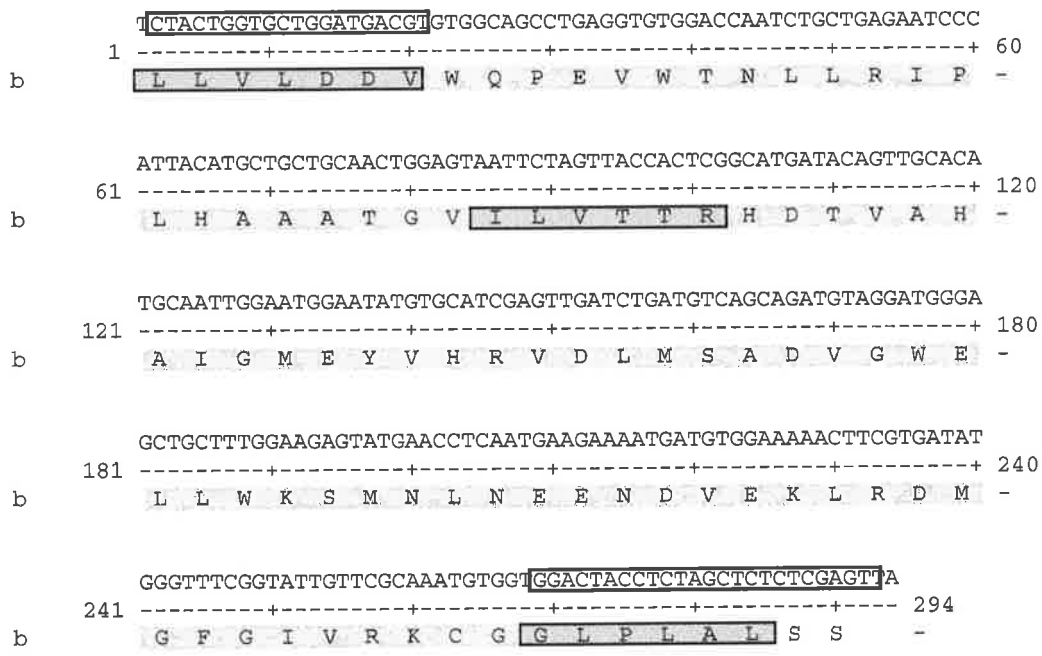
(G) DNA and deduced amino acid sequence of the insert of atm39



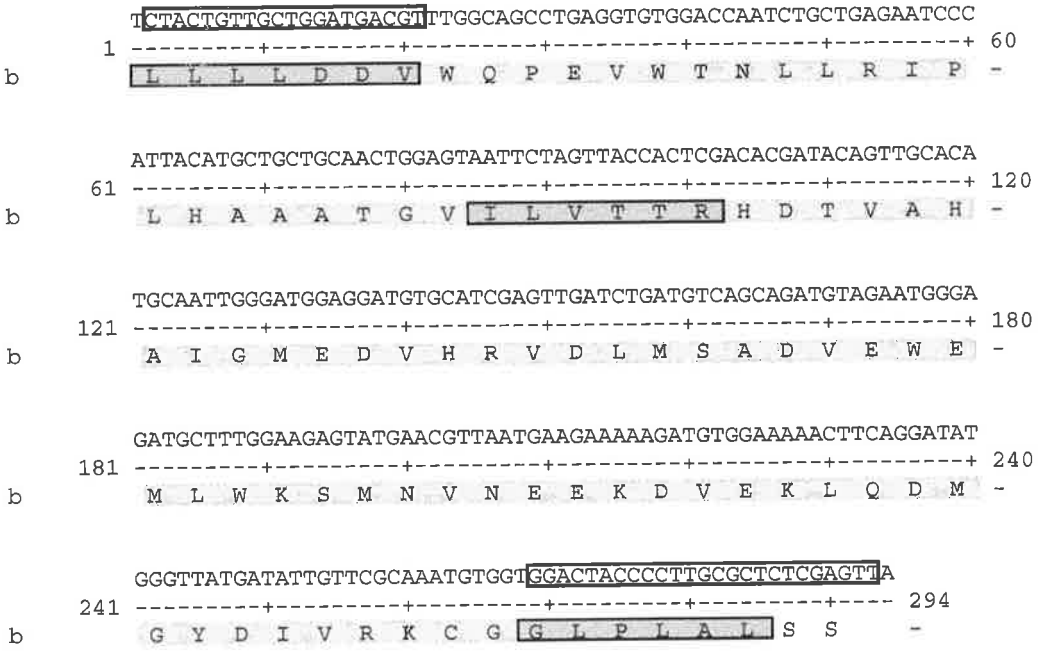
(H) DNA and deduced amino acid sequence of the insert of atm44



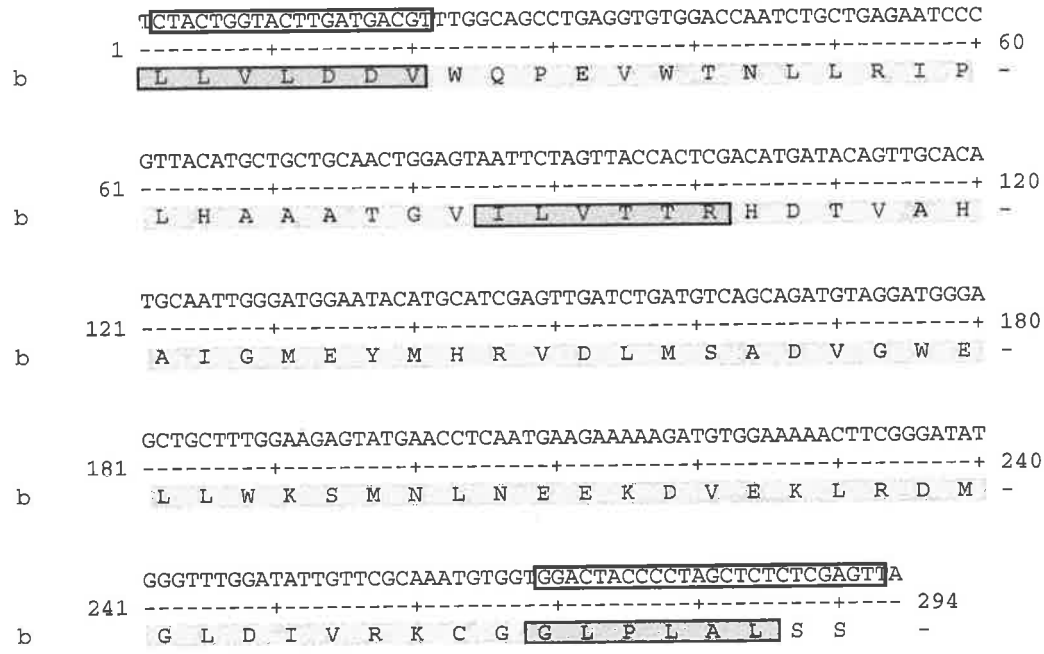
(I) DNA and deduced amino acid sequence of the insert of atm53



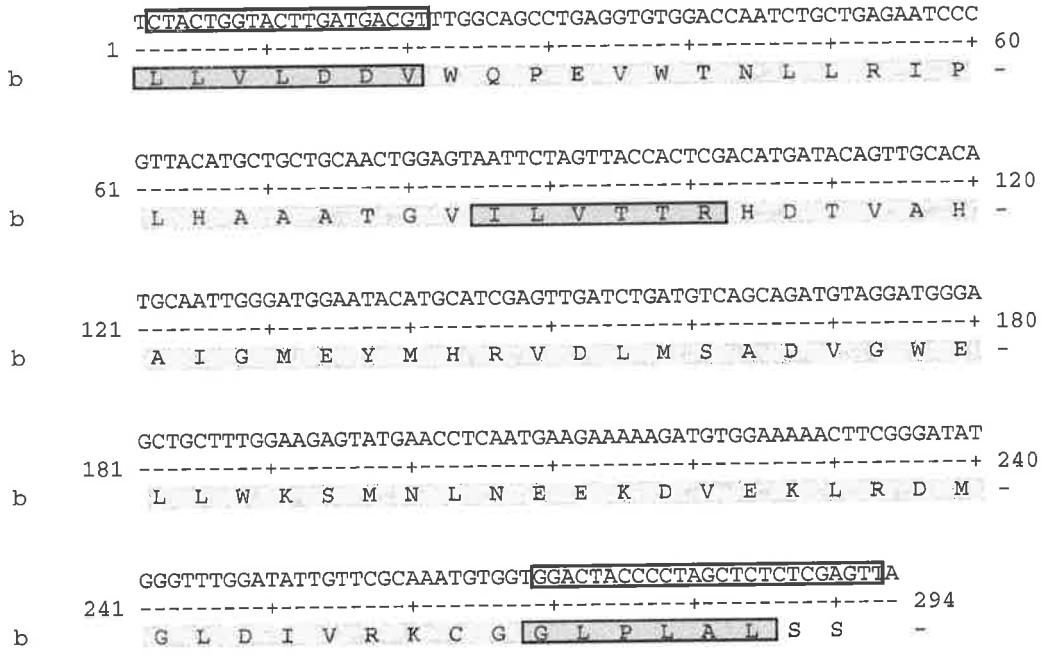
(J) DNA and deduced amino acid sequence of the insert of atm55



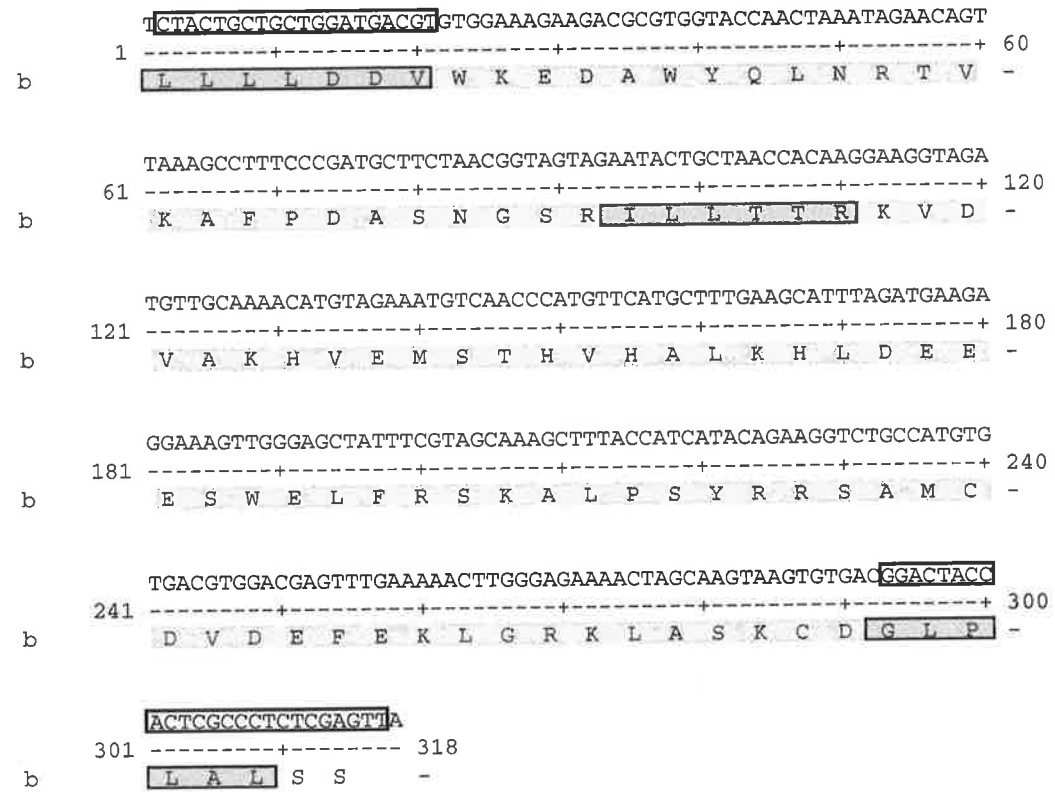
(K) DNA and deduced amino acid sequence of the insert of atm56



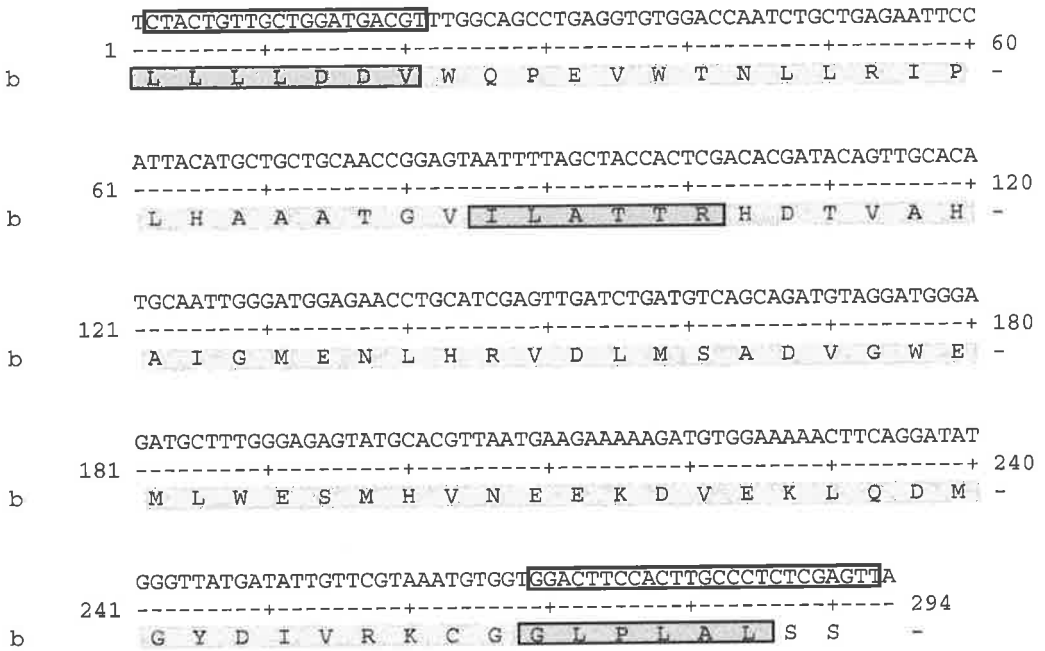
(L) DNA and deduced amino acid sequence of the insert of atm59



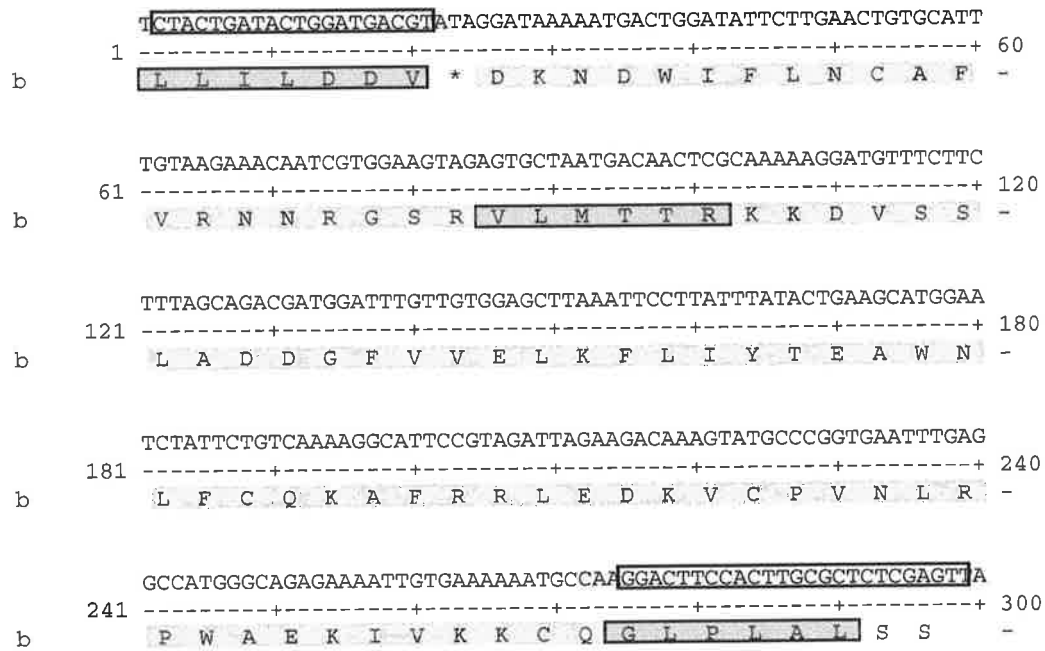
(M) DNA and deduced amino acid sequence of the insert of atm67



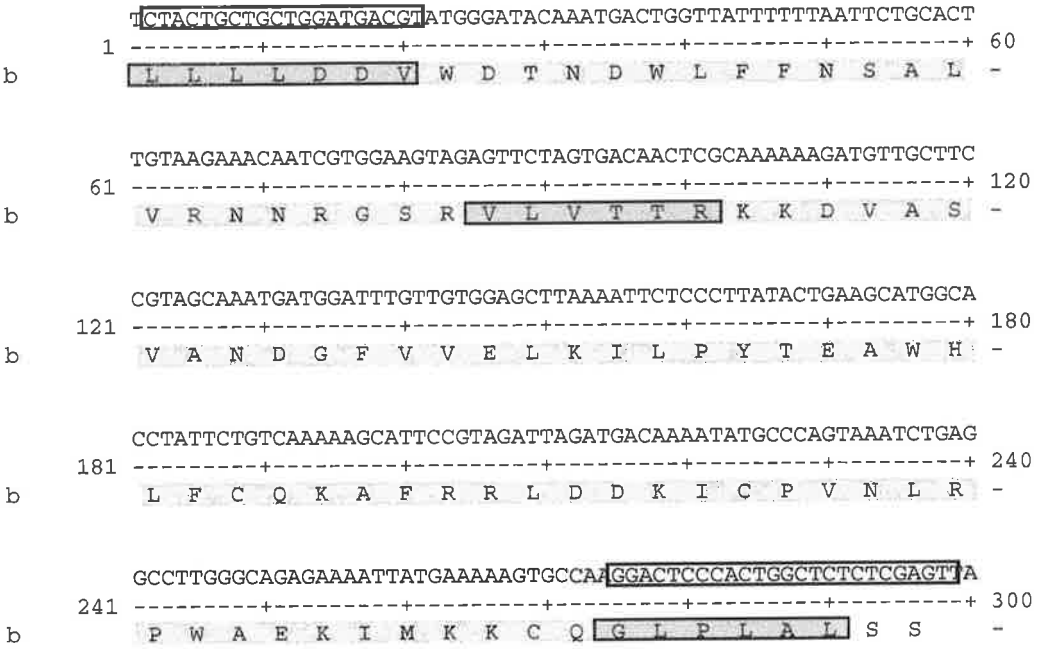
(N) DNA and deduced amino acid sequence of the insert of atm68



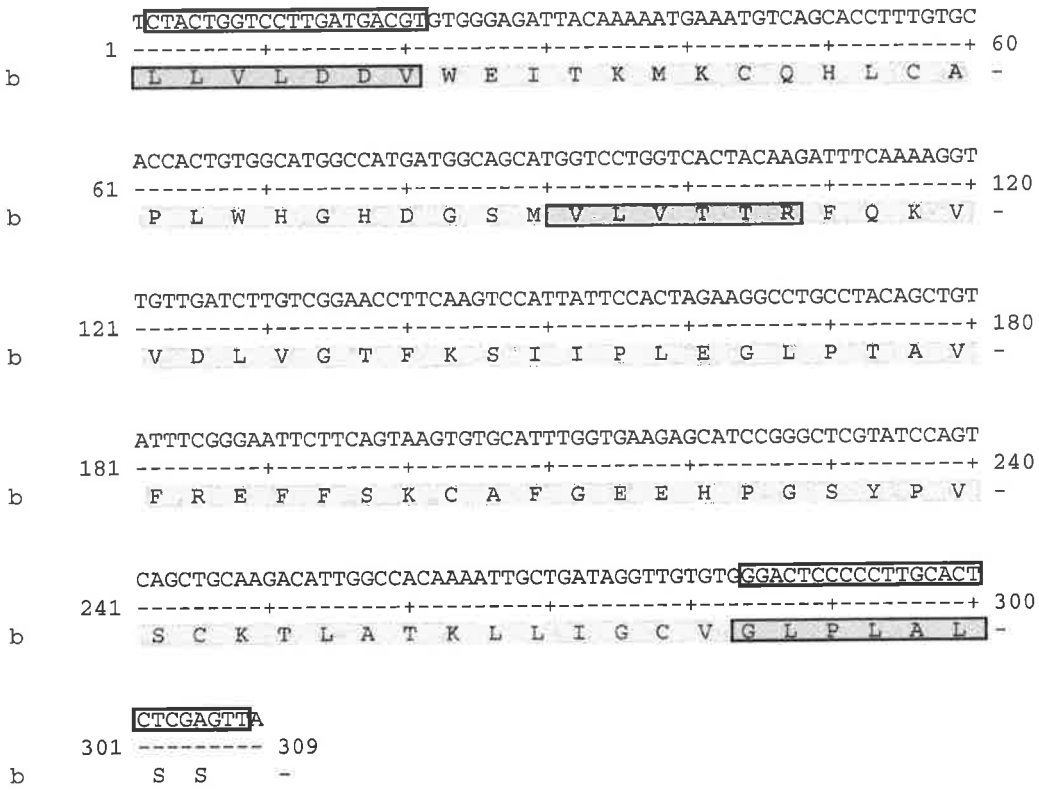
(O) DNA and deduced amino acid sequence of the insert of atm79



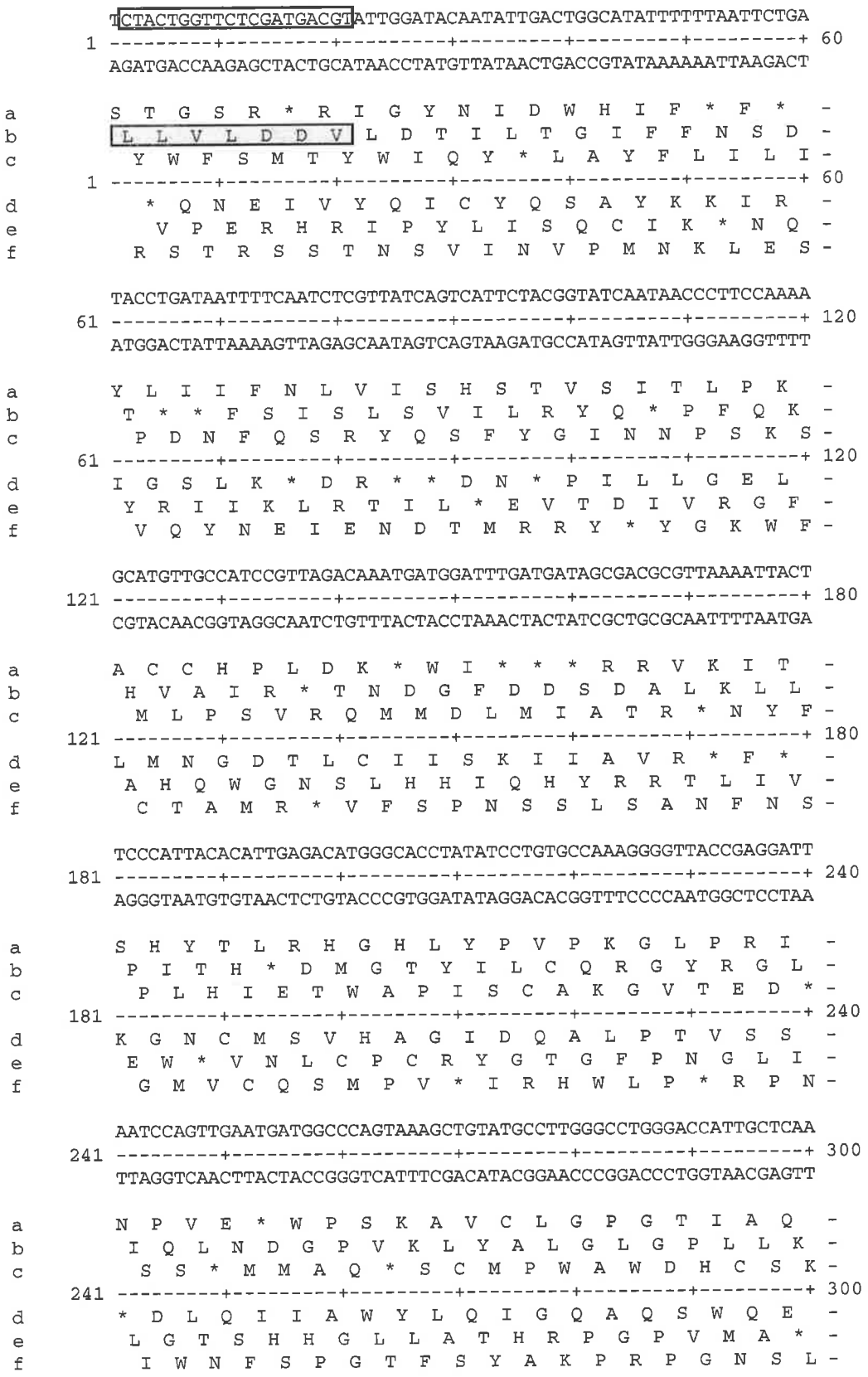
(P) DNA and deduced amino acid sequence of the insert of atm7



(Q) DNA and deduced amino acid sequence of the insert of atm84



(R) DNA and deduced amino acid sequence of the insert of atm8



(S(i)) DNA and deduced amino acid sequence of the insert of atm62

(S(ii)) DNA and deduced amino acid sequence of the insert of atm62(cont.)

Table 5.3: Shared identity between DNA sequence of inserts of atm clones.
Values shown represent the percent identity of the genomically derived sequences contained within the atm clones listed at the top and left hand side of the table. Sequence comparisons were madeusing the GCG program 'dnadistances'. Boxes shaded black represent 100% identity, dark gray represent 90-99% identity and light gray 80-89% identity.

	59	67	32	35	36	44	53	22	55	56	79	15	16	68	25	28	7	39	84	1	8	62
59																						
67																						
32	99	99																				
35	98	98	98																			
36	97	97	97	96																		
44	97	97	97	96																		
53	96	96	97	96	99	99																
22	96	96	96	96	96	96	96															
55	96	96	96	96	97	97	96	99														
56	95	95	96	94	94	94	94	93	94													
79	93	93	95	92	91	91	91	90	90	96												
15	93	93	93	94	94	94	93	94	94	95	92											
16	31	31	31	30	32	32	32	31	31	31	30	31										
68	31	31	31	30	32	32	32	31	31	31	31	31	83									
25	31	31	31	32	32	32	32	32	32	31	30	31	43	40								
28	31	31	31	32	32	32	32	31	32	31	30	31	43	40								
7	31	31	31	32	32	32	32	31	32	31	30	31	43	40								
39	35	35	31	35	35	35	35	35	35	34	33	34	43	41	87	87	87					
84	34	34	35	34	34	34	34	34	34	33	32	33	42	40	87	87	87	97				
1	34	34	35	35	35	35	35	34	35	34	33	34	42	40	87	87	87	95	96			
8	31	31	31	31	31	31	30	30	30	31	32	31	36	36	44	44	44	44	44	44		
62	27	27	28	28	28	28	27	29	28	28	29	29	25	24	30	30	30	30	30	31	24	

that motifs encoded by primers were present but motifs corresponding to the kinase-3 domain were absent. Therefore, atm62 appeared not to contain RGA sequences.

5.2.5 Deduced Amino Acid Sequence Conservation in *L. rigidum* Between RGA Sequences

As can be been in Figure 5.7, the most striking region of similarity between all atm RGA clones was the kinase-3 domain (residues 31-36 in atm8). There were no continuous regions of conservation greater than 6 amino acids. The region immediately preceeding the membrane spanning domain residues (residues 89-93 in atm8) also contained a conserved aliphatic amino acid (L/I/V/M) (residue 89) and an invariant Cysteine (residue 93). Aliphatic amino acids (L/I/V/M) at residues 40, 44, 50, 53, 56 (in atm8) were conserved in all deduced *L. rigidum* RGA sequences.

A phylogenetic tree was constructed to examine the relationship between the deduced amino acid sequences of genomically derived atm sequences (Figure 5.8). Four distinct RGA classes (1 to 4) appeared to be present among the 21 isolated RGA sequences. Classification of atm clones into class members corresponded to clones sharing high levels of DNA sequence similarity (Table 5.3).

5.2.6 Deduced Amino Acid Similarity Within *L. rigidum* RGA Classes

Due to the diversity of deduced amino acid sequence of the isolated RGA sequences, multiple sequence alignments were carried out on members within the classes identified by amino acid sequence analysis (Figure 5.9). The majority of the residues were conserved

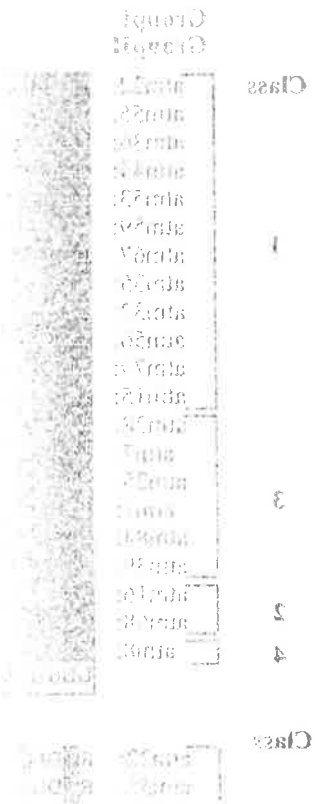
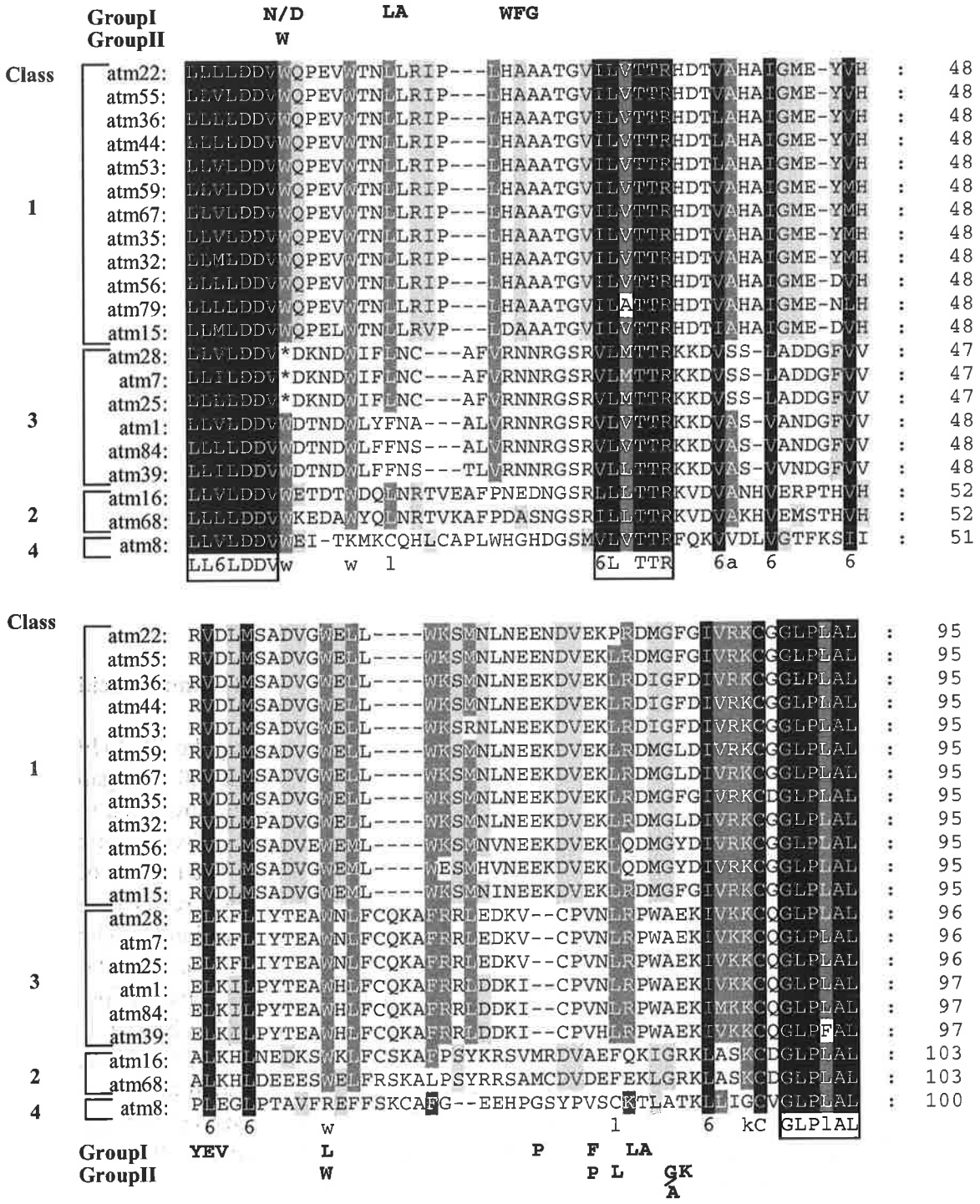


Figure 5.7: Multiple sequence alignment of deduced amino acid sequences of RGA containing atm clones.

Sequences of RGA containing atm clones were aligned using the PILEUP program with the default settings of gap creation penalty at 3.0 and gap extension penalty at 0.1. Gaps represented by dashes (-) were introduced to maximise homology. Classification of each clone based on phylogenetic analysis (Figure 5.8) is presented on the left of the alignment. The regions of homology are shown in black (100% conservation), dark gray (greater than 80% conservation), and light gray (60% to 80% conservation). The conserved residues are indicated at the bottom of each alignment as a capital letter for 100% conservation, lower case for greater than 80% conservation. Stop codons in deduced protein are represented by an asterix (*). Numerals represent sequence similarity: 1= D/N, 2= E/Q, 3= S/T, 4= K/R, 5= F/Y/W, 6= L/I/V/M. Motifs conserved in RGAs are boxed and correspond to Kinase 2, Kinase 3 of the NBS and the Hydrophobic Domain. Conserved residues identified by Pan *et al.,* (2000) in Group I and Group II RGAs are shown in bold above and below the alignment.


GroupI
GroupII
N/D
W
LA
WFG
Class
atm22:
atm55:
atm36:
atm44:
atm53:
atm59:
atm67:
atm35:
atm32:
atm56:
atm79:
atm15:
atm28:
atm7:
atm25:
atm1:
atm84:
atm39:
atm16:
atm68:
atm8:
1
3
2
4
LL6LDDVw w 1 6L TTR 6a 6 6
GroupI YEV L P F LA
GroupII W P L GK A

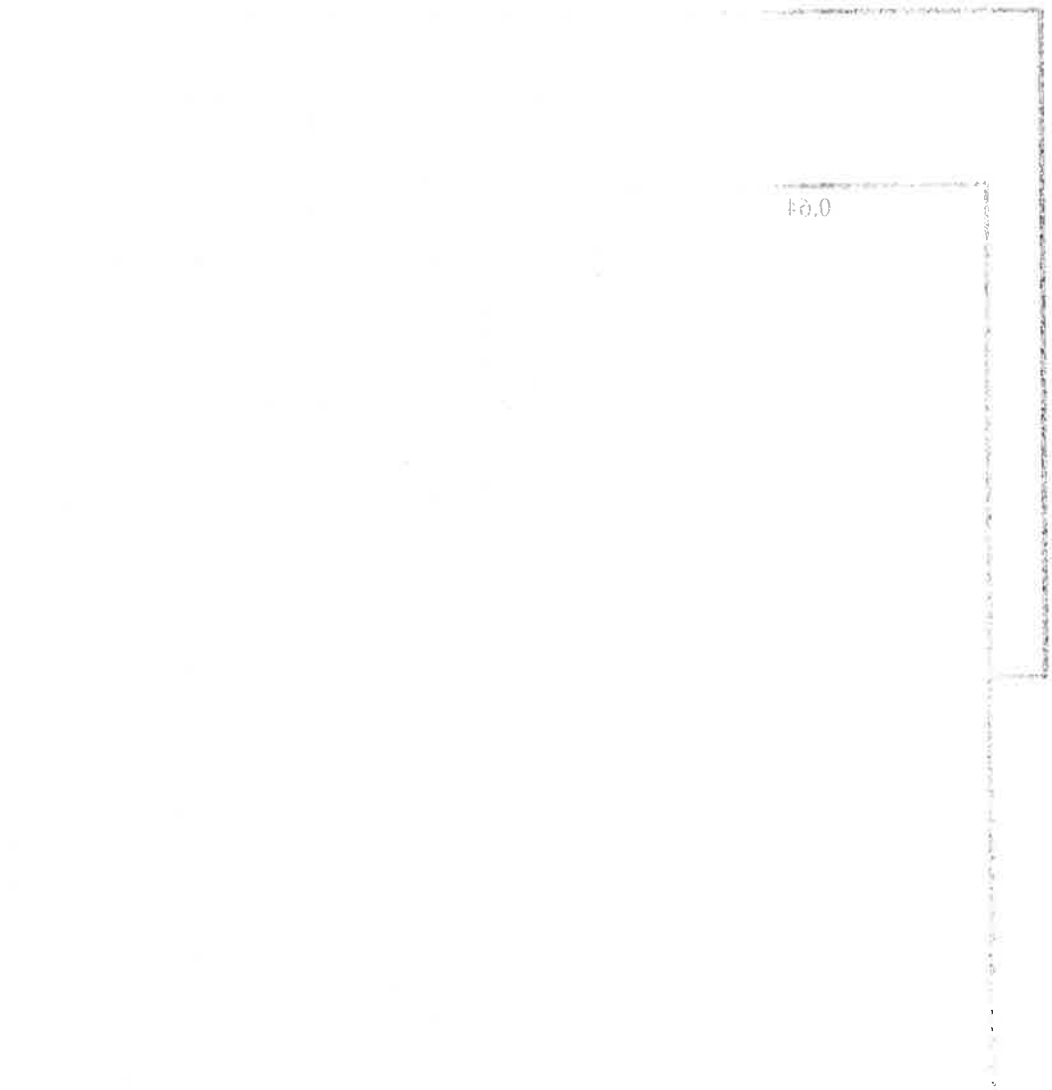


Figure 5.8: Phylogenetic tree based on the deduced amino acid sequence of atm clones

Deduced amino acid sequences from the genomically derived DNA sequences of all RGA containing clones were aligned together with the corresponding region of the human *Apaf*l gene using the GCG program 'PILEUP'. The resultant alignment was analysed using the neighbor-joining method of the GCG program 'eneighbor' to produce the phylogenetic tree. Numbers below the lines indicate the branch length. Lines without a corresponding branch length have an effective branch length of (0). Numbers to the right of the tree indicate ryegrass RGA class to which the atm clones belong.

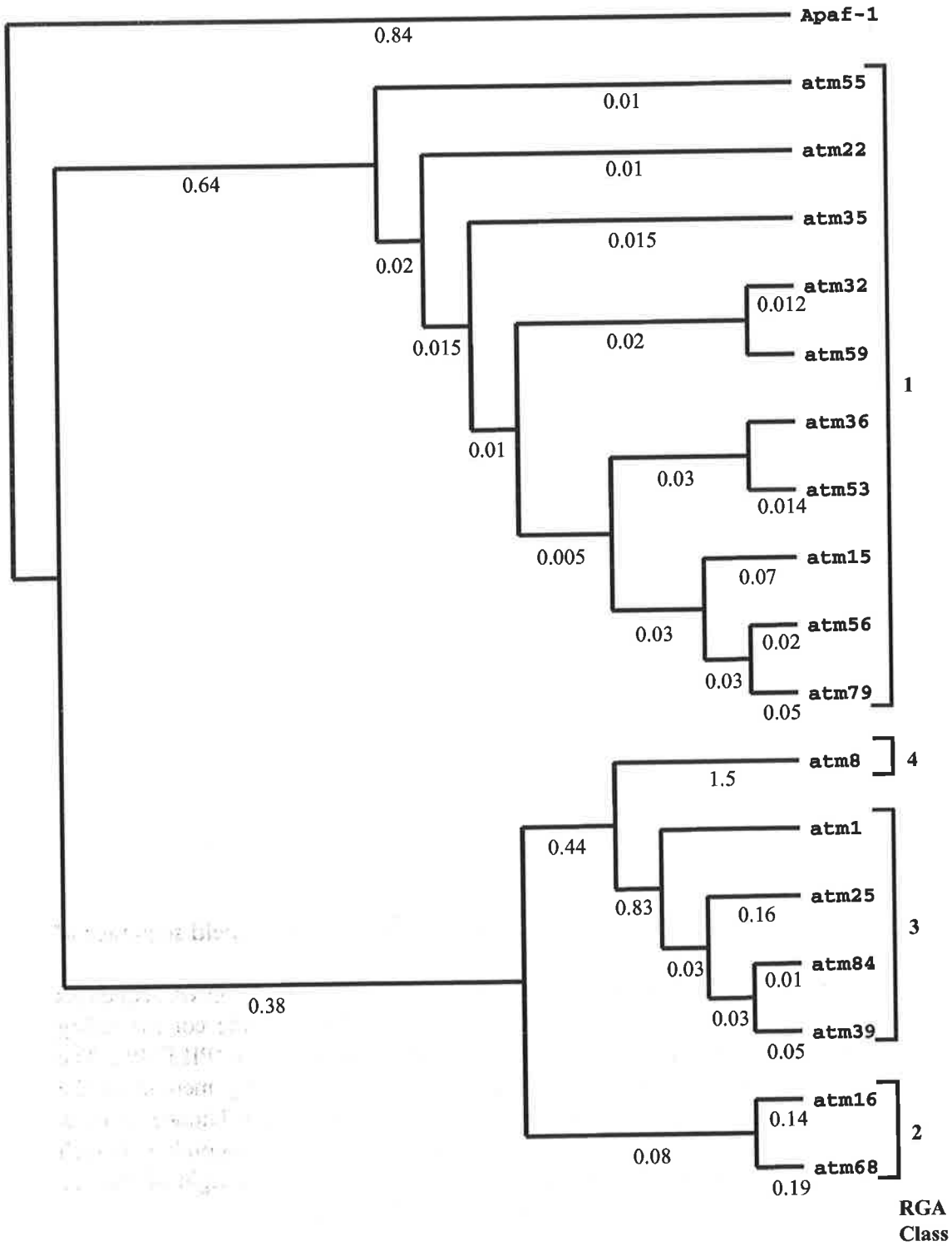
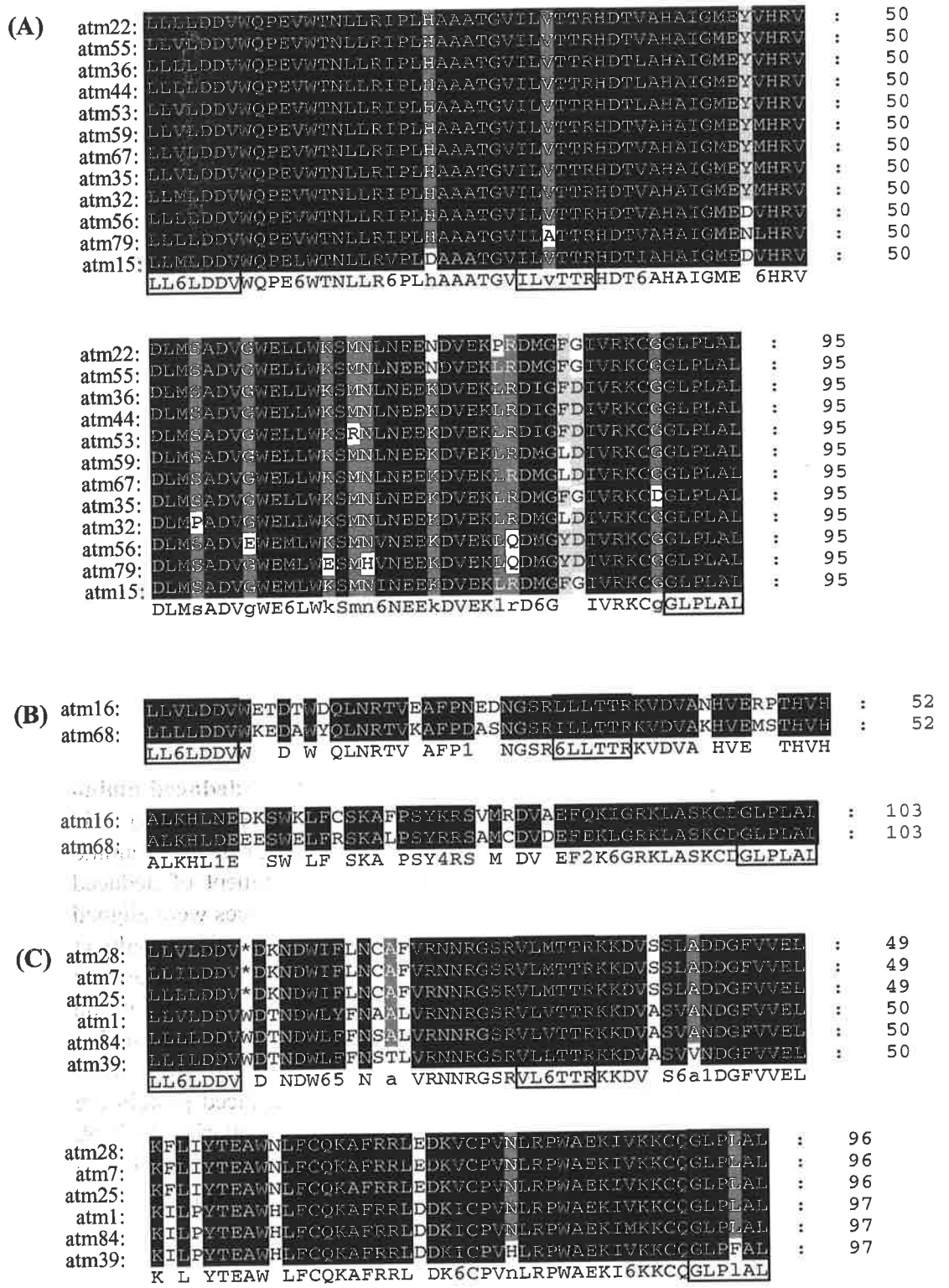

Apaf-1
0.84
atm55
0.01
atm22
0.01
0.64
atm35
0.015
0.02
atm32
0.012
0.015
0.02
atm59
1
atm36
0.01
0.03
atm53
0.014
0.005
atm15
0.07
0.03
atm56
0.02
0.03
atm79
0.05
atm8
4
1.5
atm1
0.44
atm25
0.83
0.16
3
0.03
atm84
0.01
0.38
0.03
atm39
0.05
atm16
0.14
2
0.08
atm68
0.19
RGA
Class

Figure 5.9: Class specific multiple sequence alignments of deduced amino acid sequences of atm clones. (**A**) Alignment of deduced amino acid sequences of class 1 classified atm clones. (**B**) Alignment of deduced amino acid sequences of class 2 classified atm clones. (**C**) Alignment of deduced amino acid sequences of class 3 classified atm clones. Sequences were aligned using the 'PILEUP' program with the default settings of gap creation penalty at 3.0 and gap extension penalty at 0.1. The regions of homology are shown in black (100% conservation), dark gray (greater than 80% conservation), and light gray (60% to 80% conservation). The conserved residues are indicated at the bottom of each alignment as a capital letter for 100% conservation, lower case for greater than 80% conservation. Stop codons in deduced protein are represented by an asterix (*). Numerals represent sequence similarity: 1= D/N, 2= E/Q, 3= S/T, 4= K/R, 5= F/Y/W, 6= L/I/V/M. Motifs conserved in RGAs are boxed and correspond to kinase-2, kinase-3 of the NBS and the Hydrophobic Domain.


(A)
atm22:
atm55:
atm36:
atm44:
atm53:
atm59:
atm67:
atm35:
atm32:
atm56:
atm79:
atm15:
LL6LDDVWQPE6WTNLLR6PLhAAATGVILvTTRHDT6AHAIGME 6HRV
DLMsADVgWE6LWkSmn6NEEkDVEK1rD6G IVRKCgGLPLAL
(B)
atm16:
atm68:
LL6LDDVW D W QLNRTV AFP1 NGSR6LLTTRKVDVA HVE THVH
ALKHL1E SW LF SKA PSY4RS M DV EF2K6GRKLASKCIGLPLAI
(C)
atm28:
atm7:
atm25:
atm1:
atm84:
atm39:
LL6LDDV D NDW65 N a VRNNRGSRVL6TTRKKDV S6a1DGFVVEL
K L YTEAW LFCQKAFRRL DK6CPVnLRPWAEKI6KKCQGLP1AL

within the open reading frame derived from genomically derived sequence contained within class 1 atm clones (Figure 5.9A). In total, 68 of the 82 (83%) amino acids were conserved between class 1 members. Among the observed 14 variant residues, 11 were conserved in 9 of the 10 (90%) clones. The deduced amino acid sequences of class 2 clones were 80% (72 of 90 residues) conserved (Figure 5.9B). Although the deduced protein sequences differed at 24 of the 90 genomic encoded residues, 6 of these differences were conservative. Three regions containing 100% conservation over 10 consecutive residues were observed. The first, spanning 15 amino acids from N28 to A42, included the core kinase-3 motif. The second region spanned 11 amino acids from T49 to E59. The third region, located immediately prior to the hydrophobic domain, spanned 14 amino acids from E84 to D97. Of the 84 amino acid residues of class 3 members of genomically derived sequences 71 (85%) were conserved. Several regions containing 100% sequence identity over 10 consecutive amino acids were observed. A region of 18 amino acids containing the kinase-3 motif spanned from residue V21 to V38. Sequence identity between class 3 members was observed over a region immediately before the hydrophobic domain motif. This region spanned 13 amino acids from L79 to Q91. The third region of sequence identity spanned 10 amino acids from L61 to L70.

5.2.7 Sequence Comparison of Class Specific *L. rigidum* RGAs

Class representative clones (Figure 5.10) were used as queries to screen for similar proteins contained within protein databases. The best three matches for each clone are presented in Table 5.4. The closest matches for all four class representative *L. rigidum* clones were RGA sequences and an R-gene isolated from other plant lines. A phylogenetic tree was constructed to examine the relationship between *L. rigidum* RGAs and their most related RGA

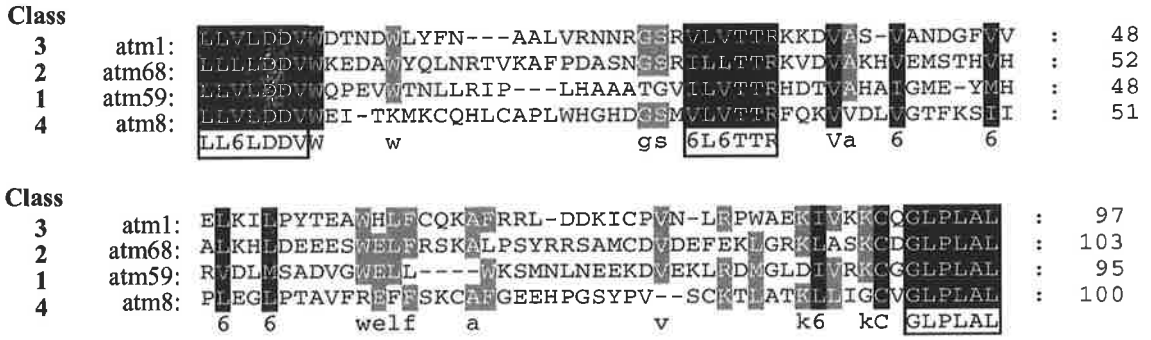


Figure 5.10: Multiple sequence alignment of deduced amino acid sequences of class representative atm clones. Deduced amino acid sequences from the atm clones atm1 (class 3), atm68 (class 2), atm59 (class1) and atm8 (class 4) were aligned using the PILEUP program with the default settings of gap creation penalty at 3.0 and gap extension penalty at 0.1. Gaps represented by dashes (-) were introduced to maximise homology. Group classification of each clone is presented on the left of the alignment. The regions of homology are shown in black (100% conservation) and gray (greater than 60% conservation). The conserved residues are indicated at the bottom of each alignment as a capital letter for 100% conservation, lower case for greater than 60% conservation. Numerals represent sequence similarity: 1= D/N, 2= E/Q, 3= S/T, 4= K/R, 5= F/Y/W, 6= L/I/V/M. Motifs conserved in RGAs are boxed and correspond to kinase-2, kinase-3 of the NBS and the hydrophobic domain.

Table 5.4: Highest scoring sequence identities to class specific RGA clones
Deduced amino acid sequences of representatives of the four RGA classes were queried against the protein database using the GCG program BLASTX. The three proteins in the database with the highest identity to each ryegrass RGA class are presented. Related proteins are detailed by their accession number (ID), source plant, the percentage identity and similarity to the queried RGA sequence (expressed both as a percentage and the number of identical or conserved residues over a given stretch of amino acids) and the probability.

Class 1-atm59 (82 aa)

Gene/Protein	Plant/ID	Identity (%)	Similarity (%)	Probability
clone II 2.17	*Avena strigosa* CAC10052	83 (68/81)	93 (76/81)	3.0×10^{-46}
rae1	*Aegilops ventricosa* CAC11100	76 (62/81)	91 (74/81)	2.6×10^{-44}
pic24	*Oryza sativa* AAF21364	71 (58/81)	85 (69/81)	1×10^{-40}

Class 2-atm68 (90 aa)

Gene/Protein	Plant/ID	Identity (%)	Similarity (%)	Probability
rae7	*Aegilops ventricosa* CAC11106	62 (58/89)	79 (71/89)	1.2×10^{-38}
rae6	*Aegilops ventricosa* CAC11105	62 (47/75)	84 (63/75)	2.5×10^{-38}
RGA6.3	*Linum usitatissimum* CAC35382	53 (26/49)	71 (35/49)	9.5×10^{-17}

Class 3-atm1 (84 aa)

Gene/Protein	Plant/ID	Identity (%)	Similarity (%)	Probability
pic11-1	*Zea mays* AAC83559	59 (50/84)	75 (63/84)	2.9×10^{-33}
pic11	*Zea mays* AAC83558	55 (47/84)	71 (60/84)	2.9×10^{-31}
pic19	*Zea mays* AAC83567	38 (32/83)	62 (52/83)	1.7×10^{-19}

Class 4-atm8 (87 aa)

Gene/Protein	Plant/ID	Identity (%)	Similarity (%)	Probability
XA1	*Oryza sativa* BAA25068	43 (10/23) 34 (10/29) 46 (7/15)	73 (17/23) 37 (11/29) 53 (8/15)	0.98 5.2×10^{-5} 0.98
YR14	*Oryza sativa* AAF43657	36 (17/47)	59 (28/47)	1.4×10^{-4}
YR18	*Oryza sativa* AAF43659	36 (17/47)	59 (28/47)	1.4×10^{-4}

sequences or R-genes in other plant species (Figure 5.11). The tree illustrated that members of any given *L. rigidum* RGA class were more closely related to RGA sequences from other species than to members of other *L. rigidum* RGA classes.

5.2.7.1 The Class 1 *L. rigidum* RGA Shares Sequence Identity with Other RGA Sequences

The class 1 representative clone, atm59, shared 83% identity (93% similarity), across all 82 amino acids queried, with clone II 2.17, an RGA isolated from *Avena strigosa* (Loarce *et al.*, 2000). This degree of identity was further increased to 88% if amino acids contained within the kinase-2 and hydrophobic domain were included in the alignment (Figure 5.12A). The clone atm59 also showed sequence identity (76%) with the RGA, *rae*I of *Aegilops ventricosa* (Lopez-Brana *et al.,* 1999) and 71% sequence identity with the deduced amino acid sequence of the *Oryza sativa* RGA, *pic*24 (Collins *et al.*, 1998 unpublished). It was noted that of the four representative *L. rigidum* RGA amino acid sequences queried against the protein database, atm59 was the clone that received the highest number of sequence matches. Thirty entries showed greater than 61% sequence identity across the entire 82 amino acid length of the deduced atm59 sequence.

5.2.7.2 The Class 2 *L. rigidum* RGA, atm68, Shares Sequence Identity with Other RGA Sequences

The class 2 representative clone, atm68, shared 62% sequence identity with the *Aegilops ventricosa* RGAs *rae*7 and *rae*6 (Lopez-Brana *et al.,* 1999). The closest match,

Figure 5.11: Phylogenetic tree based on the deduced amino acid sequence of atm clones and their closest database matches

Deduced amino acid sequences from the genomically derived DNA sequences of all RGAcontaining clones were aligned together with the corresponding regions of their closest databasematches; clone II 2.17 of *Avena strigosa* (II 2.17), *rae*7 of *Aegilops ventricosa* (rae7), pic11-1 of *Zea mays* (pic11-1) and XA1 of *Oryza sativa* (XA1) in addition to the human *Apaf*1 gene using the GCG program 'PILEUP'. The resultant alignment was analysed using the neighbor-joining method of the GCG program 'eneighbor' to produce the phylogenetic tree. Numbers below horizontal lines indicate the branch length. Horizontal lines without a corresponding branch length have an effective branch length of (0).

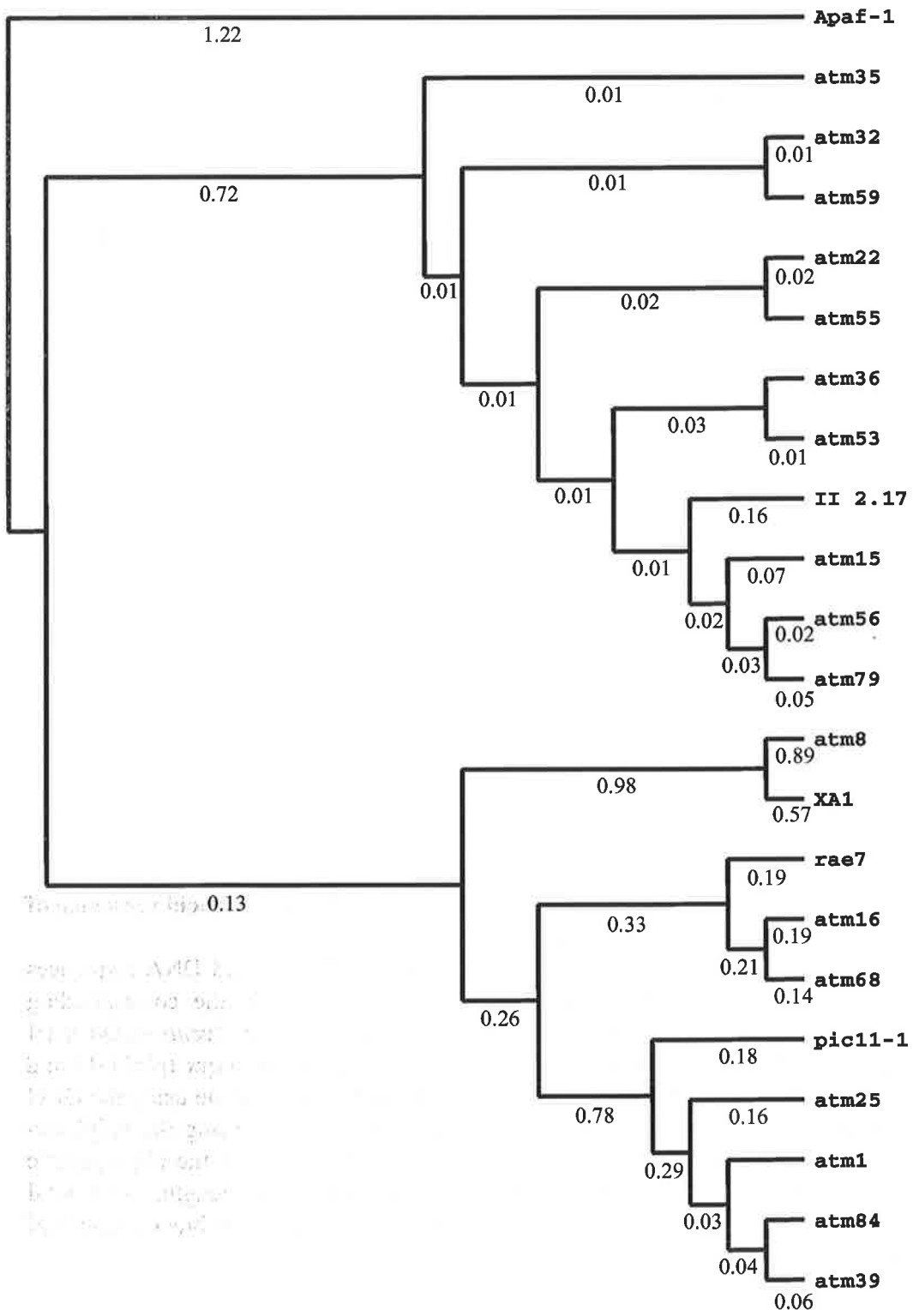

Apaf-1
1.22
atm35
0.01
atm32
0.01
0.01
atm59
0.72
atm22
0.02
0.01
0.02
atm55
atm36
0.01
0.03
atm53
0.01
0.01
II 2.17
0.16
atm15
0.01
0.07
atm56
0.02
0.02
0.03
atm79
0.05
atm8
0.89
0.98
XA1
0.57
rae7
0.19
0.13
0.33
atm16
0.19
0.21
atm68
0.14
0.26
pic11-1
0.18
atm25
0.78
0.16
0.29
atm1
0.03
atm84
0.04
atm39
0.06

Figure 5.12: Sequence alignments of deduced amino acid sequences of class representative RGA clones with database matches of greatest probability.

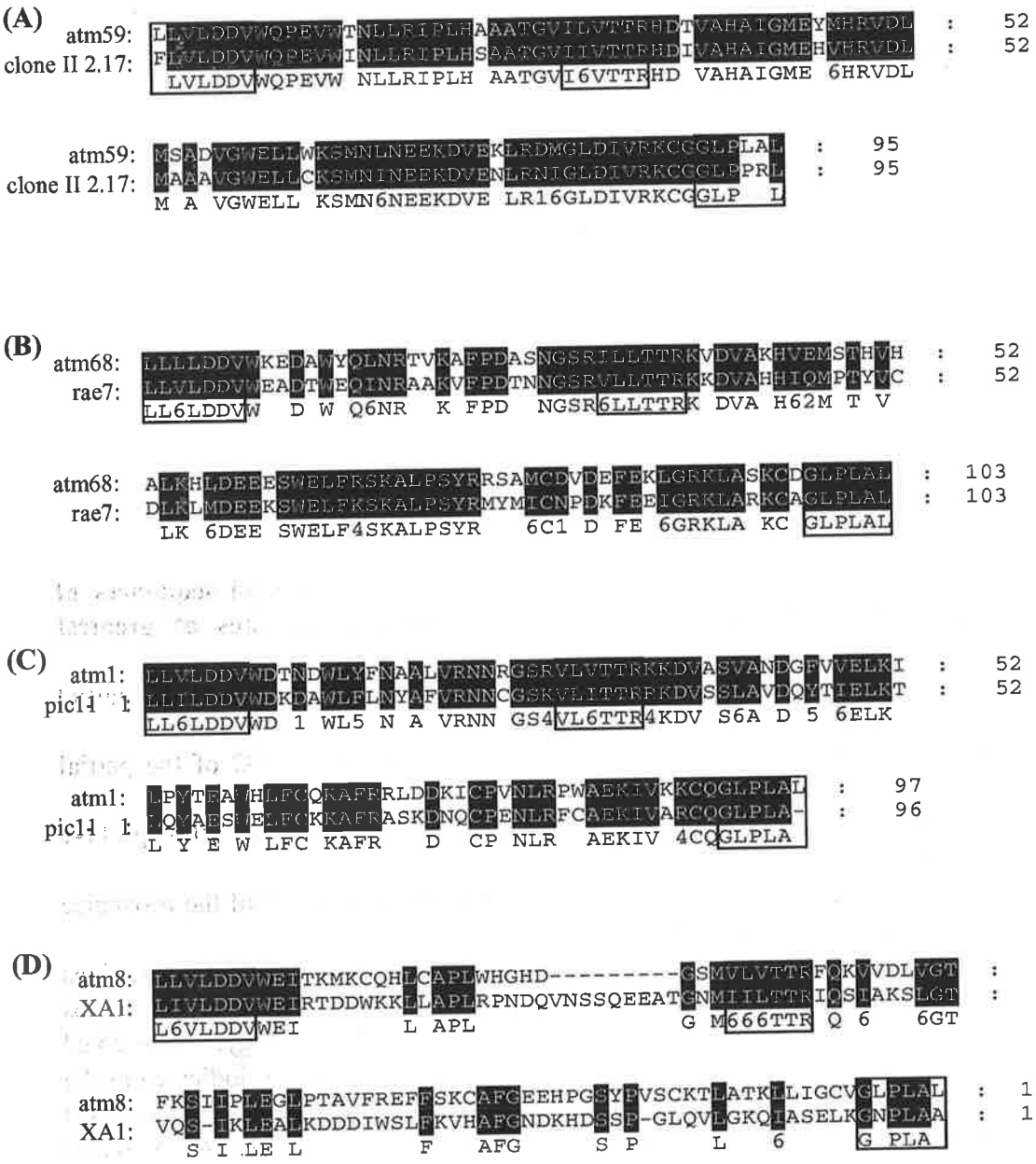
(A) Alignment of atm59 with amino acid residues 73 to 167 of the partial resistance gene II 2.17 of *Avena strigosa* (CAC10052).

(B) Alignment of atm68 with amino acid residues 80 to 182 of the partial RGA rae7 of *Aegilops ventricosa* (CAC11106).

(C) Alignment of atm1 with amino acid residues 1 to 96 of the RGA pic11-1 of *Zea mays* (AAC83559).

(D) Alignment of atm8 with amino acid residues 402 to 508 of the resistance gene XA1 of *Oryza sativa* (BAA25068).

Sequences were aligned using the PILEUP program with the default settings of gap creation penalty at 3.0 and gap extension penalty at 0.1. Gaps represented by dashes are introduced to maximise homology. Conserved amino acids are shown in black. The conserved residues are indicated at the bottom of each alignment as a capital letter for 100% conservation. Numerals represent sequence similarity: 1= D/N, 2= E/Q, 3= S/T, 4= K/R, 5= F/Y/W, 6= L/I/V/M. Motifs conserved in RGAs are boxed and correspond to kinase-2, kinase-3 of the NBS and the hydrophobic domain.


(A)
atm59:
clone II 2.17:
LLVLDDVWQPEVWTNLLRIPLHAAATGVILVTTRHDTVAHAIGMEYMHRVDL : 52
FLVLDDVWQPEVWINLLRIPLHSAATGVILVTTRHDIVAHAIGMEHVHRVDL : 52
LVLDDVWQPEVW NLLRIPLH AATGVI6VTTRHD VAHAIGME 6HRVDL
atm59:
clone II 2.17:
MSADVGWELLWKSMNLNEEKDVEKLRDMGLDIVRKCGGLPLAL : 95
MAAAVGWELLCKSMNINEEKDVENLRNIGLDIVRKCGGLPPRL : 95
M A VGWELL KSMN6NEEKDVE LR16GLDIVRKCGGLP L
(B)
atm68:
rae7:
LLLLDDVWKEDAWYQLNRTVKAFPDASNGSRILLTTRKVDVAKHVEMSTHVH : 52
LLVLDDVWEADTWEQINRAAKVFPDTNNGSRVLLTTRKKDVAHHIQMPTYVC : 52
LL6LDDVW D W Q6NR K FPD NGSR6LLTTRK DVA H62M T V
atm68:
rae7:
ALKHLDEEESWELFRSKALPSYRRSAMCDVDEFEKLGRKLASKCDGLPLAL : 103
DLKLMDEEKSWELFKSKALPSYRMYMICNPDKFEEIGRKLARKCAGLPLAL : 103
LK 6DEE SWELF4SKALPSYR 6C1 D FE 6GRKLA KC GLPLAL
(C)
atm1:
pic11 1:
LLVLDDVWDTNDWLYFNAALVRNNRGSRVLVTTRKKDVASVANDGFVVELKI : 52
LLILDDVWDKDAWLFLNYAFVRNNCGSKVLITTRFKDVSSLAVDQYTIELKT : 52
LL6LDDVWD 1 WL5 N A VRNN GS4VL6TTR4KDV S6A D 5 6ELK
atm1:
pic11 1:
LPYTEAWHLFCQKAFRRLDDKICPVNLRPWAEKIVKKCQGLPLAL : 97
LQYAESWELFCKKAFRASKDNQCPENLRFCAEKIVARCQGLPLA- : 96
L Y E W LFC KAFR D CP NLR AEKIV 4CQGLPLA
(D)
atm8:
XA1:
LLVLDDVWEITKMKCQHLCAPLWHGHD---------GSMVLVTTRFQKVVDLVGT : 46
LIVLDDVWEIRTDDWKKLLAPLRPNDQVNSSQEEATGNMIILTTRIQSIAKSLGT : 55
L6VLDDVWEI L APL G M666TTR Q 6 6GT
atm8:
XA1:
FKSITPLEGLPTAVFREFFSKCAFGEEHPGSYPVSCKTLATKLLIGCVGLPLAL : 100
VQS-IKLEALKDDDIWSLFKVHAFGNDKHDSSP-GLQVLGKQIASELKGNPLAA : 107
S I LE L F AFG S P L 6 G PLA

deemed by the probability, favoured *rae*7. Insertion of a gap in the 90 amino acids of atm68 with *rae*6 but not *rae*7 was required to maximise the sequence alignment (Figure 5.12B). An alignment of the full, deduced atm68 protein sequence with the deduced *rae*7 protein sequence indicated large stretches of identity existed, not only confined to the known kinase-3 domain but also extended to a region further downstream. Identity of 53% (71% similarity) of atm68 with the RGA6.3 of *Linum usitatissimum* was observed. However, this degree of identity was only shown over 49 residues of the total 90 amino acids of the atm68 query. Overall, only two database entries had homology over the entire length of the queried 90 amino acids of atm68.

5.2.7.3 The Class 3 *L. rigidum* RGA, atm1, Shares Sequence Identity with Other RGA Sequences

The three closest matches to the class 3 representative, atm1, were all RGA sequences from *Zea mays*; *pic*11-1, *pic*11 and *pic*19 (Collins *et al.,* 1998). The highest level of sequence identity (59%) was observed between atm1 and *pic*11-1 (Table 5.3). The greatest stretch of continuous conservation between atm1 and *pic*11-1 was observed at the kinase-3 domain (Figure 5.12C).

5.2.7.4 The Class 4 *L. rigidum* RGA, atm8, Shares Sequence Identity with an R-gene.

The closest database match to atm8, the *Oryza sativa* R-gene XA1 (Yoshimura *et al.,* 1998), showed sequence similarity over short stretches of between 15 to 29 amino acids of protein (Figure 5.12D). To achieve maximum alignment of the conserved kinase-3 domain

with XA1, a gap of 9 residues was introduced into the atm8 sequence. Sequence identity of 36% was observed between 47 of the 87 amino acids of the queried atm8 sequence with the corresponding region of *Oryza sativa* RGAs YR14 and YR18 (Yang *et al.*, 2000). Other than the conserved kinase-2, kinase-3 and hydrophobic domains, only short sequences of conservation of up to three amino acid residues were observed.

5.2.8 Classification of Remaining atm Clones by Slot Blot Analysis

To establish how representative the relative numbers of class members present amongst the sequenced clones were in relation to the total number of isolated clones, the overall hybridisation profile of each clone by class specific probes was examined (Figure 5.13A-D; Table 5.5). The sequenced atm62, known to contain an insert not related to RGA sequences, served as a useful control to ensure the observed hybridisation was not due to binding of the probe to primer sequences alone. The atm clones, atm4, atm34 and atm43, were found not to contain insert sequence (data not shown). These clones most likely represented false positive clones.

The class 1 representative probe atm59(1f-G3) hybridised to a total of 30 of the 91 clones (Figure 5.13A). Of the 22 sequenced atm clones, 12 (55%) were classified as class 1 based on their degree of sequence identity. On the basis of the hybridisation of the class 1 specific probe, 30 of the 88 insert containing atm clones (34%) were categorised as class 1 members. This indicated class 1 clones were over-represented in the subset of clones chosen for sequencing.

The class 2 representative probe, atm68(2f-G3), hybridised to a total of 25 of the 91 clones (Figure 5.13B). However, based on the overall hybridisation profile by all 4 class

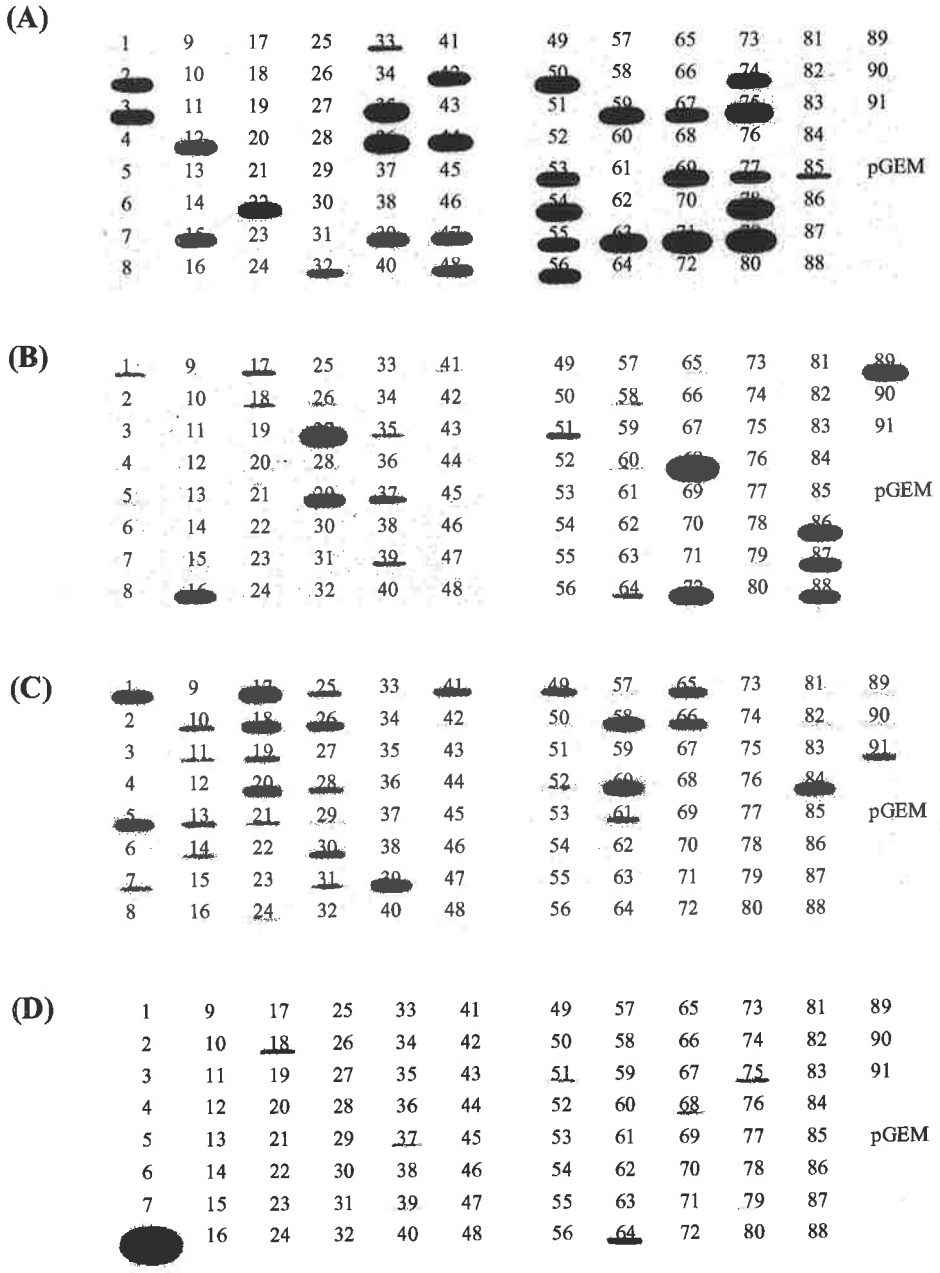


Figure 5.13: Hybridisation of class representative probes to slot blots of atm clones
Class representative probes were hybridised to identical slot blots containing DNA isolated from 91 atm clones.
(**A**) Hybridisation of class 1 representative probe, atm59(1f-G3)
(**B**) Hybridisation of class 2 representative probe, atm68(2f-G3).
(**C**) Hybridisation of class 3 representative probe, atm1(3f-G3).
(**D**) Hybridisation of class 4 representative probe, atm8(4f-G3)
pGEM represents a hybridisation negative control in the form of a non-rccombinant cloning vector

Table 5.5: Summary of hybridisation of class specific RGA probes to slot blots of atm clones. Each probe was scored on its level of hybridisation to the 91 atm clones; strong hybridisation (++), weak hybridisation (+), or no hybridisation (-). Based on the overall hybridisation pattern, each atm clone was assigned to one of the four RGA classes (Class 1, 2, 3 or 4). Clones to which no probes hybridised were designated unclassified (UC). Sequenced clones are presented in shaded rows. An asterix (*) denotes clones lacking inserts.

atm clone	Hybridisation to class specific probes				Overall classification
	Class1	Class2	Class3	Class4	
1	-	+	++	-	3
2	++	-	-	-	1
3	++	-	-	-	1
4	-	-	-	-	UC*
5	-	+	++	-	3
6	-	-	-	-	UC
7	-	-	+	-	3
8	-	-	-	++	4
9	-	-	-	-	UC
10	-	-	+	-	3
11	-	-	+	-	3
12	++	-	-	-	1
13	-	-	+	-	3
14	-	-	+	-	3
15	++	-	-	-	1
16	-	++	-	-	2
17	-	+	++	-	3
18	-	+	++	+	3
19	-	-	+	-	3
20	-	-	++	-	3
21	-	-	+	-	3
22	++	-	-	-	1
23	-	-	-	-	UC
24	-	-	+	-	3
25	-	-	+	-	3
26	-	+	++	-	3
27	-	++	-	-	2
28	-	+	+	-	3
29	-	++	+	-	2
30	-	-	++	-	3
31	-	-	+	-	3
32	+	-	-	-	1
33	+	-	-	-	1
34	-	-	-	-	UC*
35	++	+	-	-	1
36	++	-	-	-	1
37	-	++	-	+	2
38	-	-	-	-	UC
39	++	+	++	+	1
40	-	-	-	-	UC
41	-	+	++	-	3
42	++	-	-	-	1
43	-	-	-	-	UC*

44	++	-	-	-	1
45	-	-	-	-	UC
46	-	-	-	-	UC
47	++	-	-	-	1
48	++	-	-	-	1
49	-	-	++	-	3
50	++	-	-	-	1
51	-	+	-	+	2
52	-	-	+	-	3
53	++	-	-	-	1
54	++	-	-	-	1
55	++	-	-	-	1
56	++	-	-	-	1
57	-	-	+	-	3
58	-	+	++	-	3
59	++	-	-	-	1
60	-	+	++	-	3
61	-	-	+	-	3
62	-	-	-	-	UC
63	++	-	-	-	1
64	-	+	-	++	4
65	-	+	++	-	3
66	-	-	++	-	3
67	++	-	-	-	1
68	-	++	-	+	2
69	++	-	-	-	1
70	-	-	-	-	UC
71	++	-	-	-	1
72	-	++	-	-	2
73	-	-	-	-	UC
74	++	-	-	-	1
75	++	-	-	+	1
76	-	-	-	-	UC
77	++	-	-	-	1
78	++	-	-	-	1
79	++	+	-	-	1
80	-	-	-	-	UC
81	-	-	-	-	UC
82	-	-	+	-	3
83	-	-	-	-	UC
84	-	-	++	-	3
85	+	-	-	-	1
86	-	++	-	-	2
87	-	++	-	-	2
88	-	++	-	-	2
89	-	++	+	-	2
90	-	-	+	-	3
91	-	-	+	-	3

specific probes only 11 of the 88 (12.5%) insert containing atm clones were classified as class 2 members. This was comparable to the observed numbers of 2 of the 22 (9%) sequenced clones classified as class 2 members.

The class 3 representative clone, atm1(3f-G3), hybridised to a total of 34 of the 91 clones (Figure 5.13C). A difference in the level of hybridisation of atm1(3f-G3) to sequenced members of class 3 was observed. The probe hybridised strongly to atm1, atm39 and atm84. However, hybridisation to class 3 members, atm7, atm25 and atm28, was only weak. The observed discrepancy could be explained by differences in the level of sequence identity with the probe, with the clones atm7, atm25 and atm28 sharing 90% sequence identity with the probe compared to atm39 and atm84 which shared 94% and 97% sequence identity, respectively. Of the 22 atm clones sequenced, 6 (27%) were classified as class 3 members. In total, 33 of the 88 (37.5%) insert containing atm clones were classified as members of class 3. This implied class 3 members were under-represented in the selected sequenced clones.

The class 4 representative probe, atm8(4f-G3), hybridised to a total of 8 of the 91 clones (Figure 5.13D). As stronger hybridisation by alternative class specific probes was observed for 6 of the clones, overall only 2 of the 88 (2%) insert containing atm clones were classified as members of class 4. As atm8 was the sole class 4 member of the 22 clones sequenced, class 4 RGAs maintained their position as relatively rare sequences in the atm set.

5.2.9 Comparison of Hybridisation of Class-Specific Probes to Genomic DNA Isolated from R799 and S1150

By analysing Southern blots containing DNA from R799 and S1150 digested with a variety of restriction enzymes and probed with class-specific probes, information regarding the relative abundance of particular RGA sequences was determined.

Hybridisation of the class1-specific probe, atm59(1f-G3), to genomic DNA isolated from R799 and S1150 indicated class 1 sequences were present in both plant lines (Figure 5.14). The probe hybridised to up to 9 *Hind*III R799 fragments and 8 *Hind*III and *Dra*I S1150 fragments, indicating the presence of at least 9 and 8 different class 1 RGA sequences in these respective plant lines. The probe hybridised to only one *Sac*I fragment, about 500 bp in size, in S1150 genomic DNA. This indicated that within S1150 genomic DNA, although class 1 sequences were present in 8 different *Dra*I fragments greater than 3 kb, all co-localised within a *Sac*I fragment of 500 bp. This may reflect the conserved nature of the *Sac*I core of class1 RGAs that may be present in many copies throughout the genome, resulting in different sizes of *Dra*I genomic DNA hybridising fragments.

Hybridisation of the class 2-specific probe, atm68(2f-G3), was observed to a single fragment of genomic DNA isolated from R799, regardless of the enzyme used in the digestion (Figure 5.15). In contrast, no hybridisation was observed to digested, genomic DNA of S1150. This indicated the probe sequence was absent in S1150 and present as a single copy in R799.

Hybridisation of the class 3-specific probe, atm1(3f-G3), was observed to digested genomic DNA of both R799 and S1150 (Figure 5.16). This indicated class 3 sequences were present in both plant lines. Variation between the plant lines existed in both the number and

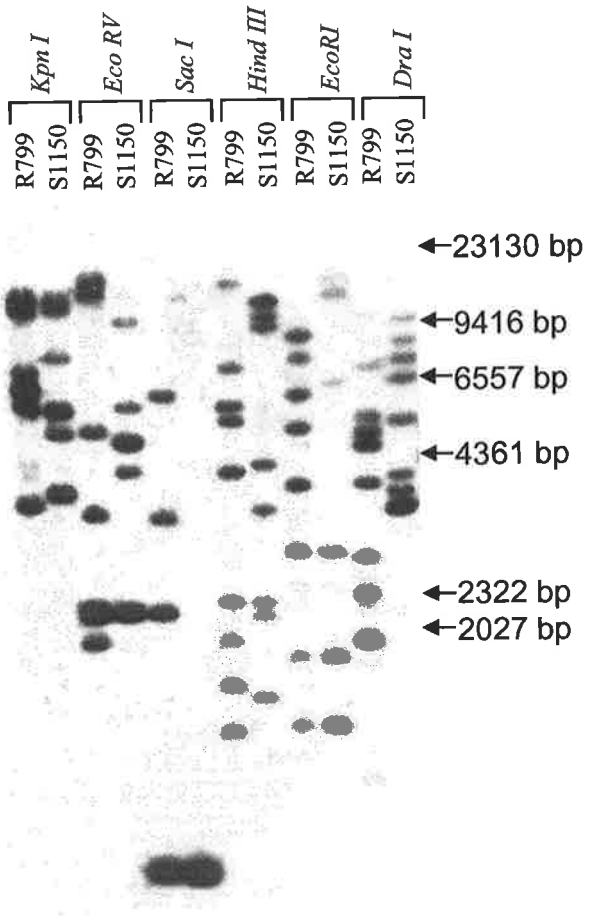


Figure 5.14: Hybridisation of class 1 representative probe, atm59(1f-G3), to genomic DNA isolated from *L. rigidum* lines R799 and S1150.

Genomic DNA isolated from R799 and S1150 was digested with six different restriction enzymes; *Kpn* I (lanes 1 and 2), *Eco* RV (lanes 3 and 4), *Sac* I (lanes 5 and 6), *Hind* III (lanes 7 and 8), *Eco* RI (lanes 9 and 10), *Dra* I (lanes 11 and 12). Digested products were separated on a 0.8% agarose gel, Southern blotted and probed with atm59(1f-G3). Lengths of marker products are indicated in base pairs (bp).

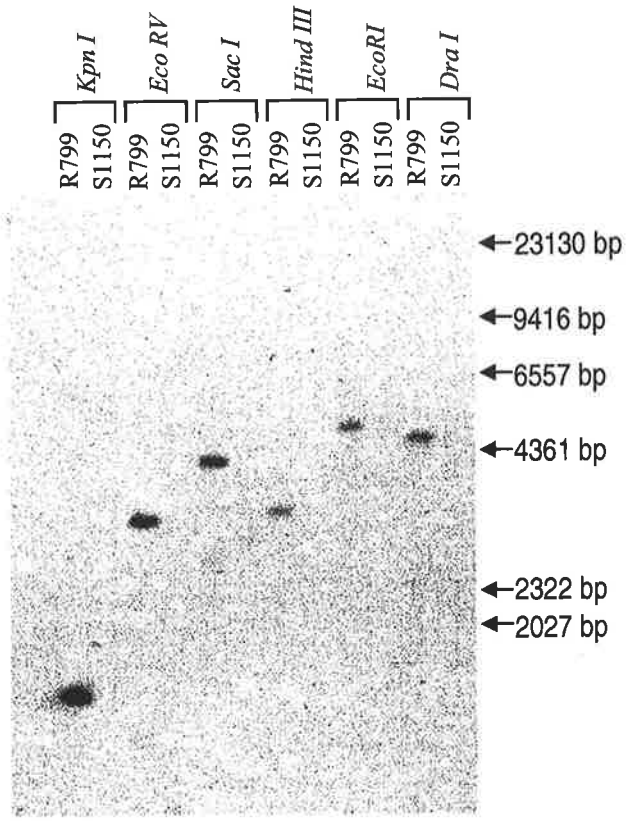


Figure 5.15: Hybridisation of class 2 representative probe, atm68(2f-G3), to genomic DNA isolated from *L. rigidum* lines R799 and S1150.

Genomic DNA isolated from R799 and S1150 was digested with six different restriction enzymes; *Kpn* I (lanes 1 and 2), *Eco* RV (lanes 3 and 4), *Sac* I (lanes 5 and 6), *Hind* III (lanes 7 and 8), *Eco* RI (lanes 9 and 10), *Dra* I (lanes 11 and 12). Digested products were separated on a 0.8% agarose gel, Southern blotted and probed with atm68(2f-G3). Lengths of marker products are indicated in base pairs (bp).

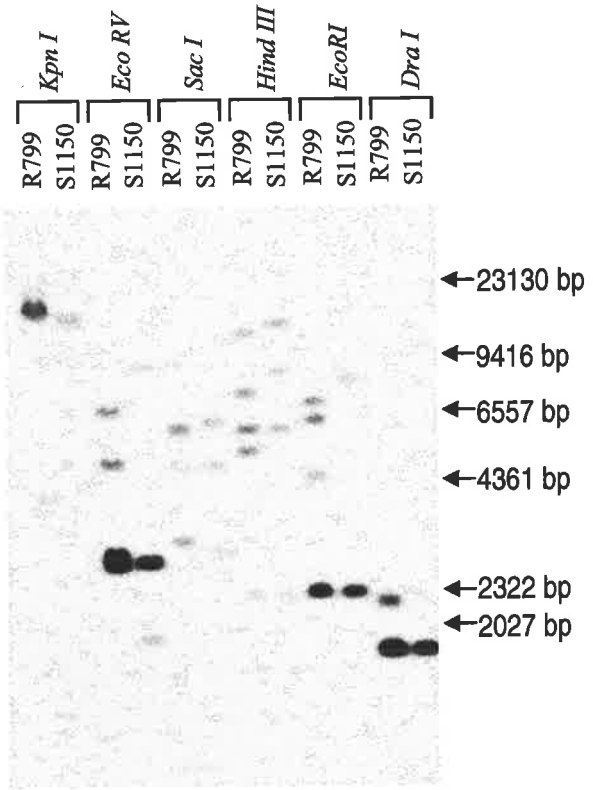


Figure 5.16: Hybridisation of class 3 representative probe, atm1(3f-G3), to genomic DNA isolated from *L. rigidum* lines R799 and S1150.

Genomic DNA isolated from R799 and S1150 was digested with six different restriction enzymes; *Kpn* I (lanes 1 and 2), *Eco* RV (lanes 3 and 4), *Sac* I (lanes 5 and 6), *Hind* III (lanes 7 and 8), *Eco* RI (lanes 9 and 10), *Dra* I (lanes 11 and 12). Digested products were separated on a 0.8% agarose gel, Southern blotted and probed with atm1(3f-G3). Lengths of marker products are indicated in base pairs (bp).

size of the fragments to which atm1(3f-G3) hybridised. This indicated the presence of more than one RGA with sequence identity to atm1(3f-G3) existed in both plant lines.

Hybridisation of the class 4-specific probe, atm8(4f-G3), was observed to a single fragment of digested genomic DNA of both R799 and S1150 (Figure 5.17). This indicated class 4 sequences were present in both plant lines and likely to exist as a single copy.

5.2.10 Comparison of Hybridisation of Class Specific Probes to the Genomic DNA of Plant Lines Susceptible and Resistant to *A. funesta*

Hybridisation of the class 1-specific probe, atm59(1f-G3), was observed to genomic DNA of all plant lines tested (Figure 5.18). However, hybridisation profiles differed for each plant line examined in both the number and size of hybridising bands. This indicated a number of copies of class 1 RGA sequences were present in the examined plant lines. The hybridisation profiles produced by atm59(1f-G3) bound to *Sac*I digested genomic DNA revealed the class 1 specific probe bound to an approximately 500 bp *Sac*I fragment in all plant lines (Figure 5.18B).

The level of hybridisaton of the class 2-specific probe, atm68(2f-G3), to genomic DNA isolated from lines varied considerably (Figure 5.19). While strong hybridisation was observed to a *Dra*I fragment of about 8 kb in size in the lines WA Ecotype, Guard II and Guard C3, weak hybridisation was observed to *Dra*I fragments present in the genomic DNA of Wimmera, Turretfield, S1150 and C19.1. As equal amounts of genomic DNA from each line were analysed, differences in the level of hybridisation may reflect differences in the copy number of class 2 RGA sequences present in the genomes. Alternatively, weaker hybridisation may be due to the hybridisation of the probe to an additional, less related class of

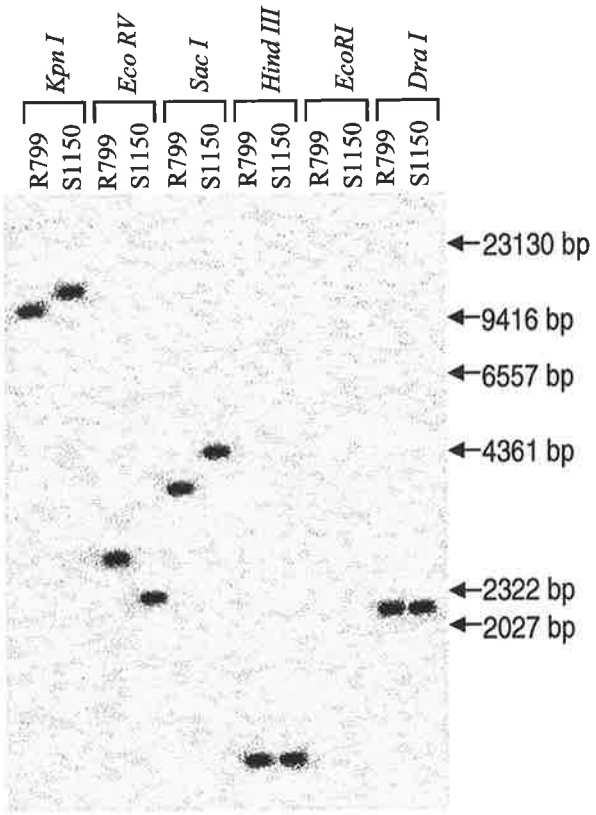


Figure 5.17: Hybridisation of class 4 representative probe, atm8(4f-G3), to genomic DNA isolated from *L. rigidum* lines R799 and S1150.

Genomic DNA isolated from R799 and S1150 was digested with six different restriction enzymes; *Kpn* I (lanes 1 and 2), *Eco* RV (lanes 3 and 4), *Sac* I (lanes 5 and 6), *Hind* lll (lanes 7 and 8), *Eco* RI (lanes 9 and 10), *Dra* I (lanes 11 and 12). Digested products were separated on a 0.8% agarose gel, Southern blotted and probed with atm8(4f-G3). Lengths of marker products are indicated in base pairs (bp).

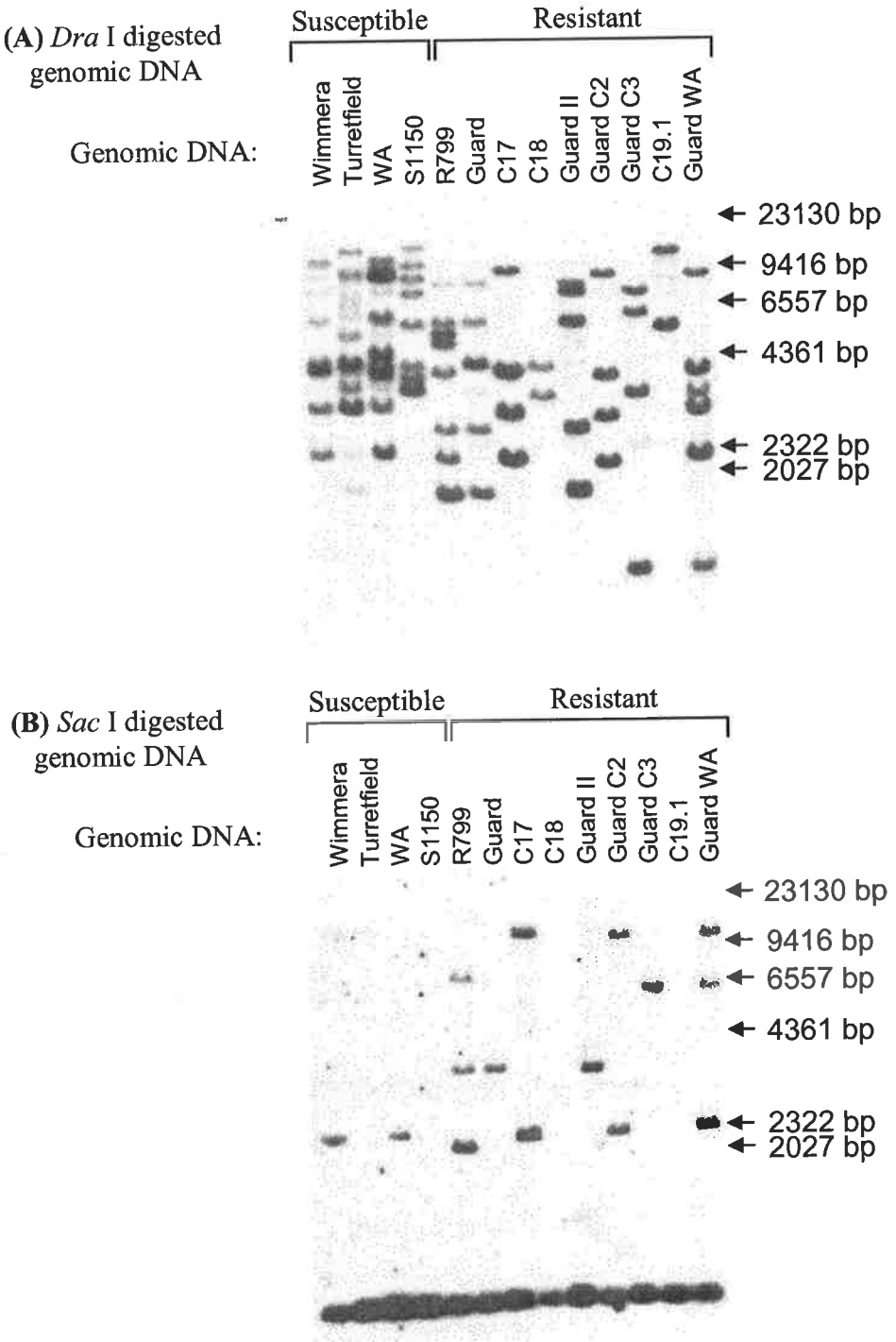


Figure 5.18: Hybridisation of the class 1 representative probe, atm59(1f-G3), to genomic DNA isolated from *L. rigidum* plant lines susceptible or resistant to nematode infection.
Genomic DNA was isolated from the susceptible lines Wimmera Ecotype, Turretfield Ecotype, Western Australia Ecotype (WA) and S1150, and the resistant lines R799, Guard, *L. rigidum* x *L. multiflorum* (C17), *L. rigidum* x *L. multiflorum* (C18), Guard II, Guard C2, Guard C3, *L. rigidum* x *L. multiflorum* (C19.1), Guard x Western Australia Ecotype (Guard WA). Genomic DNA was digested with either *Dra* I **(A)** or *Sac* I **(B)**, separated on 0.8% agarose gel which was subsequently Southern blotted and the membrane probed with the class specific probe.

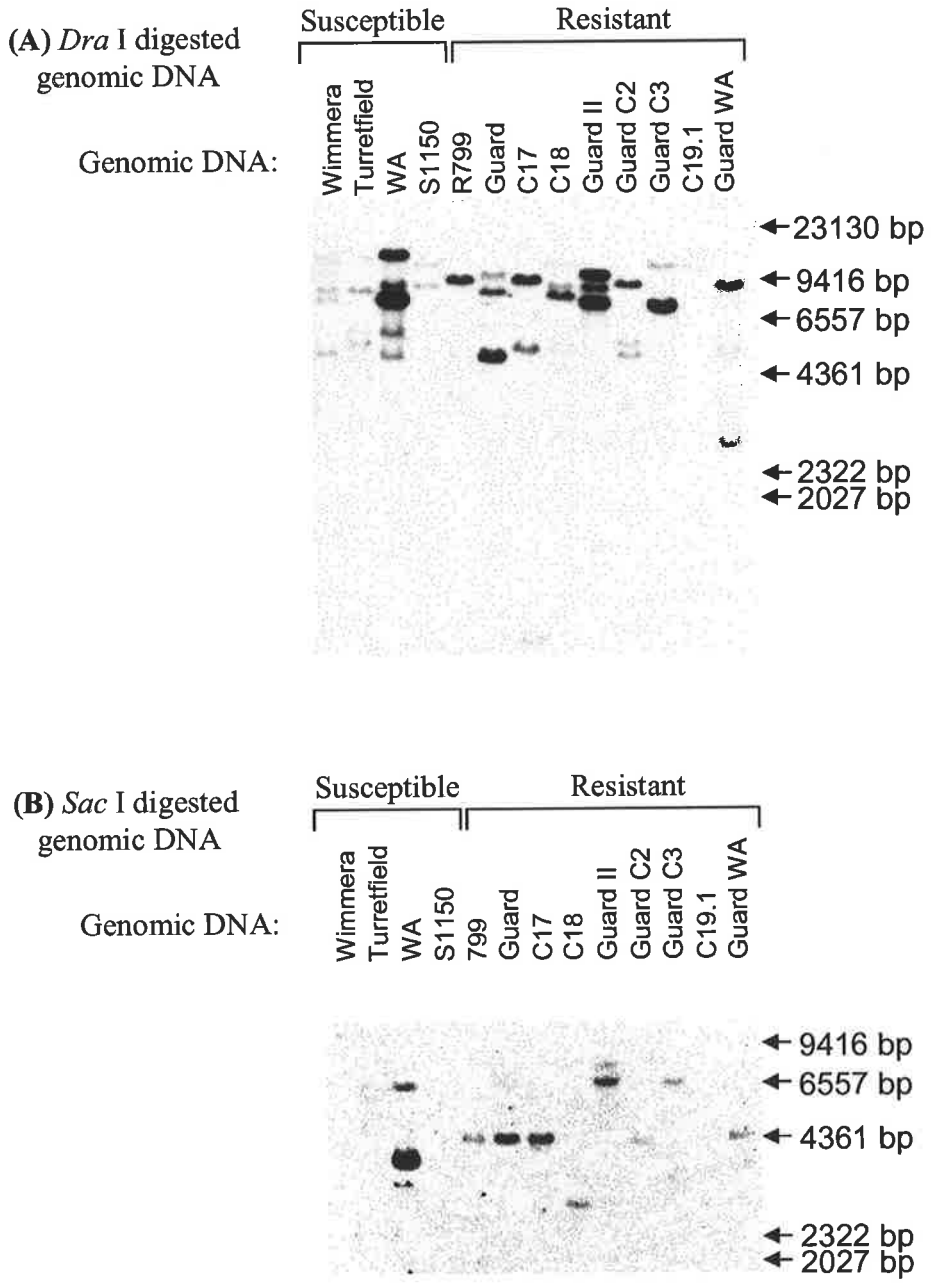


Figure 5.19: Hybridisation of the class 2 representative probe, atm68(2f-G3), to genomic DNA isolated from *L. rigidum* plant lines susceptible or resistant to nematode infection.

Genomic DNA from the susceptible lines Wimmera Ecotype, Turretfield Ecotype, Western Australia Ecotype (WA) and S1150, and the resistant lines R799, Guard, *L. rigidum* x *L. multiflorum* (C17), *L. rigidum* x *L. multiflorum* (C18), Guard II, Guard C2, Guard C3, *L. rigidum* x *L. multiflorum* (C19.1), Guard x Western Australia Ecotype (Guard WA). Genomic DNA was digested with either *Dra* I **(A)** or *Sac* I **(B)**, separated on 0.8% agarose gel which was subsequently Southern blotted and the membrane probed with the class specific probe.

RGA. Across all examined plant lines, the class 2-specific probe hybridised to relatively fewer *Dra*I and *Sac*I fragments than the class 1 specific probe. It was concluded that this was a reflection of the relative abundance of class 1 compared to class 2 RGA sequences within the *L. rigidum* genome.

The class 3-specific probe, atm1(3f-G3), hybridised to *Dra*I and *Sac*I fragments of genomic DNA from all tested plant lines (Figure 5.20). This indicated that class 3 RGA sequences were present in all examined plant lines. It was noted that the level of hybridisation to a number of fragments varied. As an example, stronger hybridisation was observed to the 4.0 kb *Dra*I fragment present in GuardII derived DNA than to the similarly sized fragment present in *Dra*I digeseted DNA of Guard C3, C19.1 and Guard WA. Two alternative explanations may account for this difference. It may be possible that hybridisation is stronger to sequences found in Guard II as class 3 sequences are present in higher copy number than the other three plant lines. Alternatively, it is possible that class 3 sequences present in Guard II share more sequence identity with the probe than similar sequences in the remaining three plant lines, resulting in stronger hybridisation.

Hybridisation of the class 4-specific probe, atm8(4f-G3), was observed to genomic DNA isolated from only 6 of the 12 lines examined (Figure 5.21). This suggested that class 4 RGA sequences were not as commonly found in *L. rigidum* genomic DNA as the RGA classes 1 and 3. Class 4 sequences were detected in genomic DNA from both lines susceptible (Turretfield and S1150) and resistant (R799, Guard, GuardC3 and Guard WA) to *A. funesta*. With the exception of Guard WA, class 4 sequences appeared to exist as single copies in the genome.

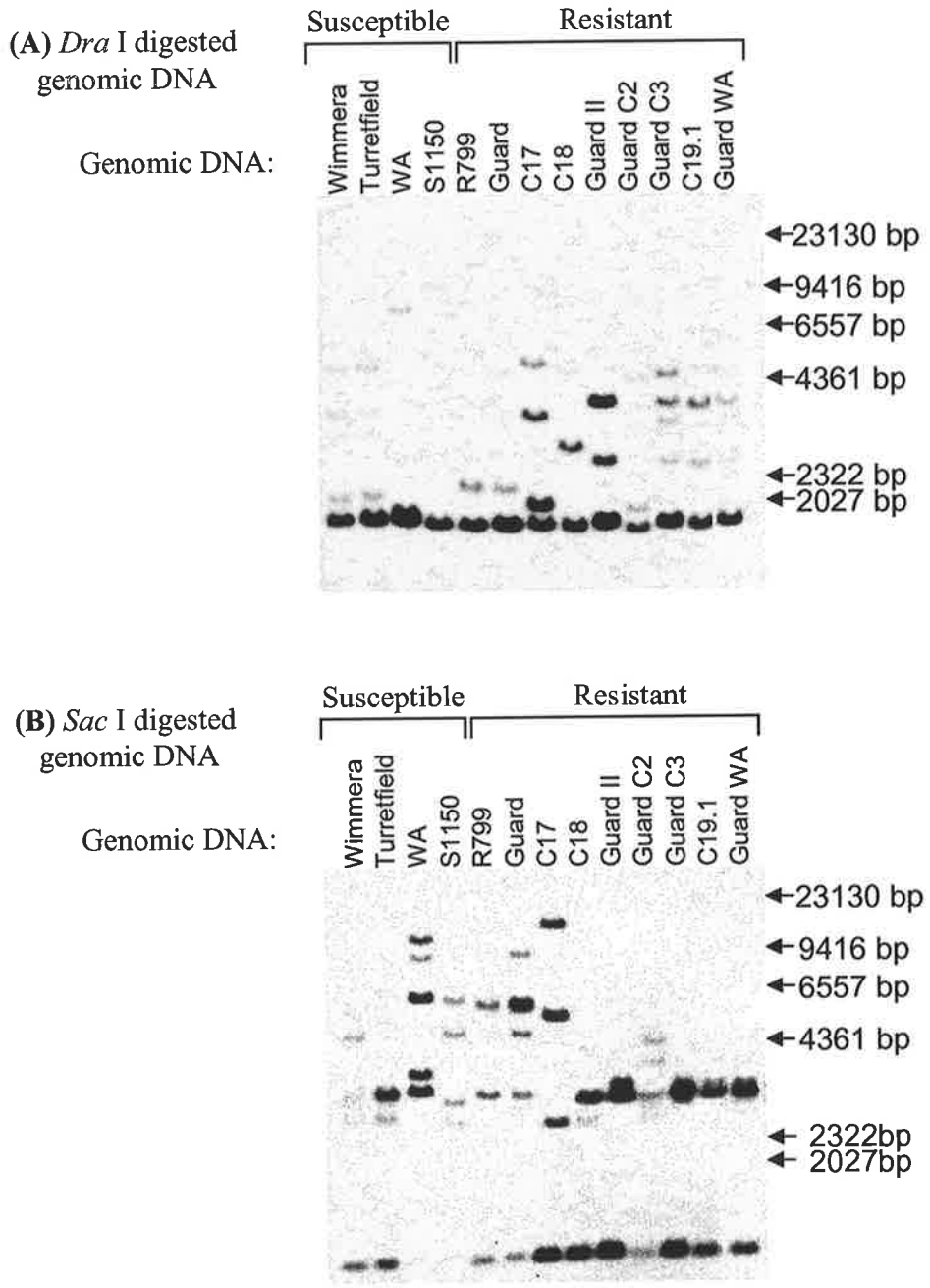


Figure 5.20: Hybridisation of the class 3 representative probe, atm1(3f-G3), to genomic DNA isolated from *L. rigidum* plant lines susceptible or resistant to nematode infection.
Genomic DNA from the susceptible lines Wimmera Ecotype, Turretfield Ecotype, Western Australia Ecotype (WA) and S1150, and the resistant lines R799, Guard, *L. rigidum* x *L. multiflorum* (C17), *L. rigidum* x *L. multiflorum* (C18), Guard II, Guard C2, Guard C3, *L. rigidum* x *L. multiflorum* (C19.1), Guard x Western Australia Ecotype (Guard WA). Genomic DNA was digested with either *Dra* I **(A)** or *Sac* I **(B)**, separated on 0.8% agarose gel which was subsequently Southern blotted and the membrane probed with the class specific probe.

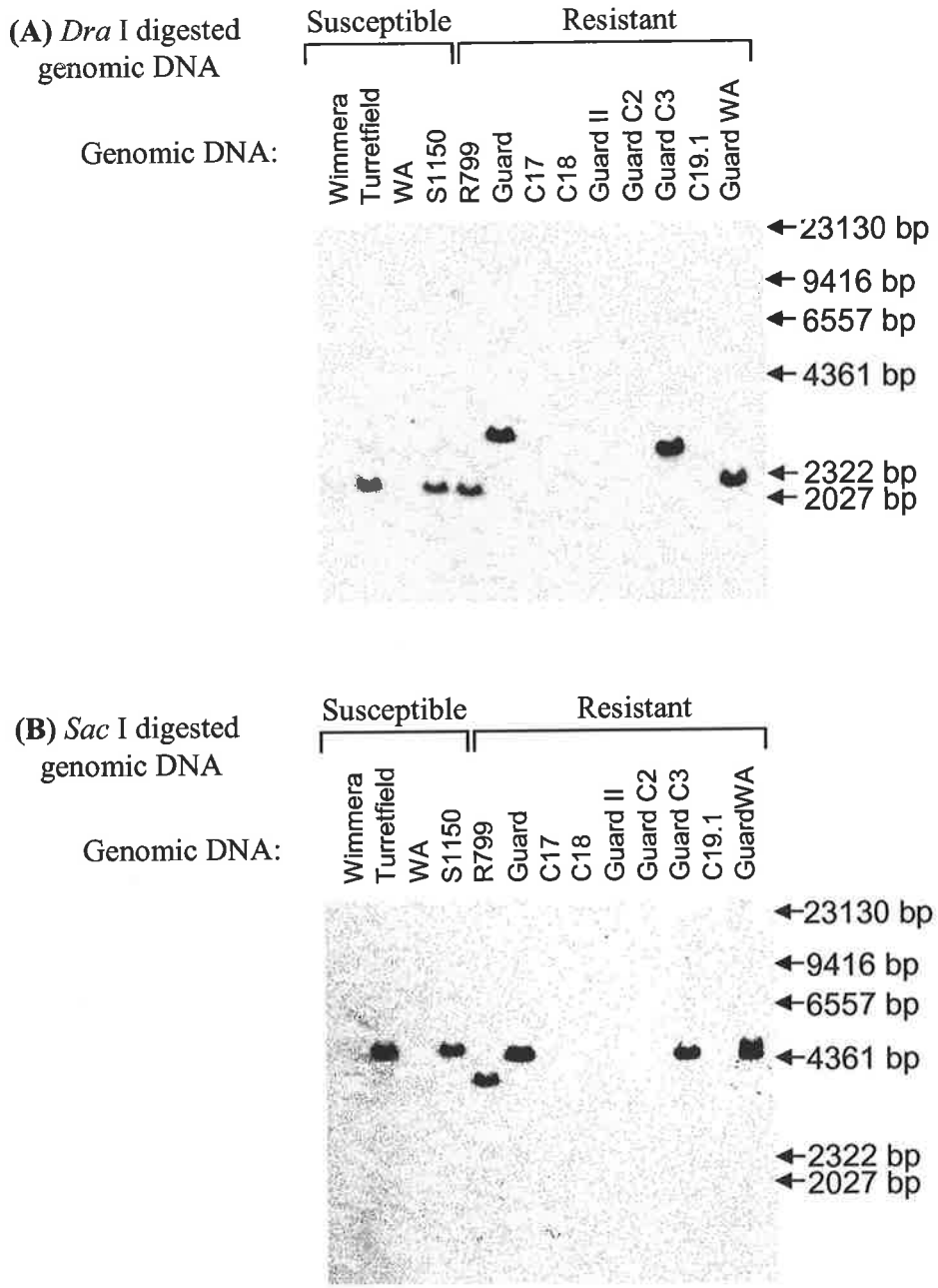


Figure 5.21: Hybridisation of the class 4 representative probe, atm8(4f-G3), to genomic DNA isolated from *L. rigidum* plant lines susceptible or resistant to nematode infection.
Genomic DNA from the susceptible lines Wimmera Ecotype, Turretfield Ecotype, Western Australia Ecotype (WA) and S1150, and the resistant lines R799, Guard, *L. rigidum* x *L. multiflorum* (C17), *L. rigidum* x *L. multiflorum* (C18), Guard II, Guard C2, Guard C3, *L. rigidum* x *L. multiflorum* (C19.1), Guard x Western Australia Ecotype (GuardWA). Genomic DNA was digested with either *Dra* I **(A)** or *Sac* I **(B)**, separated on 0.8% agarose gel which was subsequently Southern blotted and the membrane probed with the class specific probe.

5.2.11 Cloning and Analysis of Class 1, 5' RACE Products.

To increase the length of RGA sequence and to examine the possible expression of RGAs in *L. rigidum*, 5' RACE was conducted on *L. rigidum* cDNA. All five clones of the 5' RACE product were found to be identical in sequence and 236 bp in length (Figure 5.22). The 5' RACE product contained 130 bp of previously uncharacterised sequence upstream of the kinase-2 encoding sequence. Analysis revealed the 3' end of the 5' RACE product was identical to the 5' end of the genomically derived sequence of the Class 1 atm clones atm53 and atm44, confirming amplification of class 1 RGA sequence had been achieved.

Theoretical translation in all six possible reading frames indicated an open reading frame encoding 78 amino acids was present which contained kinase-2 and kinase-3 motifs. The closest database matches for the deduced 78 amino acid sequence were the *Avena strigosa* RGA clone II 2.17 (Loarce *et al.*, 2000) and the *A. ventricosa* RGA, *rae1* (Lopez-Brana *et al.*, 1999). The deduced amino acid sequence of atm5Rclass1 was 88% identical (92% similar) to the corresponding region of clone II 2.17 and 88% identical (93% similar) to *rae*1 (Figure 5.23). Both clone II 2.17 and *rae*1 were previously shown to be the closest database matches for class 1 atm clones (Section 5.3.6).

5.3 DISCUSSION

5.3.1 The Nature of RGAs in *L. rigidum*

In similar PCR-based attempts to isolate RGAs from plants, varying numbers of classes of RGA sequence were identified. Leister *et al.* (1996) identified 7 RGA classes in potato, Collins *et al.* (1998) identified eleven non-cross-hybridising RGA classes in maize and

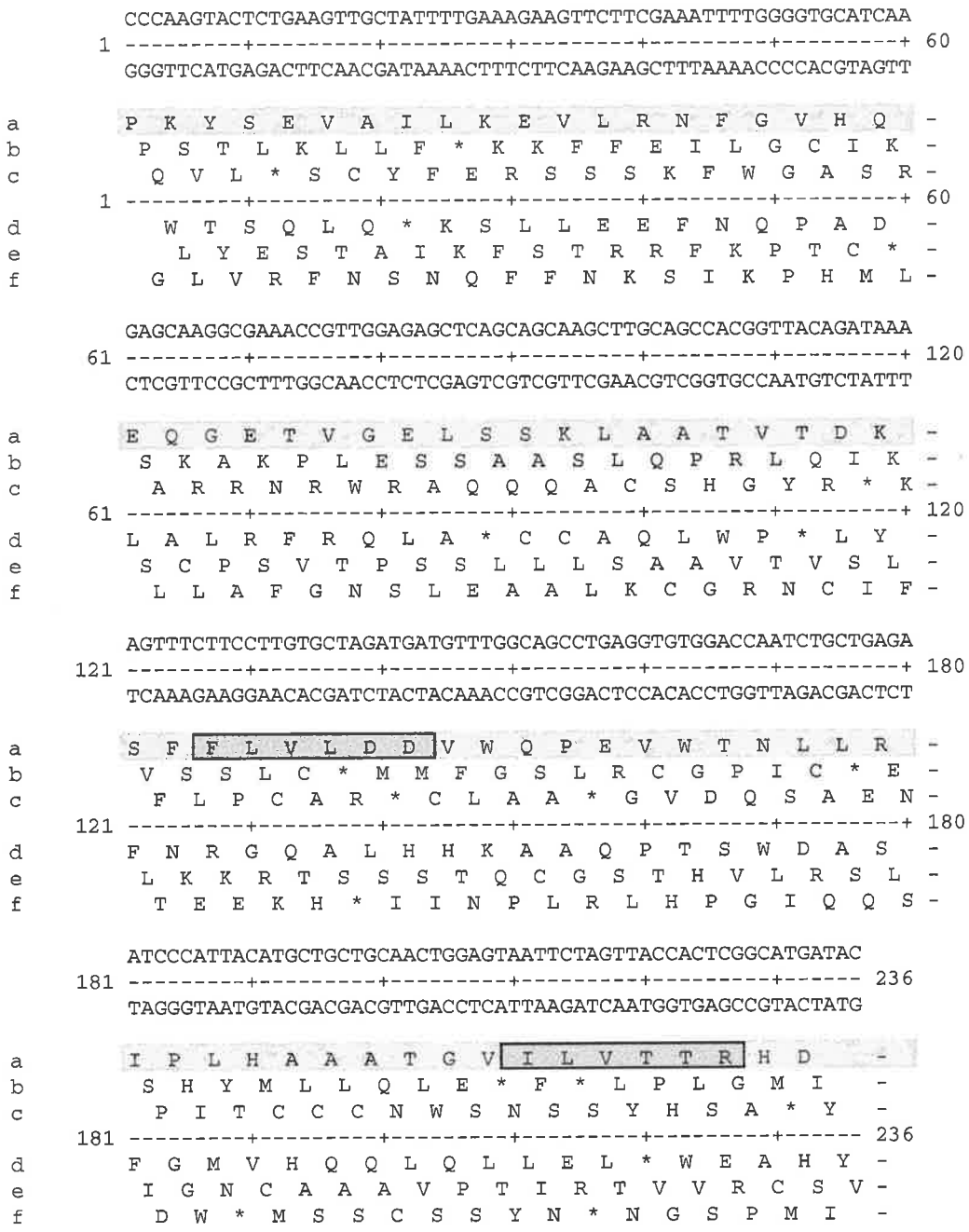
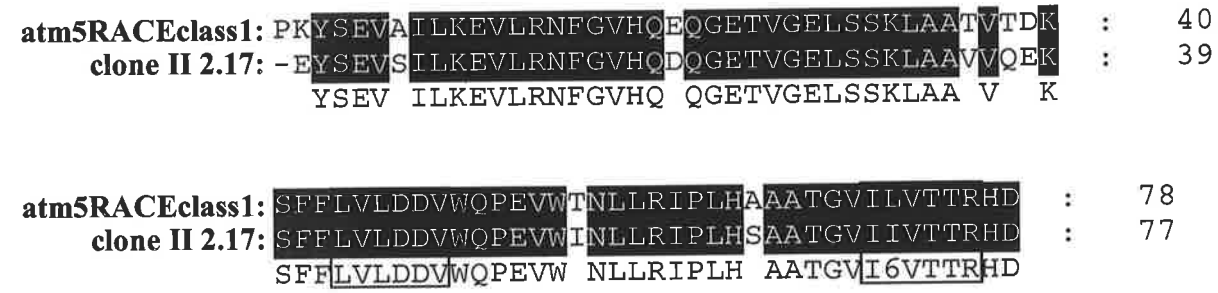


Figure 5.22: DNA and deduced amino acid sequences of class 1 5' RACE product, atm5RACEclass1.

The DNA sequence is presented on the upper line. Deduced amino acid sequence of the first forward reading frame (a) is presented directly below the DNA sequence and shaded gray to represent a continuous open reading frame. Conserved core motifs of the kinase-2 domain (L/V/L/IVLDDV) and the kinase-3 domain (XXXTTR) are boxed and shaded dark grey.

(A)



(B)

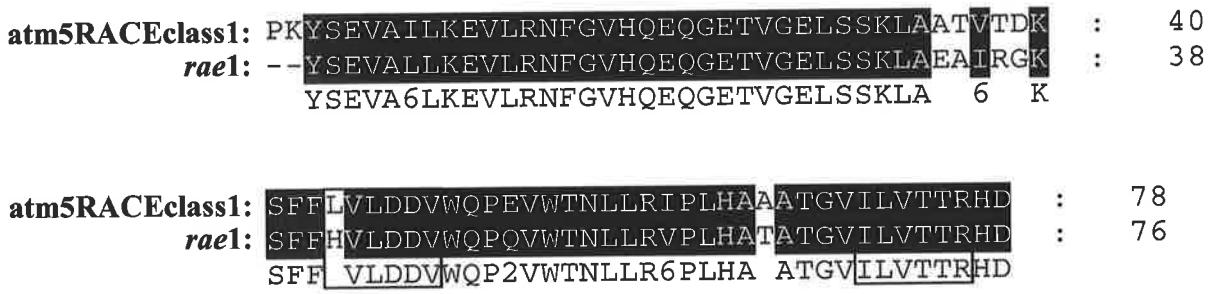


Figure 5.23: Sequence alignments of the deduced amino acid sequence of the class 1, 5' RACEproduct, atm5RACEclass1, with the database matches of highest probability

(A) Alignment of atm5RACEclass1 with residues 32 to 108 of the partial resistance gene, clone II 2.17 of *Avena strigosa* (Accession number CAC10052). **(B)** Alignment of atm5RACEclass1 with residues 32 to 109 of the *Aegilops ventricosa* RGA, *rae*1 (Accession number CAC11100). Sequences were aligned using the GCG, PILEUP program with the default settings of gap creation penalty at 3.0 and gap extension penalty at 0.1. Gaps are represented by dashes and were introduced to maximise homology. Conserved amino acids are shaded black and are presented underneath the alignment as a capital letter for 100% identity. Numerals represent sequence similarity; 1=D/N,2=E/Q, 3=S/T, 4=K/R, 5=F/Y/W, 6=L/I/V/M. Motifs conserved in RGAs are boxed and correspond to the kinase-2 and kinase-3 motifs.

Mago *et al.* (1999) identified 14 classes in rice. Based on the hybridisation of class specific probes to slot blots containing DNA isolated from the 91 *L. rigidum* atm clones, 74 clones were classified into one of the four RGA classes.

Of the 88 atm clones containing inserts, 14 (16%) were not hybridised by any of the four RGA class specific probes. These may represent clones that contained inserts of about 300 bp that were not RGA sequences such as atm62. Non-RGA sequences have previously been amplified using the same approach in maize (Collins *et al.,* 1998). Of the 68 sequenced clones sequenced by Collins *et al.,* (1998), only 26 contained RGA sequences. Alternatively, additional classes of RGA may exist in *L. rigidum* as the unclassified clones may contain RGA sequences sufficiently diverged from the 4 classes identified to prevent detection by the class specific probes. While capable of isolating RGA sequences, a PCR based approach cannot ensure all RGA sequences contained within a genome have been amplified. An exhaustive analysis of RGA sequences was not carried out due to uncertainty concerning the total number of RGA classes within *L. rigidum*. A truly exhaustive search for RGA sequences within the genome of any given plant is only possible for plants, such as *Arabidopsis*, in which the entire genome has been sequenced, allowing for thorough searching of RGA sequences (Pan *et al.,* 2000).

The identification of RGA sequences with high sequence identity to three of the four *L. rigidum* classes in other plants indicated these three RGA classes did not evolve from one RGA sequence within *L. rigidum*. It suggested the existence of a number of RGA sequences in a common ancestor.

The presence of a stop codon within the RGA sequence of atm7, 25 and 28 suggested they might represent a pseudogene. RGA sequences containing stop codons have been reported in soybean (Kanazin *et al.,* 1996), tomato (Ohmori *et al.,* 1998) and *Arabidopsis* (Pan

et al., 2000). The ability to amplify RGA sequence from a *L. rigidum* cDNA library provided evidence that a least one RGA sequence is expressed in *L. rigidum*.

5.3.2 Comparison of *L. rigidum* Putative RGA Protein Sequences with Group Specific Conserved Residues.

Exhaustive, evolutionary analysis by Pan *et al.* (2000) on all known R-genes and RGA sequences present in sequence databases concluded that two distinct types of NBS sequence exist. Analysis of conserved residues within these proteins uncovered conserved domains and group-specific conserved residues (Pan *et al.*, 2000). The RGAs isolated in *L. rigidum* corresponded to conserved domains III to VI of the NBS region described by Pan *et al.*, (2000). This region contained 14 Group I specific amino acids and 6 Group II specific amino acids (Figure 5.7). Overall, the majority of Group II specific residues were conserved in a number of *L. rigidum* RGA classes, but only one of the 14 Group I specific residues was conserved. Based on the model proposed by Pan *et al.* (2000), the conservation of the majority of the Group II key residues in *L. rigidum* RGAs predicts an association with a coiled-coil domain at the N-terminus of their predicted amino acid sequence. Further isolation and characterisation of full-length RGA genes would be required to confirm this predicted association.

5.3.3 RGA Sequences Representing Potential R-gene Markers

As resistance to *A. funesta* arose from a common origin amongst all the resistance plant lines analysed, RGA sequences were required to be present in all resistant plant lines to

be considered potential R-gene markers. Under this criterion, class 1, 2 and 3 RGA sequences were potential R-gene markers as homologous sequences were identified in the genomic DNA of all resistant plant lines examined. As the class 4 probe hybridised to only 4 of the 8 resistant plant lines it was eliminated as being a potential R-gene marker.

To be considered as potential R-gene markers, RGA sequences were also required to be absent in the genomic DNA isolated from S1150. The class 2 and 3 RGA sequences matched this criterion as weak or no hybridisation of the class 2 specific probe, atm68(2f-G3), was noted to S1150 genomic DNA. The class 3 specific probe, atm1(3f-G3) failed to hybridise to a 1.5 kb *Sac*I fragment present in all resistant plant lines.

5.3.4 RGA Sequences Representing Potential R-genes

It was expected that if a given RGA class member was one of the two R-genes responsible, each resistance plant line would be expected to contain such sequences. This eliminated the possibility of the class 4 RGA, atm8, as an R-gene as it was present in only 4 of the 8 resistant plant lines. Under this criterion all three remaining RGA classes were still potential candidates as their sequences were detected in all resistant plant lines examined.

Potential R-gene candidates require further investigation as, unlike R-gene markers, R-gene sequences would not be expected to be absent in all susceptible plant lines. Sequences homologous to the R-gene may be present at the same locus in susceptible plant lines, as was the case for the rice R-gene, *Xa1* (Yoshimura *et al.*, 1998). Alternatively, other RGA sequences, high in sequence identity with the R-gene may be closely linked to the R-gene and indistinguishable from the true R-gene using hybridisation techniques. This was the case in tomato, in which two tightly linked RGA sequences, Mi1.1 and Mi1.2 shared 95% sequence

identity (Rossi *et al.*, 1998). Through transformation of susceptible plants, it was shown that only the Mi1.1 gene could confer resistance to nematode infection. Under the mode of inheritance present in *L. rigidum*, it would be possible for either of the resistance genes to be present in susceptible carrier plants. Hybridisation of any given RGA probe to genomic DNA of a susceptible plant line would not exclude the possibility of the RGA being an R-gene.

5.3.5 Future Directions

5.3.5.1: Development of RGAs as Markers

Isolated RGA sequences have the potential to be used as markers for both plant identification and further investigation towards isolating genes responsible for resistance to *A. funesta* infection in *L. rigidum*. A number of group specific probes revealed differences in the hybridisation profiles produced. These probes, in combination, have the potential to be used for identification purposes. A more extensive examination of the presence of RGA sequences in a wider variety of *L. rigidum* lines may be necessary before this can be considered as suitable for use as a diagnostic tool on *L. rigidum* types within a pasture.

A more time efficient diagnosis may be developed based on the technique of Chen *et al.* (1998). In this technique, primers directed towards conserved RGA motifs are used to amplify products from plant genomic DNA. PCR products are separated on high resolution, acrylamide gels enabling the staining of up to 130 discrete products and the separation of products differing by as little as a single base pair. As RGAs differing in size were isolated from *L. rigidum*, it may be possible to apply the technique to plant identification in *L. rigidum*.

5.3.5.2: The Path to R-gene Confirmation in *L. rigidum*

This study has presented the first successful step towards the isolation of R-genes in *L. rigidum*. Using a PCR based approach, RGA sequences were isolated from the *L. rigidum* genome and shown to exist in a variety of *L. rigidum* plant lines. A number of potential R-genes candidates were presented. This step represents only the first of many that must be undertaken if R-genes are to be identified in *L. rigidum*.

Isolation of full length RGA sequences from *L. rigidum* cDNA libraries could limit R-gene candidates to expressed RGA sequences. Northern analysis on RNA isolated from plant tissue may need to be carried out to confirm the expression of full length RGA genes. Further investigation towards isolating R-genes in *L. rigidum* could also make use of the identified RGAs as markers. The linkage of any given RGA to resistance to nematode infection could be examined in extensive crossing experiments. As a number of isolated R-genes have been found to exist in clusters in the genome, isolation of large fragments of genomic DNA through hybridisation to the RGA probes could be used to clone such clusters. This would assist in both the isolation of full length RGA sequences and the full variety of RGA sequences present in *L. rigidum*.

Any current attempt to map potential R-genes to resistance loci in *L. rigidum* would be a large undertaking as only few phenotypic loci or molecular markers have been mapped. Therefore, mapping of RGA sequences would require an extensive genome project to be carried out to determine the relative location of RGAs to other molecular markers and resistance loci.

Confirmation of R-gene status would require the transformation of plant material to either knockout R-gene function in a resistant plant or to provide resistance to a susceptible plant. Currently there is no reliable, routine DNA transformation procedure in operation for *L.*

rigidum. An effective transformation technique would need to be established before advancing to this stage. While transformation to knock out R-gene function in a resistant plant would be possible, transformation of a susceptible line to gain resistance would require the transformation of a susceptible plant line known to be a carrier of the second resistance gene.

Chapter 6

General Discussion

6.1: Importance of Plant Breeding in Pasture Species

Plant breeding has traditionally focussed on improving traits effecting both yields and pathogen resistance in food crops and crops used for fibres. Unfortunately, the effect of new virulent pathogen races on the resultant susceptible monocultures has had devastating effects on yields. In contrast, pastures are usually mixtures of heterogenous grass species, containing resistance to a diverse range of pathogens and pathogen races. As a result, the effect of a single pathogen race is more difficult to observe on a single plant species in a mixed pasture than in a homogeneous crop. The overall effect on yield is also less severe, as sufficient plant material remains for animal feed. This is one of the reasons relatively less study has been devoted to the understanding of resistance to pathogens in pasture species relative to crop species.

A number of biological factors contribute to the severe impact ARGT has on grazing animals. The impact of the combination of pathogens in pastures containing *L. rigidum* is more apparent as the ingestion of toxin containing plant material is toxic to the grazing animal. Secondly, due to the prolific growth rate and reproductive capacity of *L. rigidum*, pastures are predominantly ryegrass rather than a mixture of grass species. Although *L. rigidum* plants within a pasture are genetically diverse rather than a monoculture, the frequency of resistance to *A. funesta* is low, which supports a high *A. funesta* replication rate. There is also low selective pressure for plants resistant to *A. funesta* as, although the nematode impacts on the reproductive capacity of susceptible lines of *L. rigidum* by displacing some seed formation for galls, there is still sufficient viable seed production to ensure survival in the next season and generation. Studies on the nature of the resistance to *A. funesta* in *L. rigidum* is of scientific and economic importance.

6.2: Impact of Low Level of Relatedness between R799 and S1150 on Success of Undertaken Approaches

The work presented in the preceding chapters contains further groundwork of investigations of both the genetic and molecular basis of resistance to *A. funesta* in *L. rigidum*. Limitations in the degree of progress made with each approach occurred partly due to the lack of previous genetic and molecular studies of *L. rigidum* as extensive as crops species and the observation that the putative NILs R799 and S1150 were not as closely related as previously assumed. Through the analysis of RAPD profiles in Chapter 4, R799 and S1150 were calculated to be only 23-38% identical rather than the assumed 93.4%. Qualitative evidence of the lower relatedness was also provided by the differences in both the abundance and genomic localisation of group specific RGA sequences in Chapter 5. As lines which shared 93.4% of their genetic background would also have a high probability of sharing SI alleles they would not be expected to set seed when crossed. In contrast, as described in Chapter 3 seed set was produced in crosses between R799 and S1150. The lack of NILs impeded the success of the strategies of using NILs to assist in the isolation of RAPD markers or RGA sequences linked to resistance to *A. funesta*, as the use of R799 and S1150 in screens had no advantage over the use of unrelated resistant and susceptible lines of *L. rigidum*. The evidence provided from the comparison of genetic relatedness between ryegrass lines based on shared RAPD band profiles, demonstrated the utility and applicability of this method for the monitoring of fidelity in crossing programmes.

6.3: Future Directions of Research with RGA Sequences in *L. rigidum*

This study is the first record of the abundance of RGA sequences in the *L. rigidum* genome. Two potentially overlapping directions are worthy of further investigation. One line of research would be aimed at the isolation of full length cDNA clones to enable the discrimination of functional genes containing RGA sequences from unexpressed, pseudogenes. While it is possible these may represent R-genes responsible for resistance to *A. funesta*, it is also possible, based on the conserved motifs present, encoded proteins may be involved in signal transduction within the plant cell and therefore also of interest to study further. An alternate line of research would continue the current focus of attempting to isolate molecular markers linked to the two putative R-genes encoding resistance to *A. funesta* in *L. rigidum*. The use of true NILs of *L. rigidum* differing in resistance to *A. funesta* would minimise the differences in the genetic backgrounds in regions other than those encoding the R-genes and therefore maximise the likelihood of detecting an association between a RGA sequence and resistance.

6.4: Future Directions of Research into the Nature of Resistance to *A. funesta* in *L. rigidum*

Future study on the nature of the resistance to *A. funesta* in *L. rigidum* would be greatly assisted by the large undertaking of extensive mapping of the *L. rigidum* genome with respect to both resistance genes and molecular markers. As many of the RAPD products observed in Chapter 4 were not amplified across all *L. rigidum* lines, they may be useful as potential markers in any future mapping project. Further RAPD screening of new NILs differing in resistance to *A. funesta* for association with resistance would be important for the mapping of

the two resistance genes responsible for resistance to *A. funesta*. As it is possible that the R-genes do not contain RGA sequences, these markers would be an important starting point in any chromosome walk or landing to isolate and characterise the genes to gain a better understanding into the molecular and cellular basis of resistance to *A. funesta* in *L. rigidum*.

Appendix A: Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 1:Statistical analysis of numbers of resistant and susceptible progeny from the cross S15782.3 x S1150.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
S15782.3		S1150	R:S	R:S				
AABb	x	*aaBb*	3:1	7.5	2.5	23	2.1×10^{-6}	✖
AaBb	x	*aaBb*	3:5	3.75	6.25	3.2	0.072	✓
AaBB	x	*aaBb*	1:1	5	5	6.4	0.011	✖
AABb	x	*aabb*	1:1	5	5	6.4	0.011	✖
AaBb	x	*aabb*	1:3	2.5	7.5	1.2	0.27	✓
AaBB	x	*aabb*	1:1	5	5	6.4	0.011	✖

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✖).

Appendix A (Cont.): Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 2: Statistical analysis of numbers of resistant and susceptible progeny from the cross R15782.3 x R1386.4.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
R15782.3		R1386.4	R:S	R:S				
AABb	x	*AABb*	3:1	7.5	2.5	0.13	0.72	✓
AABb	x	*AaBb*	3:1	7.5	2.5	0.13	0.72	✓
AABb	x	*AaBB*	all R	10	0	-	-	✗
AaBB	x	*AABb*	all R	10	0	-	-	✗
AaBB	x	*AaBb*	3:1	7.5	2.5	0.13	0.72	✓
AaBB	x	*AaBB*	3:1	7.5	2.5	0.13	0.72	✓
AaBb	x	*AABb*	3:1	7.5	2.5	0.13	0.72	✓
AaBb	x	*AaBb*	9:7	5.625	4.375	2.3	0.13	✓
AaBb	x	*AaBB*	3:1	7.5	2.5	0.13	0.72	✓

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✗).

Appendix A (Cont.): Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 3:Statistical analysis of numbers of resistant and susceptible progeny from the cross R15782.3 x R15794.1.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
R15782.3		R15794.1	R:S	R:S				
AABb	x	*AABb*	3:1	7.5	2.5	0.13	0.72	✓
AABb	x	*AaBb*	3:1	7.5	2.5	0.13	0.72	✓
AABb	x	*AaBB*	all R	10	0	-	-	✗
AaBB	x	*AABb*	all R	10	0	-	-	✗
AaBB	x	*AaBb*	3:1	7.5	2.5	0.13	0.72	✓
AaBB	x	*AaBB*	3:1	7.5	2.5	0.13	0.72	✓
AaBb	x	*AABb*	3:1	7.5	2.5	0.13	0.72	✓
AaBb	x	*AaBb*	9:7	5.625	4.375	2.3	0.13	✓
AaBb	x	*AaBB*	3:1	7.5	2.5	0.13	0.72	✓

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✗).

Appendix A (Cont.): Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 4: Statistical analysis of numbers of resistant and susceptible progeny from the cross R15782.3 x R15789.5.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
R15782.3		R15789.5	R:S	R:S				
AABb	x	*AABb*	3:1	5.25	1.75	3.9	0.05	✖
AABb	x	*AaBb*	3:1	5.25	1.75	3.9	0.05	✖
AABb	x	*AaBB*	all R	7	0	-	-	✖
AaBB	x	*AABb*	all R	7	0	-	-	✖
AaBB	x	*AaBb*	3:1	5.25	1.75	3.9	0.05	✖
AaBB	x	*AaBB*	3:1	5.25	1.75	3.9	0.05	✖
AaBb	x	*AABb*	3:1	5.25	1.75	3.9	0.05	✖
AaBb	x	*AaBb*	9:7	3.94	3.06	0.51	0.48	✓
AaBb	x	*AaBB*	3:1	5.25	1.75	3.9	0.05	✖

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✖).

Appendix A (Cont.): Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 5: Statistical analysis of numbers of resistant and susceptible progeny from the cross R15789.5 x R1386.4.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
R15789.5		R1386.4	R:S	R:S				
AABb	x	*AABb*	3:1	6.75	2.25	0.037	0.85	✓
AABb	x	*AaBb*	3:1	6.75	2.25	0.037	0.85	✓
AABb	x	*AaBB*	all R	9	0	-	-	✗
AaBB	x	*AABb*	all R	9	0	-	-	✗
AaBB	x	*AaBb*	3:1	6.75	2.25	0.037	0.85	✓
AaBB	x	*AaBB*	3:1	6.75	2.25	0.037	0.85	✓
AaBb	x	*AABb*	3:1	6.75	2.25	0.037	0.85	✓
AaBb	x	*AaBb*	9:7	5.06	3.94	1.7	0.19	✓
AaBb	x	*AaBB*	3:1	6.75	2.25	0.037	0.85	✓

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✗).

Appendix A (Cont.): Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 6: Statistical analysis of numbers of resistant and susceptible progeny from the cross R1386.4 x R799.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
R1386.4		R799	R:S	R:S				
AABb	x	*AABb*	3:1	5.25	1.75	0.43	0.51	✓
AABb	x	*AaBb*	3:1	5.25	1.75	0.43	0.51	✓
AABb	x	*AaBB*	all R	7	0	-	-	✗
AaBB	x	*AABb*	all R	7	0	-	-	✗
AaBB	x	*AaBb*	3:1	5.25	1.75	0.43	0.51	✓
AaBB	x	*AaBB*	3:1	5.25	1.75	0.43	0.51	✓
AaBb	x	*AABb*	3:1	5.25	1.75	0.43	0.51	✓
AaBb	x	*AaBb*	9:7	3.94	3.06	2.5	0.12	✓
AaBb	x	*AaBB*	3:1	5.25	1.75	0.43	0.51	✓

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✗).

Appendix A (Cont.): Statistical Analysis of Progeny in Crosses Presented in Table 3.5.

Table 7: Statistical analysis of numbers of resistant and susceptible progeny from the cross 1150 x 799.

Genetic Model			Model	Expected		χ^2_1	*P*	Accept/ Reject Model
S1150		R799		R:S				
aaBb	x	*AABb*	3:1	6.75	2.25	3	0.083	✓
aaBb	x	*AaBb*	3:5	3.38	5.63	15	0.00011	✗
aaBb	x	*AaBB*	1:1	4.5	4.5	9	0.0026	✗
aabb	x	*AABb*	1:1	4.5	4.5	9	0.0026	✗
aabb	x	*AaBb*	1:3	2.25	6.75	27	2.0×10^{-7}	✗
aabb	x	*AaBB*	1:1	4.5	4.5	9	0.0026	✗

Expected ratios of R:S are derived from genetic models that may account for the genotypes of parental lines which in turn are responsible for expected numbers of resistant (R) and susceptible (S) progeny plants. These values are used in conjunction with observed numbers to calculate the chi-squared (χ^2_1) and corresponding probability (p) value. On the basis of the value of p, the model is either accepted (✓) or rejected (✗).

Appendix B: Binary Matrix of RAPD Profiles

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
OPAM-01																						
10.19	0	0	1	1	1	0	0	0	0	0	1	1	1	1								
10.20	0	0	1	0	0	0	1	0	1	1	0	0	1	1								
799	0	0	1	1	1	0	0	1	0	0	1	1	1	1								
1150	1	1	0	0	0	1	1	0	1	1	0	0	0	1								
OPAM-03																						
10.19	1	1	1	1	0	1	1	0	0	0	0	1	0									
10.20	0	1	0	0	1	0	1	0	1	0	1	1	0									
799	1	1	1	1	0	1	1	1	1	1	1	1	1									
1150	0	0	0	1	1	0	1	0	1	0	1	0	0									
OPAM-07																						
10.19	1	1	0	1	0	1	0	1	0													
10.20	1	1	1	1	1	1	1	1	1													
799	1	1	0	1	0	0	0	1	0													
1150	1	0	1	1	1	1	1	1	1													
OPAM-12																						
10.19	1	0	1	1	0	0	1	0	0	0	1	1	1	1	1							
10.20	0	0	0	0	0	1	0	1	0	0	1	1	1	1	1							
799	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1							
1150	0	1	1	1	0	1	0	1	0	0	0	1	0	0	0							
OPD-15																						
10.19	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1							
10.20	1	0	1	1	1	0	0	0	0	0	1	1	0	1	0							
799	0	1	0	0	0	0	1	0	1	0	1	1	0	1	1							
1150	1	0	1	1	1	1	0	1	1	1	1	1	1	1	0							
OPE-06																						
10.19	0	0	1	0	1	0	0	1	0	0	1	1	0	1								
10.20	1	1	1	1	0	1	0	1	1	0	0	1	1	1								
799	1	1	1	1	0	0	1	1	1	1	0	1	1	1								
1150	1	0	1	0	1	1	0	1	0	0	1	1	0	1								
OPE-12																						
10.19	1	1	0	1	0	0	0	1	1	1	0	1	1									
10.20	1	1	0	0	0	1	1	1	0	1	1	1	0									
799	1	1	0	0	0	1	1	0	0	1	1	1	0									
1150	1	1	1	1	1	0	0	1	1	1	0	0	1									
OPE-13																						
10.19	1	0	1	1	0	1	0	1	0	1	1	1	1	0	1	1	0	1	1	0	0	1
10.20	1	0	0	1	0	1	0	1	0	1	0	1	1	1	0	0	1	0	0	0	1	1
799	1	0	0	1	0	1	1	1	0	1	0	1	1	1	0	0	1	0	0	0	0	1
1150	1	1	1	0	1	0	0	0	1	0	1	0	1	0	1	1	0	1	1	1	1	1
OPW-08																						
10.19	1	0	1	1	0	1	0	1	1	1	1	1	1									
10.20	1	1	0	0	1	0	0	1	0	1	1	1	1									
799	1	0	0	1	0	1	0	1	0	1	1	1	1									
1150	1	1	1	0	1	0	1	0	1	1	0	1	1									

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Corrections

Chapter 1.1.2.1, p5, The phrase "nucleotide binding site (NBS) and leucine -rich repeat (LRR) motifs"should read "nucleotide binding site (NBS) and leucine -rich repeat (LRR)".

Chapter 1.1.2.2, p5, the feature "nucleotide binding site" should read "NBS" and "leucine rich repeats" should read "LRR".

Chapter 1.1.2.5, p9, the text "rice" should read "*Oryza sativa*", "tomato" should read "*Lycopersicon esculentum*", and "lettuce" should read "*Lactuca serriola*".

Chapter 1.1.3.1, p11, the text "tomato" should read "*Lycopersicon esculentum*", "tobacco" should read "*Nicotiana glutinosa*" and "flax" should read "*Linum usitatissimum*".

Chapter 1.2.1.5, p18, the following text should be inserted after the last paragraph on the page "Recently, other *Lolium* maps have been published and these include Hayward *et al*., (1998) and Jones et al., (2002)."

Chapter 2.1.9, p32, the name "Dr Alan McKay's" should read "Dr. Alan McKay's"

Chapter 3.2.3, p58, the phrase "consistent with *L rigidum"* should read: "consistent with *L. rigidum*".

Chapter 3.3.8.3, p73, the phrase "This approach may" should read "The approach, as described in present chapter, may".

Table 4.1 "Amplificatio n" should read "Amplification" and "Artefacts" should read "Artifacts"

Chapter 4.3.3, p80, the phrase "It is possible the RAPD" should read "It is probable that the RAPD".

Chapter 4.5, p84, the phrase "This provides" should read "The lack of sufficient genetic relatedness between R799 and S1150 provides".

Chapter 5.2.8, p95, the name "atm 28" should read "atm28"and "atm 39" should read "atm36".

Chapter 5.2.10, p98, the name "GuardII" should read "Guard II".

Chapter 5.3.4, p102, the phrase "being a potential R-gene markers" should read "being a potential R-gene marker".

References p9, the authors "Allen, J and Bywater, B" should read "Allen, J. and Bywater, B.".

For the remainder of the references the correct and consistent format for the citation of authors of a reference is for example "Bird, A.F." rather than "Bird, A. F." as is shown.

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